Strain selection and optimization of *Ulva* spp. for land-based recirculating cultivation systems

Isabel Luísa de Avelar Cardoso

Dissertation

in fulfilment of the requirements for the degree of Doctor of Natural Sciences (Dr. rer. nat.) at the Faculty 02 – Biology and Chemistry of the University of Bremen, Germany.

Thesis supervised by Prof. Dr. Bela H. Buck and Prof. Dr. Laurie C. Hofmann

Submitted on 08.04.2024 and reviewed by
Prof. Dr. Bela H. Buck
Dr. Ricardo Bermejo Lacida
Colloquium held on 15.05.2024

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Title Figure germling of *Ulva lacinulata*
Cultivated under laboratory conditions at the Alfred Wegener Institute, Germany. Photo by Isabel Cardoso.

This study has been conducted at the Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research in Bremerhaven, Germany, and was funded through the Mak-Pak Scale-Up Project with funds from the Federal Ministry of Food and Agriculture (BMEL) based on a decision of the parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support program (Funding Reference Number 281DL02B20).

ALFRED-WEGENER-INSTITUT
HELMHOLTZ-ZENTRUM FÜR POLAR- UND MEERESFORSCHUNG

HELMHOLTZ

Universität Bremen

With support from

Federal Ministry of Food and Agriculture

by decision of the German Bundestag

Project manager

Federal Office for Agriculture and Food

Thesis reviewers

First Reviewer: Prof. Dr. Bela H. Buck
Marine Aquaculture, Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research
and Bremerhaven University of Applied Sciences,
Bremerhaven, Germany

Second Reviewer: Dr. Ricardo Bermejo Lacida
Ecology and Geology Department, University of Málaga, Málaga, Spain

Examination commission

Commission chair: Prof. Dr. Kai Bischof
Marine Botany, University of Bremen
and MARUM – Centre for Marine Environmental Sciences,
Bremen, Germany

Examiner: Prof. Dr. Bela H. Buck
Marine Aquaculture, Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research
and Bremerhaven University of Applied Sciences,
Bremerhaven, Germany

Examiner: Prof. Dr. Laurie C. Hofmann
Marine Aquaculture, Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research and Bremerhaven University of Applied Sciences,
Bremerhaven, Germany

Examiner: Dr. Inka Bartsch
Rocky Shore Ecology, Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research,
Bremerhaven, Germany

Faculty member: Dr. Nora Diehl
Marine Botany, University of Bremen,
Bremen, Germany

Student member: Janka Jokay
Bachelor's student, University of Bremen,
Bremen, Germany

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Summary

Ulva species are intertidal macroalgae spread worldwide, known for their biochemical composition (e.g., *ulvan*) that grants them interesting properties to explore in sectors such as food, feed, cosmetics, pharmaceutical, and bioplastics. They are known for their high growth rates, bioremediation capacity, and plasticity, which can be associated with the formation of harmful green tides. Nevertheless, *Ulva* is considered a good candidate to cultivate on a large scale. Recirculating aquaculture systems (RAS) are well-established systems for the cultivation of fish, shrimp, and other aquatic species and can be operated as a partly flow-through or even complete closed system. Thus, allowing the cultivation of non-native species in land-based recirculating systems without introducing these species in the wild. Land-based systems present high costs and *Ulva* cultivation still presents several bottlenecks that limit the possibility of a profitable scale-up process (e.g., variation in biochemical composition, different adjustments, and responses to the environment). The works presented in this dissertation aimed to select *Ulva* strains for later cultivation in a RAS with artificial seawater and optimize them for their later use as the main constituent of food packaging. Four strains of *Ulva* from two geographical origins (*Ulva flexuosa*, *Ulva lacinulata*, from the Mediterranean Sea, and *U. lacinulata* and *Ulva linza* from the NE-Atlantic Ocean) were tested and compared for their robustness and capacity to grow in high temperatures and low salinities.

To test the importance of strain selection for *Ulva* cultivation, eco-physiological experiments were conducted. The relative growth rates (RGR) of different *Ulva* species and strains under different salinity (**Publication I**) and temperature conditions were tested (**Chapter 6**). Strains that presented RGRs below a 7 % day^{-1} RGR threshold, were considered not ideal. *Ulva linza* and both strains of *U. lacinulata* presented elevated growth rates in the temperature range tested. In the salinity experiment (**Publication I**) it was estimated that RGRs of the adult *U. lacinulata* strains would be above the 7 % day^{-1} threshold, even if salinity was reduced to 12 PSU. For this reason, *U. lacinulata* strains, particularly the NE-Atlantic strain, were considered potential candidates for vegetative cultivation in a RAS under lower salinity settings. To test the same salinity treatments as a potential method for strain optimization, the antioxidant activity (AA) from the NE-Atlantic *U. lacinulata* was measured for 10 days. AA showed a tendency to increase in the lowest and highest treatments (10 and 30 PSU), suggesting that biomass quality can be increased by further reducing the salinity for a short period before harvesting. Not all the species tested were good candidates for the system, thus

corroborating the importance of strain selection before cultivation. Cultivating *U. lacinulata* strains at lower salinities could amount to a reduction in the costs for the system and an increase in biomass quality.

High-quality biomass can increase the profitability of the system by enabling the biomass to be sold for a higher price. Antioxidant activity was considered a necessary property in the strains, as high levels of antioxidants can increase food's shelf-life. The effect of irradiance on the AA of the two *U. lacinulata* strains was evaluated (**Publication II**). Both strains were grown under a saturating irradiance treatment for 5 days and their AA and photosynthetic efficiency were evaluated. Both strains showed the capacity to adapt to the saturating irradiance, but only the NE-Atlantic strain had a significant increase in AA under the saturating irradiance treatment. Thus, suggesting that a significant light increase can improve the quality of the biomass. The lack of differences found in the AA of the Mediterranean strain between the control group and the saturating treatment indicates that light dose (kept the same between the treatments) also plays a role in determining the AA of a strain. The different results from both strains corroborate once more the importance of strain selection and optimization.

The NE-Atlantic *Ulva lacinulata*, often and spontaneously degraded without signs of fertility, and attempts at inducing reproduction were unsuccessful. As a first attempt to overcome these limitations, a successful protoplast isolation method was developed based on the methods reported in the literature. Two eco-physiological pre-treatments (light and salinity) were tested to be used before the isolation of protoplasts to reduce the costs of the isolation method. Only the 40 PSU pre-treatment reduced the cell wall of *Ulva*-1 (**Chapter 6)**. To understand the constant biomass loss, the degradation process of the NE-Atlantic *U. lacinulata* was followed (**Publication III)**. The degradation caused the removal of the cell wall and the release of protoplasts into the water that were able to regenerate and regrow. Protoplasts grew into three morphologies (cell masses, unattached discs, and unattached germlings), two of which very soon after regeneration and germination, released gametes into the water. A similar experiment was performed with wild material from *Ulva compressa*. This species became fertile and released swarmers, but a portion of the cells released were found to be protoplasts. These findings suggest that *Ulva* species have an unexplored asexual reproduction strategy. This is the first time that the natural formation of protoplast has been reported in *Ulva* species as a method of reproduction. This knowledge can help us understand *Ulva* species and how to deal with future degradation events that limit *Ulva* cultivation.

Zusammenfassung

Ulva ist eine weltweit in den Gezeitenzonen vorkommende Gattung von Makroalgen, welche für ihre biochemische Zusammensetzung (z.B. *ulvan*) bekannt ist und damit Eigenschaften aufweist, deren Erforschung für Bereiche wie Lebensmittel, Tierfutter, Kosmetik, Pharmazeutik und Bioplastik interessant sein könnten. *Ulva* Arten sind für ihre hohen Wachstumsraten, ihre Fähigkeiten zur biologischen Schadstoffeliminierung und ihre phänotypische Plastizität bekannt, wobei letztere auch mit schädlichen Algenblüten in Zusammenhang gebracht werden kann. Dennoch wird *Ulva* als geeigneter Kandidat für die Kultivierung in großem Maßstab vorgeschlagen. Die Aquakultur in Kreislaufanlagen (KLA) ist ein etabliertes Verfahren zur Zucht von Fischen, Krebstieren und anderen aquatischen Arten und kann als sowohl als Teil-Durchlaufsystem als auch als komplett geschlossene Einheit betrieben werden. Dieses erlaubt die Zucht nichtheimischer Arten in landbasierten komplett-geschlossenen KLA, da die Gefahr, dass diese Arten in das heimische Ökosystem eingebracht werden, ausbleibt. Landbasierte KLA sind mit hohen Betriebs- und Investment-Kosten verbunden. Außerdem ist die Kultivierung von *Ulva* noch immer von diversen Engpässen geprägt, die einen profitablen Hochskalierungsprozess beschränken (z.B. Variationen der biochemischen Zusammensetzung, unterschiedliche Anpassungen oder Reaktionen auf Umwelteinflüsse).
Das Ziel dieser Dissertation war es, *Ulva* Stämme so zu selektieren, dass sie sich für eine spätere KLA-Zucht mit künstlichem Meerwasser eignen und diese wiederum für eine geplante Nutzung als Hauptbestandteil in Lebensmittelverpackungen genutzt werden können. Vier *Ulva* Stämme aus zwei Herkunftsorten (*Ulva flexuosa* und *Ulva lacinulata* [Mittelmeer], sowie *U. lacinulata* und *Ulva linza* [Nordostatlantik] wurden Tests bezüglich ihrer Robustheit und dem Wachstumsverlauf bei hohen Temperaturen sowie geringen Salzgehalten unterzogen. Um die Bedeutung der Stammselektion für die Kultivierung von *Ulva* zu prüfen wurden ökophysiologische Experimente durchgeführt. Die relativen Wachstumsraten der *Ulva*-Arten und Stämme wurden unter verschiedenen Salzgehalten (Veröffentlichung I) und Temperaturen untersucht (Kapitel VI). Hierbei wurden Stämme mit relativen Wachstumsraten unter einem Wert von 7 % Tag-1 als nicht ideal beurteilt. *Ulva linza* sowie die beiden betrachteten *U. lacinulata* Stämme zeigten erhöhte Wachstumsraten in dem untersuchten Temperaturspektren. In den Salinitätsexperimenten (Veröffentlichung I) wurden bei adulten *U. lacinulata*-Stämmen selbst bei Salzgehalten unter 12 PSU relative Wachstumsraten oberhalb der Grenze von 7 % Tag-1 ermittelt. Aus diesem Grund wurden *U. lacinulata*-Stämme, inbesondere der aus dem Nordostatlantik bezogene Stamm, als vielversprechende Kandidat für die vegetative KLA bei geringer Salinität identifiziert. Um die gleichen Salinitätsbehandlungen als potentielle Methode zur Stammoptimierung zu testen, wurde die antioxidative Aktivität der nordostatlantischen *U. lacinulata* über zehn Tage gemessen. Für die antioxidative Aktivität wurde hierbei eine Tendenz des Anstiegs im höchsten sowie niedrigsten Salzgehalt (10 bzw. 30 PSU) festgestellt, was die Vermutung erlaubt, dass die Qualität der Biomasse durch eine Herabsenkung des Salzgehalts kurz vor der Ernte erhöht werden kann. Es zeigte sich, dass nicht alle der untersuchten Arten und Stämme für das genutzte Verfahren geeignet sind, was die Bedeutung der Stammesselektion im Vorwege der Kultivierung hervorhebt. Geringe Salzgehalte bei der Zucht von *U. lacinulata* könnten hier erheblich zur Senkung der Kosten bei gleichzeitiger Erhöhung der Biomassequalität beitragen. Qualitativ hochwertige Biomasse kann somit zusätzlich die Profitabilität der Zucht steigern und höhere Verkaufspreise rechtfertigen. Die antioxidative Aktivität wurde hierbei als eine entscheidende Eigenschaft der Stämme bewertet, da hohe Antioxidanzgehalte die

Haltbarkeit der Lebensmittel erhöht. Zusätzlich wurde der Einfluss der Bestrahlungsstärke auf die antioxidative Aktivität der zwei *U. lacinulata*-Stämme untersucht (Veröffentlichung II). Beide Stämme wuchsen über fünf Tage bei gesättigter Strahlungsdichte und sowohl ihre antioxidative Aktivität als auch die Effizienz der Photosynthese wurde bewertet. Hierbei zeigten beide Stämme ihre Fähigkeit zur Anpassung an eine gesättigte Strahlungsdichte, wobei sich aber nur für den Stamm aus dem Nordostatlantik ein signifikanter Anstieg der antioxidativen Aktivität feststellen ließ. Eine starke Erhöhung der Lichtbestrahlung kann demnach die Qualität der Biomasse steigern. Zugleich zeigten die fehlenden Unterschiede in der antioxidativen Aktivität des Mittelmeer-Stamms zwischen der Kontrollgruppe und der Behandlung mit gesättigter Strahlungsdichte, dass die Strahlungsdosis (welche zwischen den Behandlungen gleichbleibend war), eine entscheidende Rolle zur Feststellung der antioxidativen Aktivität eines Stammes spielen kann. Die unterschiedlichen Ergebnisse zwischen den Stämmen sind ein weiterer Hinweis auf die Wichtigkeit der Selektion und Optimierung von Stämmen.
Der nordostatlantische *Ulva lacinulata*-Stamm zeigte eine häufig stattfindende spontane Degradation ohne erkennbare Anzeichen von potentieller Fertilität. Versuche, die Reproduktion zu induzieren, blieben erfolglos. Für einen ersten Ansatz, diese Limitierung zu umgehen, wurde eine erfolgreiche Isolierungsmethode für Protoplasten entwickelt, die auf den in der Literatur beschriebenen Methoden basierte. Dazu wurden zwei ökophysiologische Vorbehandlungen (Lichteinstrahlung und Salzgehalt) getestet, die der Protoplastenisolation vorangestellt wurden, um die Kosten dieser Isolationsmethode zu senken. Allein die Vorbehandlung mit 40 PSU zeigte dabei einen Abbau der Zellwände von *Ulva*-1 (Kapitel VI). Um den konstanten Biomasseverlust ohne zugehörige Reproduktion nachzuvollziehen, wurde der Degradationsprozess des nordostatlantischen Stammes von *U. lacinulata* untersucht (Veröffentlichung III). Die Degradation war der Grund für die Auflösung der Zellwände und die Entlassung der Protoplasten in das Wasser, welche in der Lage waren, sich zu regenerieren und nachzuwachsen. Die Protoplasten wuchsen in drei verschiedenen Morphologien (Zellmassen, unbefestigte Scheiben und freie Gameten), von denen zwei zügig nach Regeneration und Keimung weitere Gameten im Wasser freisetzten. Ein ähnliches Experiment wurde mit dem Wildtyp-Material von *Ulva compressa* durchgeführt. Diese Art wurde fertil und entließ Schwärmer, wobei sich ein Teil der Zellen als Protoplasten identifizieren ließen. Diese Funde lassen vermuten, dass die Arten der Gattung *Ulva* über eine bisher nicht bekannte asexuelle Fortpflanzungsstrategie verfügen. Erstmalig wurde die natürliche Freisetzung von Protoplasten als Methode der Fortpflanzung bei *Ulva* Arten dokumentiert. Dieses Wissen kann für das weitere Verständnis der *Ulva*-Spezies und den Umgang mit weiteren Degradationsereignissen genutzt werden.

Abbreviations

α (alpha)	Photosynthetic efficiency – slope of the light curve
AA	Antioxidant activity
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AGMPFs	Algal growth and morphogenesis promoting factors
ANOVA	Analysis of variance
ASW	Artificial seawater
Bio-PE	Bio-based polyethylene
CFW	Calcofluor white
Chl a	Chlorophyll a
CO_2	Carbon dioxide
DLI	Daylight integral
DMS	dimethyl sulfide
DMSP	Dimethylsulfoniopropionate
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DW	Dry weight
ETR	Electron transport rate
FRAP	Ferric Reducing Ability of Plasma
Fv/Fm	Optimum quantum yield
FW	Fresh weight
GeO_2	Germanium dioxide
HAB	Harmful algal blooms
HL	High light
I_k	Saturation irradiance of photosynthesis
IMTA	Integrated multitrophic aquaculture
LL	Low light
MACR	Macroalgal cultivation rigs
N (statistics)	Number of replicates per treatment
NSW	Natural seawater
PAM	Pulse-amplitude modulation
PERMANOVA	Permutational multivariate analysis of variance
PES	Provasoli enriched seawater
pH	Negative decimal logarithm of the hydronium ion activity in a solution
PHBV	Poly(3-hydroxybutanoic acid- co -3-hydroxyvaleric acid)
PSU	Practical salinity unit
PUFA	Polyunsaturated fatty acid
RAS	Recirculating aquaculture system
rETR	Relative electron transport rate
RGR	Relative growth rate
ROS	Reactive oxygen species
SE	Standard error
SI	Sporulation Inhibitor
STSM	Short-term scientific mission
UV	Ultraviolet (radiation)

[All the photographs presented in this work were taken by the author unless stated otherwise]

1. Introduction

1.1. Seaweed production around the world

With the increase in population (UN, 2022), and the cumulative impacts of fossil fuels, sustainable alternatives are required to give answers to the population's needs (e.g., food demand) and to combat pollution levels, that increase CO_2 emissions, and consequently increase the planet's temperature (OECD, 2022, n.d.; Statista, 2023a, 2023b). The direct and indirect impact of non-renewable sources and unsustainable production methods are visible nowadays throughout the world and have raised the debate about climate change (Dhakal et al., 2022). The introduction of new concepts such as the Circulating economy or the ocean-focused Blue economy (Bari, 2017; Rizos et al., 2017; Lee et al., 2020; Joniver et al., 2021; Ngo et al., 2022), and the inclusion of new and sustainable practices to produce food, pharmaceuticals, cosmetics, and plastic substitutes can help delay the negative impacts of non-renewable fossil-based products and climate change. For this reason, seaweeds are seen nowadays as potential resources and sustainable alternatives to fossil products and can reduce the stress from intensive farming required to feed the world's population (Behera et al., 2022; Jagtap & Meena, 2022; Buck & Shpigel, 2023; Maar et al., 2023; El-Gendy et al., 2023).

With sustainability and ecological concerns in mind, in Europe, there has been an increase in companies, level of investment, and research projects for the exploration of seaweeds (European Commission, 2022, 2023a, b; FutureBridge, 2023; Hermans, 2023). All of these projects fall under the Blue biotechnology umbrella, as an emerging sector of the Blue economy (The Ocean Foundation, n.d.), essential to create sustainable ways to use the ocean.

The global macroalgae industry has been on the rise since the 1950's (Cai & Galli, 2021). Even though seaweed was found to be part of ancient European diets (Cai & Galli, 2021; Buckley et al., 2023), nowadays the consumption of macroalgae occurs mostly in Asian countries (Déléris, 2016), responsible for 99.1 % of farmed and harvested macroalgae (Behera et al., 2022). However, other countries around the world are increasing their interest from eating to cultivating macroalgae as well (Cai & Galli, 2021), and the demand for macroalgae-based products is increasing in Europe (Lähteenmäki-Uutela et al., 2021). Macroalgae are known for their bioactivity and properties such as their antioxidant activity (Tretiak et al., 2021; Michalak et al., 2022), anti-viral (Ahmadi et al., 2015; Lomartire & Gonçalves, 2022b), antibacterial (Pérez et al., 2016; Cardoso et al., 2019), and anti-cancer properties (Shao et al., 2013; Moussavou et al., 2014), with potential applications in the pharmaceutical and medical sectors (Mo'o et al., 2020;

Lomartire & Gonçalves, 2022a). They can potentially provide a healthy source of food for the growing population (Peñalver et al., 2020; de Sousa et al., 2023) and be used as feed (Michalak & Mahrose, 2020). Moreover, they can act as bioremediators, and be used for carbon fixation (Thomas et al., 2021; Filbee-Dexter et al., 2023), therefore being interesting to be explored as climate change mitigators. Lastly, the development of the macroalgae industries provides opportunities for research and sustainable farming that can have a positive impact, especially in developing countries, by reducing poverty and helping with ecosystem management (Sievanen et al., 2005; Mantri et al., 2017; Behera et al., 2022).

Despite the visible potential and increasing interest, the production of seaweeds in top-producing (and long-time producing) countries such as Indonesia, Tanzania, Malaysia, and the Philippines is currently in decline (Hermans, 2023). The reasons are the fluctuations in price (Valderrama et al., 2015), the increase in genetically diverse cultivated crops (Loureiro et al., 2015), and climate change (Kim et al., 2017). In areas where seaweed cultivation occurs at the coastline seaweed production and quality are dependent on the quality of the water which can change based on environmental conditions and seasonality (Behera et al., 2022; Langford et al., 2022). Climate change has an impact on the water quality and is one of the reasons for the reduced production of seaweed in Tanzania (Ndawala et al., 2021). The decline of established seaweed farming in these countries, at the same moment when the seaweed industry is rising globally, shows the importance of finding solutions to mitigate the impacts that climate change has or can have on nearshore and onshore seaweed cultivation facilities (Amri & Arifin, 2016; Largo et al., 2017). It is important to develop technologies that can optimize seaweed cultivation to be profitable, sustainable, and resilient to variations in the environment, and that can reduce the pressure on coastal waters.

There are several ways to cultivate seaweed which depend on the species to be cultivated or the aim of cultivation (e.g., polysaccharide extraction or feed production). Large-scale mariculture is done near-shore and uses methods such as seabeds, ropes, lines, or nets to which the seaweeds are attached and can grow until harvesting time (Titlyanov & Titlyanova, 2010; Hendri et al., 2018; Rathour et al., 2021). The main limitation of this method is the fact that it is labor intensive and dependent on the environment (which can lead to fluctuations in the seaweed quality and occurrence of pathogens; Hendri et al., 2018; Gutow et al., 2020; Olsson et al., 2020a, b; Steinhagen et al., 2022a). In intensive cultivation, seaweeds are cultivated in natural or artificial tanks, ponds, or raceways, and

the cultivation conditions can be evaluated and adjusted depending on how the system was built (Neori et al., 1998; Rathour et al., 2021; Revilla-Lovano et al., 2021). Such intensive cultivation systems can be developed in tanks on land, or they can use small water bodies (e.g., lakes or ponds). The light can be natural or artificial, and temperature and salinity can be adjusted (Rathour et al., 2021). The nutrients used can be the effluents of animal aquaculture (e.g., fish or abalone) or added fertilizers (Schuenhoff et al., 2003; Robertson-Andersson et al., 2008; Nobre et al., 2009; Mata et al., 2016; Rathour et al., 2021; Schmitz & Kraft, 2021; Qiu et al., 2022). To avoid pathogens, bacterial inhibitors and UV-pumps can be used to keep the water clean (Bychkova et al., 2020; Rathour et al., 2021; Revilla-Lovano et al., 2021; Angelo et al., 2022; Schol, 2022;).

Offshore cultivation of seaweed is also possible (Buck et al., 2017; Grote & Buck, 2017; Buck & Grote, 2018; Steinhagen et al., 2021; Jagtap & Meena, 2022). This method allows for the cultivation of seaweed at increased depths because of the larger photic layer (Buck & Buchholz, 2005), but can also produce them horizontally over a larger area allowing them to grow (Buck et al., 2018). Because of the currents, the dilution of wastewater with high levels of chemicals and pathogens reduces the probability of disease in the organisms being cultivated (Buck et al., 2005; Pogoda et al., 2012; Buck et al., 2018), something that it is still a problem in near-shore cultivations (Buck et al., 2005). Additionally, the cultivation of macroalgae in offshore areas can be used to remove inorganic compounds from the waters and reduce their negative impact on the environment whilst transforming them into valuable products (Buck et al., 2018; Califano et al., 2020).

The two most advanced seaweed cultivation techniques are Integrated Multitrophic Aquaculture (IMTA) systems and offshore Macroalgal cultivation rigs (MACR; Bak et al., 2018; Jagtap & Meena, 2022). IMTA integrates the cultivation of several organisms at once, becoming a sustainable way of multi-trophic farming, which can include seaweed (Schuenhoff et al., 2003; Robertson-Andersson et al., 2008; Nobre et al., 2009; Jagtap & Meena, 2022; Qiu et al., 2022). This type of system can be implemented in different locations, from land to offshore (Neori et al., 2004; Cahill et al., 2010; van Khoi & Fotedar, 2011; Buck et al., 2018; Buck & Grote, 2018; Oca et al., 2019; Jagtap & Meena, 2022) and in fresh or seawater areas (Neori et al., 2004). While the IMTA system is used for the cultivation of several organisms, the MACR method is aimed at the cultivation of macroalgae specifically (Bak et al., 2018). This method allows for the vertical cultivation of different macroalgae species between harvesting periods,

following the multiple partial harvesting method. This non-destructive harvesting method ensures the regrowth of the macroalgae, therefore reducing the costs of cultivation, reducing the need for re-seeding, and increasing the profitability of the system (Jagtap & Meena, 2022). The cultivation is done in exposed or deep-water locations and can withstand exposed conditions typical of offshore areas (Buck, 2002; Buck et al., 2006; Bak et al., 2018). Both methods reduce the pressure on coastal waters, by bringing the cultivation either in-land or offshore (Buck et al., 2018; Jagtap & Meena, 2022). Land-based cultivation, associated with IMTA systems (Hurtado, 2022) has shown its potential during the cultivation of *Chondrus crispus* Stackhouse, in Canada (Acadian Seaplus, 2024), *Ulva pertusa* Kjellman in Israel (Seakura, n.d.) and Ulva spp. South Africa (Bolton et al., 2006, 2008) and *Ulva* sp. and *Gracilaria* sp. in Israel (SeaOr Marine Enterprise; Neori et al., 2004; Chopin et al., 2008).

The most cultivated macroalgae nowadays are *Kappaphycus/Euchema* spp., *Gracilaria* spp., *Porphyra* spp., from the phylum Rhodophyta, and *Laminaria/Saccharina* (Kelp) and *Undaria* spp. from the class Phaeohphycea. The phylum Chlorophyta comprises only 0.05 % of all seaweeds cultivated in 2019 (Cai et al., 2021). The five most cultivated green seaweeds, as reported by Cai et al. (2021), were *Caulerpa* spp., *Monostroma nitidum* Wittrock, *Ulva prolifera* O. F. Müller, *Capsosiphon fulvescens* (C.Agardh) Setchell & N.L.Gardner, and *Codium fragile* (Suringar) Hariot. But for its particular exploration in Europe, *Ulva* species have been considered the most suitable macroalgae to produce (Buck & Shpiegel, 2023).

1.2. Introduction to *Ulva*

1.2.1. Morphology and Taxonomy

The *Ulva* Genus consists of a widespread group of green macroalgae, from the phylum Chlorophyta, characterized by multicellular macroscopical individuals. Macroscopic features associated with *Ulva* species are: the shape (foliose or tubular), colour (from dark green to light green), texture of the thallus, presence or absence of perforations and dentations, the size of the holdfast (as a disc-like structure) and the presence and size of the stipe (Woolcott & King, 1993; Maggs et al., 2007; **Fig. 1.1**). Microscopically, the cell's shape and size (polygonal to rounded, at the surface view, with more than 10 μm), as well as their organization in the thallus (in rows or irregular) are also diagnostic features of the different *Ulva* species (Woolcott & King, 1993; Maggs et al., 2007; Mantri et al., 2020). Inside the cell, the arrangement of chloroplasts (one single

chloroplast per cell), and distribution of the pyrenoids (1 to 15 per cell) are used as well to distinguish *Ulva* species (Woolcott & King, 1993; Maggs et al., 2007). Species of this genus begin their development as a single cell that can divide symmetrically in a disc, or grow as a row of cells, branching to create a strong rhizoid system (Wichar, 2015). At such early stages of development, *Ulva* germlings present similarities between those from species that will mature into distromatic blade forms and those that will mature to become monostromatic long tubular structures.

Ulva spp. are often associated with one of the two morphologies (blade-like/foliose morphology or a tubular morphology, with a hollow center separating the two cell layers (Hayden et al., 2003; **Fig. 1.1**). Moreover, tubular forms of *Ulva* can grow as a single long tube or develop branches (Hayden et al., 2003). Both morphologies can be found usually attached to solid substrates by rhizoid structures but a free-floating strategy is also known in *Ulva* (Malta et al., 1999; Shimada et al., 2003; Wan et al., 2017). However, *Ulva's* species morphology has been considered unstable (Tan, 1999; Blomster et al., 2002; Hofmann et al., 2010; Steinhagen, 2018), with the same species presenting the two common morphotypes or different species presenting the same morphotype. This morphological plasticity is influenced by factors such as salinity (Reed & Russell, 1978; Tan et al., 1999; Hofmann et al., 2010; Steinhagen et al., 2019c), nutrient concentration (Blomster et al., 2002; Steinhagen, 2018), and presence of certain bacteria (Spoerner et al., 2012; Wichard, 2015).

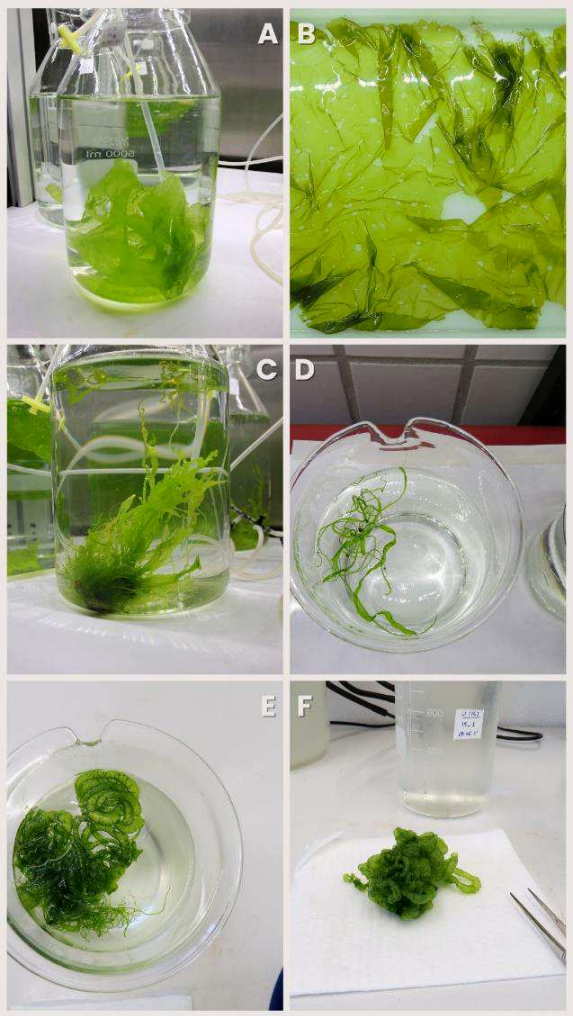

Figure 1.1 – *Ulva* species used in this thesis and their morphologies. A and B: *Ulva lacinulata*, as an example of the foliose morphology with a distromatic blade; C and D: *Ulva linza*, as an example of a monostromatic tubular morphology; E and F: *Ulva flexuosa*, as an example of a monostromatic inflated tubular morphology.

A known community integrated by *Ulva* and two bacteria (*Roseovarius* sp. and

Maribacter sp.) has been used to study the interactions between host and microbiome. The morphogen released by *Maribacter*, named *thallusin*, was found responsible for inducing cell differentiation, the formation of the rhizoid, and the development of the cell wall in *Ulva compressa* Linnaeus (Wichard, 2015; Alsufyani et al., 2020). *Ulva*'s microbiome enables the adaptation of *Ulva* species to environmental stresses and releases algal growth and morphogenesis promoting factors (AGMPFs) that are essential for the *Ulva*'s healthy growth and correct development (Ghaderiardakani et al., 2020). But *Ulva*, as the host, can release photosynthates (nutrients) and bacterial attractants (e.g., dimethylsulfoniopropionate, DMSP) to the water to attract bacteria, therefore having an active role in the arrangement of the bacterial community surrounding itself. This allows *Ulva* to make changes in the environment based on its preferences and needs (Kessler et al., 2017, 2018; Califano et al., 2020).

Ulva has a high tendency for mutagenesis (Wichard, 2015; Wichard et al., 2015) and a single mutation can change the morphology of the species (Løvlie, 1968). For this reason, (while under its previous name, *Ulva mutabilis* Föyn; Wichard et al., 2015; Steinhagen et al., 2019a) has become a model organism to study morphogenesis (Wichard et al., 2015) but also, the bacteria-macroalgal interactions and microbiome-dependent responses to changes in the environment (Spoerner et al., 2012; Wichard, 2015; Wichard et al., 2015; Ghaderiardakani et al., 2020). Such interactions will be explored further in the section **Geographic distribution and plasticity**.

For the reasons detailed above, correct identification of different *Ulva* species based on morphology can be difficult and cause taxonomic complications (Kirkendale et al., 2012). Molecular identification techniques through DNA barcoding, and particularly the use of the *tuf*A marker gene (Saunders & Kucera, 2010) lead to the detection of wrongful identifications within the *Ulva* genus. These techniques also lead to the consolidation between *Ulva* and *Enteromorpha* genus (Hayden et al., 2003). Historically, the two genera had been taxonomically separated based on their morphology. From the *Enteromorpha* genus, species presented tubular forms while the *Ulva* genus was characterized only for the blade forms. However, as predicted by Linnaeus (1753) and later proved by Hayden et al. (2003) through molecular identification, both genera are phylogenetically similar and were combined into the genus *Ulva*. Nowadays, several species of *Ulva* have been synonymized, as is the case of *Ulva fasciata* Delile (currently *Ulva lactuca* Linnaeus; Hughey et al., 2019) and *Ulva rigida* C. Agardh (currently *Ulva lacinulata* (Kützing) Wittrock; Hughey et al., 2021). Hughey et al. (2019), separated *U.*

lactuca from *Ulva fenestrata* Postels & Ruprecht based on their geographical occurrence. *U. lactuca* can be found in warmer regions of the globe while *U. fenestrata* is present in colder areas, in the northern hemisphere (Hughey et al., 2019). Despite the importance of DNA barcoding for the correct identification of *Ulva* species, the combination between molecular and morphological methods still needs to improve as several DNA sequences deposited in GenBank are still associated with the incorrect species names (Kirkendale et al., 2012; Hughey, 2022). Currently, there are more than 550 species names, 186 are regarded as synonyms, 66 with a pending taxonomic verification, 74 with an uncertain taxonomic status, and 103 are taxonomically accepted species (Tran et al., 2022; Steinhagen et al., 2023; Guiry & Guiry, 2024).

1.2.2. Reproductive biology

Ulva species can reproduce sexually or asexually and have an isomorphic haplodiplontic life cycle, separated between a sporophytic (2n) and a gametophytic (n) generation (**Fig. 1.2**; Wichard et al., 2015; Balar & Mantri, 2020). The gametophytes (n) can be separated into "-" and "+" and release motile "mt-" and "mt+" gametes (n) that can either fuse together (syngamy) to form a zygote and consequently a new sporophyte (2n) or grow parthenogenically into a haploid gametophyte (n; see ** in **Fig. 1.2**). Often, the gametes developing parthenogenically can go through spontaneous diploidization and become homozygotic sporophytes (2n; see * in **Fig. 1.2**) (Hoxmark, 1975; Wichard et al., 2015, Balar & Mantri, 2020). Sporophytes release zoospores (n) "mt-/+" that can develop into gametophytes (n) or can also develop parthenocarpically into a new sporophyte (2n; see *** in **Fig. 1.2**; Wichard et al., 2015; Balar & Mantri, 2020). Because the life cycle is isomorphic, only small morphological and behavioral differences can be found between the different generations. Gametes are slightly smaller than zoospores (Balar & Mantri, 2020), only have two flagella, and are positively phototactic (swim towards the light; Kuwano et al., 2012) while zoospores are larger, have four flagella, and are negatively phototactic (swim against the light; Hiraoka et al., 2003; Ma et al., 2009; Mantri et al., 2011). Sporophytes and gametophytes occur at the same time and can be found together in the wild showing different stages of vegetative or reproductive development. The change between generations can occur within one season and the ratio gametophyte:sporophyte varies non-proportionally (Phillips, 1990). Haploid and diploid germlings are similar in morphology (**Fig. 1.2**). Gametes and zoospores have a spindle shape and are only motile for some time (minimum of 6 h; Jones & Babb, 1968; Kuwano

et al., 2012), after which they settle, lose their flagella and become round. The cells start to elongate and divide into either a single tube that will differentiate into the rhizoid and thalli (Wichard, 2023) or multiple tubes with a radial distribution (Kim et al., 2021). The process of reproduction and germling development is explained in **Fig. 1.3** based on the author's observations while working with different *Ulva* strains.

Because of the complexity of the *Ulva*'s life cycle, crucial research has been done to successfully determine its underlying processes. The first species having their life cycle studied in detail under laboratory conditions were *U. lactuca* and *U. compressa* (Wichard et al., 2015; Hughey et al., 2019). Though, more recently, a total of 14 species were studied for their life cycles' characteristics such as type and size of zoospore, type of life history (sexual or asexual), and phototactic behavior (Balar & Mantri, 2020).

Several abiotic factors such as temperature, light, and desiccation influence reproduction in *Ulva* species, often when combined (Brawley & Johnsons, 1992; Lüning et al., 2008; Balar & Mantri, 2020). However, the impact of each factor depends on the species, and can cause different responses (Brawley & Johnsons, 1992; Lüning et al., 2008, Balar &

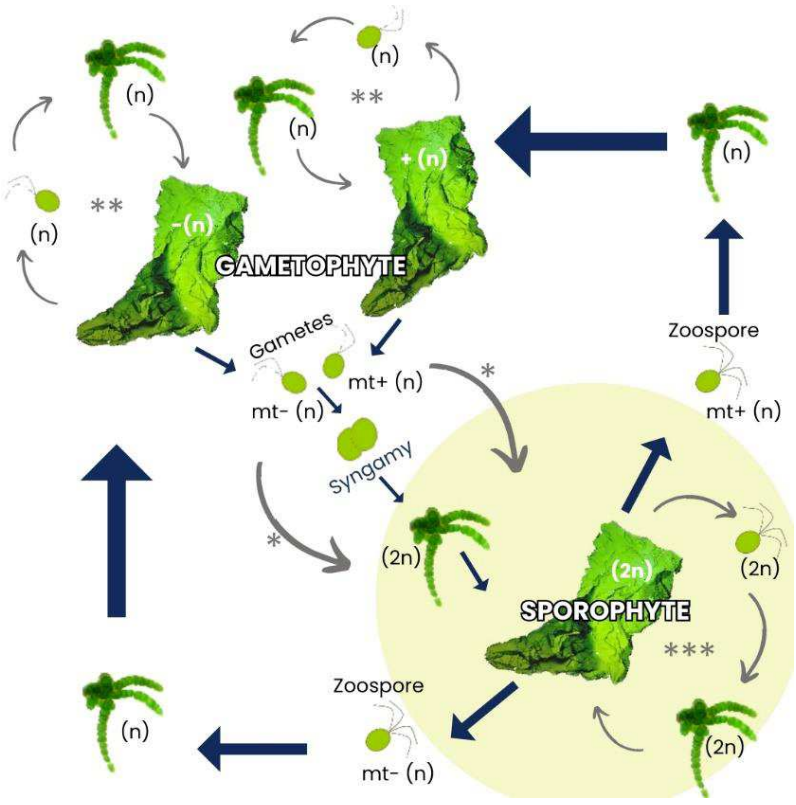

Figure 1.2 – *Ulva* sp. life cycle. The life cycle is divided primarily between a gametophytic (n) and a sporophytic (2n) generation. The complete sporophytic generation is underlined by the yellow circle at the bottom right. Zoospores are represented with four flagella, gametes are represented with two flagella. The blue squared arrows follow the typical life cycle with the sexual reproduction process and the change between generations. The grey rounded arrows follow asexual reproduction processes. Legend: *: spontaneous diploidization of individual gametes; **: parthenogenesis; ***: parthenocarpy. [Graphical design created in Canva (Canva, n.d.)].

Mantri, 2020). Therefore, these influencing factors can induce or inhibit reproduction, enabling control over the life-cycle. Rybak & Gabka (2018) found that the same abiotic factors that regulate growth also induce the release of swarmers (gametes or zoospores), connecting growth with maturation (Singh et al., 2011; Vesty et al., 2015; Wichard et al., 2015).

Other factors such as thallus fragmentation, the age of the thallus, the presence of specific microorganisms, or the presence and amount of sporulation and swarmer inhibitors (related to the age of the thalli) also play a role during the reproduction process (Hiraoka & Enomoto, 1998; Singh et al., 2011; Carl et al., 2014b; Vesty et al., 2015; Wichard et al., 2015; Balar & Mantri, 2020). Sporulation inhibitors (SI-1 and SI-2) are directly associated with reproduction, as the concentration of those inhibitors directly determines the occurrence of sporulation and swarmer release (Stratmann et al., 1996). As the name of these molecules suggests, they inhibit sporulation from occurring, thus guaranteeing the vegetative growth of the thallus and controlling cell differentiation. However, it is necessary to keep the concentration of the two inhibitors above a minimum inhibitory concentration to avoid *Ulva* becoming fertile.

In other words, both inhibitors have to be below the concentration threshold for the thallus to become fertile and release swarmers (Stratmann et al., 1996; Wichard & Oertel, 2010). Once the synthesis of the sporulation inhibitor SI-1, in the cell wall, stops or any changes to the environment reduce its concentration, the thalli will start to differentiate, and sporogenesis/gametogenesis begins (Stratmann et al., 1996). But, the determination of which cells undergo this process depends on sporulation inhibitor SI-2, also known as the "swarming inhibitor" (Wichard & Oertel, 2010). SI-1 starts being produced at an earlier stage of the germling development and increases in concentration until maturity, while the concentration levels of SI-2 stay similar throughout the development of the thallus. Only in areas where the level of SI-2 is below its minimum inhibitory concentration (e.g., between the cell layers), will the cells be induced to form swarmers (Stratmann et al., 1996). This explains why one of the most common induction techniques requires the fragmentation of the thallus, which causes the release of SI-2 from the damaged areas into the water (Wichard & Oertel, 2010). The biological cycles of *Ulva* vary between species and are influenced by several factors (e.g., abiotic conditions). Therefore, a clear timeline of *Ulva*'s reproduction cycle is difficult to elaborate. However, under natural circumstances, different *Ulva* species can become fertile after weeks or even months since their germination (Huang et al., 2023). Under

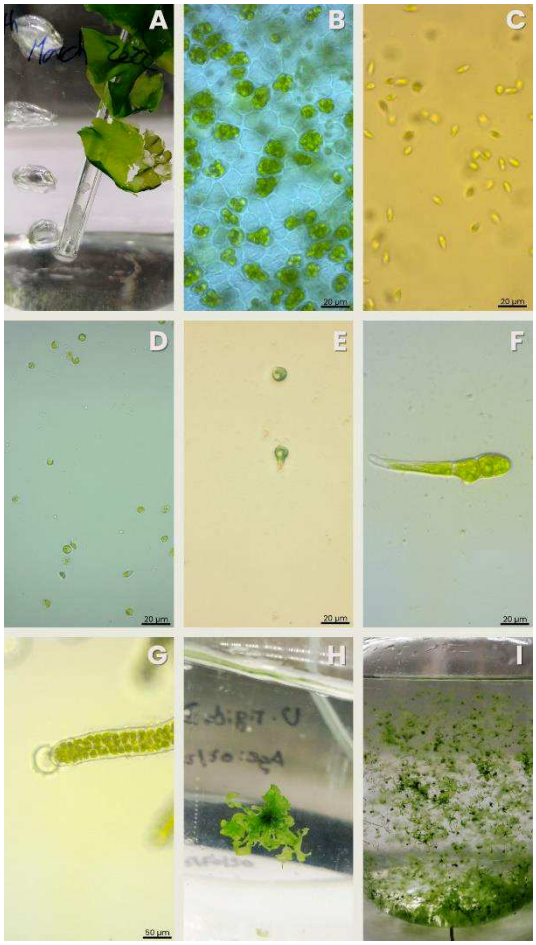

Figure 1.3 – Sexual reproduction and stages of development in *Ulva*-3 A: fertile piece of *Ulva* (brown/pale green area) with a foliose morphology; B: microscopic observation of swarmers (gametes or zoospores) inside a fertile piece of the thallus, before being released into the water; C: Swarmers after being released into the water with the characteristic spindle shape. D: Germinative cells - swarmers after settling at the bottom of the Petri dish. The flagella is lost and the cell shape becomes circular. E: Elongation of the first cell; F: Elongation and cell division; G: germling's cell structure two and a half weeks after germination; H: Macroscopic branched germling; I: Scale-up of multi-tubular germlings into a 10 L bottle, one month after germination. The pictures were taken from different fertile *Ulva* specimens under lab conditions over 8 months.

semi-natural conditions *Ulva* can become fertile at the age of five weeks (Stratmann et al., 1996). Under laboratory conditions, following common methods of induction of reproduction, a minimum 3-day waiting period is needed until swarmers are released (Hiraoka et al., 2003; Wichard & Oertel, 2010; Kuwano et al., 2012; Steinhagen et al., 2021). For cultivation purposes, reproduction can be avoided if *Ulva* is grown in mixed-age cultures, thus guaranteeing the production of SI by the younger thalli, keeping the levels above the inhibitory threshold (Obolski et al., 2022).

1.2.3. Geographic distribution and plasticity

Species of *Ulva* are distributed worldwide and in a variety of environments, being present from the tropics to the polar regions (Carl et al., 2014a, b; Wichard et al., 2015; Mantri et al., 2020; Roleda et al., 2021; Abo-Raya et al., 2023). They can be found in the intertidal, in marine and freshwater environments (salinities between < 0.5 and 49 PSU) and in different bodies of water (e.g., sea, river, lake; Hofmann et al., 2010; Rybak, 2018; Rybak & Gabka, 2018; Mantri et al., 2020; Rybak, 2021; Hu et al., 2022; van der Loos et al., 2022). At present, only *Ulva shanxiensis* Chen, J. Feng & S. L. Xie, in China (Chen et al., 2015; Xie et al., 2020), and *Ulva pilifera* (Kützing) Škaloud & Leliaert, in Poland (Rybak, 2021), have been reported in freshwater (< 0.5 PSU) ecosystems (Mareš et al., 2011; Rybak et al., 2014; Rybak, 2018).

Ulva's presence around the globe is an indicator of its capacity to adapt to important environmental factors, such as temperature, salinity and light. For this reason, the geographical distribution, seasonality, and daily variations in the environment can have an impact on *Ulva*'s physiological and biochemical properties. The same species in different geographical locations can report different morphologies, levels of compounds, or growth rates (Fort et al., 2019; Steinhagen et al., 2019b; Fort et al., 2020b; Olsson et al., 2020a; Moreira et al., 2021; Jansen et al., 2022).

Considering *Ulva*'s growth rates, Fortes & Lüning (1980) determined that the optimal growth for *U. lactuca* from Helgoland (nowadays named *U. fenestrata*; Hughey et al., 2019) Germany was achieved between the temperatures of 10 and 15 ˚C. However, Enright (1979) reported the optimal temperature for the same species in Canada to be 20 ˚C. For *U. pertusa* in Japan, a different species in a different location, the optimal growth was found to be between 20 and 25 ˚C (Ohno, 1977). Regarding salinity, tubular species have a higher tolerance for lower salinity compared to foliose species (Rybak, 2018). However, the inverse observations were found for *U. compressa*. This species, known to present the two morphologies (Wichard et al., 2015), depending on the presence/absence of its microbiome (Wichard, 2015; Wichard et al., 2015) was found to show a foliose morphology under low salinity (Hofmann et al., 2010; Steinhagen, 2018, Steinhagen et al., 2019c) and a tubular morphology under higher salinity conditions (Steinhagen et al., 2019c). However, in Steinhagen et al. (2019c) both morphologies were found at higher salinities, with the foliose morphology being spread over a wider range of salinities than the tubular form (only found in areas with salinities above 17 PSU). Additionally, in the work from Steinhagen et al. (2019c) it was found that the foliose specimens were almost exclusively unattached (except for one collection site). Thus, suggesting that other factors, such as attachment to a substrate versus free-floating, can play a role in the morphology of *Ulva*.

In general, under laboratory conditions, *Ulva* presents suitable growth rates under light intensities of up to 70 µmol photons m^{-2} s^{-1} and under a day length of up to 16 h, at which point the growth becomes saturated (Fortes & Lüning, 1980; Mantri et al., 2020). However, while a clear picture of the *Ulva* genus' preferences facilitates research and its cultivation, inter- and intra-specific qualities should be considered when working with *Ulva* (Fort et al., 2019; Fort et al., 2020a). Different species and strains have different responses to changes in the environment and to stressful conditions thus representing variations in their growth and biochemical composition when under different conditions

(e.g., accumulation of sucrose, free amino acids and antioxidants; Edwards et al., 1987; Lee & Chen, 1998; Shiu & Lee, 2005; Lu et al., 2006). While such plasticity can be seen as a positive characteristic in *Ulva* (discussed further in **Cultivation methods**), there are cases where this trait can have a negative impact on the environment (e.g. green tide events).

Furthermore, *Ulva*'s holobiont should be considered when referring to *Ulva*'s adaptations to different environments or to variations in the environment. It was found that only when both the host (*Ulva*) and its bacteria, together, show a stress response to environmental changes, morphogens, and other chemicals will be released into the water. Thus, making host-bacteria relations important for *Ulva*'s acclimation to environmental changes (Dittami et al., 2015; Ghaderiardakani et al., 2020). The importance of *Ulva*'s microbiome has been highlighted in the work with *U. compressa* under low temperatures (2˚C). While axenic gametes from *U. compressa* do not grow under such low temperatures, it was found that non-axenic gametes were able to grow and present a normal morphotype (Ghaderiardakani et al., 2020). This capacity of *U. compressa* to adapt to such an environment has been attributed to the presence of psychrophilic bacteria that release AGMPFs under extreme cold conditions (Ghaderiardakani et al., 2020). Additionally, *thallusin,* a molecule produced by bacteria associated with *Ulva*, has been described as a scavenger of metal ions, increasing *Ulva*'s bioremediation potential (Alsufyani et al., 2020; Ghaderiardakani et al., 2020; for more information about bacterial interactions with *Ulva* see **Morphology and taxonomy**).

The qualities that make *Ulva* such an interesting genus to explore are the same qualities responsible for green tide events (e.g., high growth rates, bioremediation properties, and plasticity). Green tide events are commonly associated with seasonality, agricultural runoff, pollution, and the fast accumulation of biomass (van Alstyne et al., 2015; Bermejo et al., 2022). Some *Ulva* species such as *U. prolifera* (Song et al., 2022), *Ulva linza* Linnaeus (Leliaert et al., 2009), *Ulva intestinalis* Linnaeus (Kim et al., 2021), *U. lacinulata* (as *Ulva rigida*; Gao et al., 2017a; Hughey et al., 2022; Bermejo et al., 2023), *U. compressa* (Bermejo et al., 2023), and *Ulva flexuosa* Wulfen (Rybak & Gabka, 2018) have been reported to cause economic and ecological damages in several places around the world (e.g., China, South Korea, Japan, the USA, Morocco and France; Nelson et al., 2003, 2008; Charlier et al., 2008; Leliaert et al., 2009; Yabe et al., 2009; Kim et al., 2021; Rahhou et al., 2023).

There is not one single cause for the occurrence of green tides, instead, their occurrence is associated with the combination of several factors. Such factors can be virtually divided into different types: abiotic, anthropogenic, and biological. Abiotic factors such as temperature, light, salinity, pH, CO_2, nutrients and water currents are known to play a role in the formation of green tides as they can facilitate access to nutrients and the growth of *Ulva* spp. (Bao et al., 2015; Rybak & Gabka, 2018; Wang et al., 2021; Bermejo et al., 2023). Anthropogenic factors, often associated with densely populated areas are agriculture runoff, overall pollution (both of which can cause eutrophication of the system; Valiela et al., 1997; Ye et al., 2011), and the presence of aquaculture facilities (e.g., *Pyropia* sp. aquaculture in rafts in the Yellow Sea; Liu et al., 2009, 2010; Han et al., 2020). Biological factors can be subdivided into the category of the overall marine organism's response to the environmental changes (caused by abiotic and anthropogenic factors) and into the characteristics that *Ulva* species possess to cause the green tide. As examples of the former, the composition and size of seaweed population depend on environmental conditions, and some might give space for *Ulva* blooms to occur (Wang et al., 2021). That is the case of the observed reduction of *Sargassum* biomass that caused a decrease in competition for *U. prolifera* enabling its expansion (Song et al., 2022). Besides the overall plasticity, high growth rates, and bioremediation qualities of *Ulva*, other properties give species of this genus the possibility to bloom into green tides. The relationship between *Ulva* species can cause multispecific green tides, with one *Ulva* species facilitating the development of another (Largo et al., 2004; Yabe et al., 2009; Bermejo et al., 2023).

Another characteristic that enables *Ulva* species to cause green tides is the possibility of free-floating specimens which seem to be determinant for the occurrence of these tides (Bermejo et al., 2019). These specimens have better access to light, and a high surface-to-volume ratio that allows for rapid nutrient uptake (making them good competitors) and, therefore, higher growth rates (Valiela et al., 1997; Yabe et al., 2009). Moreover, *Ulva* propagules and somatic cells were found over the sediment during winter, when the conditions for growth were not ideal. The amount of settled propagules determines the number of *Ulva* individuals and the size of the bloom during the following seasons, as they can be brought back to the surface by vertical water movements and re-grow once the conditions improve (Kamermans et al., 1998; Gao et al., 2010; Zhang et al., 2011; Smetacek & Zingone, 2013; Gao et al., 2017b; Rybak & Gabka, 2018). Despite green tides seemingly being caused by a combination of the events described above,

each situation is different. Yabe et al. (2009) reported that two species of *Ulva* were responsible for a green tide (*Ulva ohnoi* Hiraoka & S. Shimada, during spring and summer and *U. pertusa*, in summer and autumn). But in this case, the blooms occurred without the eutrophication of the system and during the process of water quality remediation (Yabe et al., 2009). Therefore, green tides are complex and their complexity causes limitations in finding solutions to the problem.

The monitoring and prediction of algal blooms through satellite remote sensing, predictive modeling, or in situ monitoring can help determine the beginning of a bloom, but fail to predict accurately the end of a bloom or its duration, and adjustments to the methods are necessary (Ren et al., 2014; Davidson et al., 2016; Yu et al., 2017). During a bloom, control measures take the form of biological, physical, and chemical methods. Biological methods use other organisms such as seagrasses or seaweeds and their bacterial community to control other blooms by secreting algicidal compounds in the water (Alamsjah et al., 2006; Zerrifi et al., 2018; Inaba et al., 2019). *U. pertusa* and *U. fasciata*, have algicidal effects that can help control species that also cause harmful algal blooms (HAB; Alamsjah et al., 2006). Additionally, bacteria and archaea might be used as well against green tides. Variations were found within the bacterial and archaeal communities during a green tide caused by *U. prolifera*, suggesting that these microorganisms might have a vital role in controlling the green tide event, speeding up its decline phase by releasing algicidal substances to the water (Zhao et al., 2022). The manual or mechanical removal of the algal biomass from the sea and coastline, or the cleaning of fouling *Ulva* from ships (as a preventive method), are examples of physical methods applied as a control measure to blooms (Fletcher, 1996; Holm et al., 2003; Charlier et al., 2007; Filipkowska et al., 2008; Wan et al., 2017). Chemical methods consist of using either natural or synthetic chemicals (including isolated algicidal compounds), with a biocide or biosurfactant activity against the blooms (Belamari & Belamarič, 2010; Randhawa et al., 2012; Tang et al., 2021). Both the physical and the chemical approaches are more expensive than the biological ones and are less effective in coastal environments. The delayed removal of *Ulva*'s biomass from the shore, because it is a labor-intensive process, can lead to an increase in toxicity caused by the degradation of the biomass (Charlier et al., 2008). Governmental measures (e.g., legislation to reduce the release of pollutants to the water) and sensibilization of the population should also be considered as effective methods to prevent and mitigate the impacts of algal blooms. Governmental initiatives, such as the funding for green tide-

associated projects can increase the understanding of the mechanisms behind such events and the direct impacts society has on their occurrence and management (Bushaw-Newton & Sellner, 1999; Anderson et al., 2001; Ye et al., 2011) and new legislation can reduce the anthropogenic impacts in the ocean.

Bloom-forming algae can be explored in a circular economy strategy to be turned into commercially interesting products (Lourenço-Lopes et al., 2020; Joniver et al., 2021; Ngo et al., 2022). This concept has been applied to *Ulva* species by exploring their economic value by cultivating them in wastewater treatment where they work as water bioremediators and can later be used for animal feed (Asino et al., 2010), the production of biofuel (Allen et al., 2013) or bioplastics (Chiellini et al., 2008). *Ulva* presented similar biomethane yields and higher net annual primary productivity than terrestrial crops (Barbot et al., 2016; Chemodanov et al., 2017). However, the use of green tide *Ulva* biomass in different industries is limited as the quality of the biomass (e.g., presence of heavy metals; Haritonidis & Malea, 1999; J. Li et al., 2018; El-Mahrouk et al., 2023; Rahhou et al., 2023) and its yields varies. Blooms often include a mixture of several species that might require different methods of processing or cause variations in productivity (Pinon-Gimate et al., 2008; Prabhu et al., 2020). Moreover, the costs of biomass transportation from the area where the bloom occurred to the processing facilities, together with processing costs and variability of the profits can render the entire strategy too expensive. Nowadays, the least expensive and most viable strategy is the use of *Ulva* from a green tide area as fertilizer (Filipkowska et al., 2008).

It has been found that green tide strains of *Ulva* are genetically different from non-green tide strains and the former showed faster growth and presented higher levels of pigments and proteins when compared with the non-green tide strains (Fort et al., 2020a). On the one hand, this represents the danger of more intense and re-occurrent green tide events, as selective pressure in *Ulva* blooming areas can cause an increase in the green tide genotype (Fort et al., 2020a). On the other hand, this shows the potential that green tide strains of *Ulva* have for cultivation, considering their fast growth and higher quality. Nevertheless, working with such dangerous strains would require the existence of a closed system, (e.g., recirculating aquaculture system, RAS) that could guarantee the proper management of the water in the system and avoid the release of green-tide forming *Ulva* strains into the environment.

1.2.4. Biochemical composition and industry applications

The genus *Ulva* was reported by Cai et al. (2021) as one of the main green seaweed species cultivated in 2019 with its major production being used as feed to abalone. Nevertheless, several *Ulva* species have become a source of interest for their attractive characteristics that can be applied in different industries such as pharmaceutical (Mao et al., 2006; Olasehinde et al., 2019), cosmetics (Morelli et al., 2019), bioethanol production (Rinastiti et al., 2022; El-Gendy et al., 2023; Kolo et al., 2023), food (Rioux et al., 2017; Leyva-Porras et al., 2021) and feed (Laramore et al., 2018; Santizo-Taan et al., 2020; Martínez-Antequera et al., 2021; Addis et al., 2023). Additionally, *Ulva* species have also been explored as bioremediators of polluted areas and wastewaters (Ho, 1990; Baumann et al., 2009; Nielsen et al., 2012; Hlihor et al., 2017; El-Naggar et al., 2018).

Commonly known as "Sea lettuce" (usually associated with *U. lactuca*) and part of traditional Asian cuisine (Rioux et al., 2017), *Ulva* spp. are nowadays found as food in America and Europe as well. European countries have approved the use of seaweeds, including *Ulva*, after premarket safety assessments for food consumption (McHugh, 2003; Marfaing & Lerat, 2007; Pereira, 2016). Besides human consumption, *Ulva* species have been thought a good additive to the diets of fish (Natify et al., 2015; Valente et al., 2016; Zhu et al., 2016; Guerreiro et al., 2018; Martínez-Antequera et al., 2021), sea-urchins (Addis et al., 2023), abalone (Mulvaney et al., 2013; Bansemer et al., 2016; Ansary et al., 2019; Santizo-Taan et al., 2020) and shrimp (Pallaoro et al., 2016; Qiu et al., 2017; Laramore et al., 2018;).

Ulva species and strains vary in their biochemical composition and growth rates (Olsson et al., 2020b; Abu Ahmed et al., 2021; Moreira et al., 2021; Jansen et al., 2022; Abo-Raya et al., 2023). Nonetheless, *Ulva* is characterized by having a protein content ranging 9-14 % from which 39 % of the total protein content in dry weight (DW) is composed of essential amino acids (Wong & Cheung, 2001), representing higher levels of essential amino acids for humans than soy (Aguilera-Morales et al., 2005). *Ulva*'s low lipid content ranges between 0.3 and 3.5 % DW (7.87 % DW has been registered by Yaich et al. (2011)), with high levels of omega-3 and -6 (Wong & Cheung, 2001; Aguilera-Morales et al., 2005; Peña-Rodríguez et al., 2011; Yaich et al., 2011). Within the fatty acids group, 35.5 % of total fatty acids are polyunsaturated fatty acids that can be explored for their nutraceutical and health benefits. Relative compounds such as diacids, long-chain aliphatic alcohols, sterols and monoglycerides are also known for

their health and nutritional benefits (Santos et al., 2015). Additionally, *Ulva* spp. have high levels of carbohydrates that range between 30 and 66 % (Ortiz et al., 2006; Rasyid, 2017; Balar et al., 2019; Jönsson et al., 2023) of their dry weight. The carbohydrate group is mostly constituted of structural and dietary fibers and polysaccharides (Holdt & Kraan, 2011). Polysaccharides can be found in the cell wall or can be used as storage polysaccharides (Holdt & Kraan, 2011). The highly sulphated polysaccharide, *ulvan*, can be found in the cell wall and has been extensively explored as it is known for its therapeutical and nutraceutical applications (Brading et al., 1954; Wijesekara et al., 2011; Cunha & Grenha, 2016; Barakat et al., 2022). *Ulvan* contribution to the total biomass (DW) can range between 9 and 36 % (Lahaye & Robic, 2007).

Ulva's bioactive peptides have shown their potential use as anti-obesity, anti-diabetic, antihypertensive molecules and can further be used to induce satiety, relaxation and help modulate the immune system (Hayes & Tiwari, 2015). Lectins were found to be useful in detecting viruses, bacteria, fungi, and parasites that can cause infections. Lectins also have bioactive characteristics such as antibiotic, anti-inflammatory, anti-adhesion, and anti-HIV (Bird et al., 1993; Hori et al., 2000; Schaeffer & Krylov 2000).

Despite the lipid content in *Ulva* species being low in comparison to the proteins and carbohydrates, the fatty acids have been studied for their potential as human food (Moreira et al., 2021), animal feed (El-Gendy et al., 2023), health treatments (Moreira et al., 2021; Ramkumar & Jayavel, 2022) and to produce biofuels (Moustafa & Batran, 2014; El-Sheekh et al., 2021; El-Gendy et al., 2023).

Ulvan, can be used as a dietary fiber, containing a water-soluble fraction and an insoluble fraction (Hayes & Tiwari, 2015; Barakat et al., 2022) and can form a gel with thermoreversible behaviour (Lahaye et al., 1998). *Ulvan* properties make it a biodegradable hydrocolloid, which can be used in some industry sectors as bioplastics, biofilms, cosmetic emulsions, or as a substitute for gelatins and food gelling agents (Ganesan et al., 2018; Guidara et al., 2019; Morelli et al., 2019; Sulastri et al., 2021; Don et al., 2022). *Ulvan*s' oligosaccharides and the presence of sulphate groups have shown antioxidant (Olasehinde et al., 2019; Barakat et al., 2022), anticancer (Kaeffer et al., 1999), antiviral (Ivanova et al., 1994), anti-thrombotic and anticoagulant activity (Mao et al., 2006) and can work as immunomodulators. As a modulator of the immune system, *ulvan* can control the effects of inflammation. Moreover, *ulvan* and its derivatives can reduce the absorption of cholesterol, having a hypocholesterolemic and

hypolipidemic effect (Smit, 2004; W. Li et al., 2018) making them interesting compounds to be explored for pharmaceutical and medicine purposes.

The *Ulva*'s antioxidant properties are associated with the presence of molecules such as chlorophyll *a* and *c*, carotenoids, vitamins, flavonoids, and phenolic compounds (El-Baky et al., 2009; Yildiz et al., 2012; Wulanjati et al., 2020; Pappou et al., 2022). These compounds confer to the macroalgae the capacity to respond to oxidative stress and excessive production of ROS (Reactive Oxygen Species) that are harmful to the organism (Sies, 1997; Bischof & Rautenberger, 2012; Halliwell & Gutteridge, 2015). Therefore, under stressful situations such as extreme variations of irradiance, salinity, temperature, or desiccation throughout the day, antioxidants help balance the negative impacts of ROS by scavenging these molecules and reducing the oxidative stress (Kranner & Birtic, 2005; Kakinuma et al., 2005; Luo & Liu, 2011; Hanelt & Figueroa, 2012; Bischof & Rautenberger, 2012; see **Publication I** and **II**). Because *Ulva* is an intertidal species, antioxidants protect the macroalgae when environmental conditions change daily, following the tides (Cruces et al., 2019; Eisman et al., 2020), but also allow *Ulva* to adjust to seasonal variations (Prasedya et al., 2019; Steinhagen et al., 2022b). Moreover, it has been found that sporogenesis is partially regulated by an antioxidant signal that is involved with spore formation (Jiang et al., 2023).

Several methods can be used for the extraction and measure of antioxidants as these compounds have different affinities to the methods of extraction and the methods of antioxidant quantification. The most common extracts are obtained via alcoholic or aqueous methods and measured through common methods such as 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, Ferric Reducing Ability of Plasma (FRAP) assay or 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation decolourization assay. While an overview of the total antioxidant concentrations in *Ulva* can be obtained by evaluating the results of the different extracts and methods, the different affinities between antioxidants allow for a more specific extraction of particular antioxidants of interest (e.g., carotenoids; Eisman et al., 2020; Pappou et al., 2022). The potential use of *Ulva* as a whole in different industry sectors (e.g., food) was previously mentioned, but its antioxidant extracts can also be incorporated in different industries such as pharmaceutical and medicine (Mezghani et al., 2013), cosmetics (Mo'o et al., 2020; Leyva-Porras et al., 2021), feed (Martínez-Antequera et al., 2021) or food (e.g., increasing the shelf-life of food products; El-Baky et al., 2009; El Fayoumy et al., 2022; Gomaa et al., 2022).

Nowadays, it is possible to find several companies that use extracted compounds from seaweed, to produce a wide range of products (**Table 1.1;** Chiellini et al., 2008), where biodegradable plastic substitutes are a clear tendency. Research is still ongoing to scale-up production and make seaweed-based products easily accessible to the average customer. Several European-funded projects have been developed based on the potential of *Ulva* species and their importance (e.g., OLAMUR, ULTFARMS, SensAlg, CirkAlg, Genialg, Seamark, BIOSEA, AquaVitae). Some recent examples of projects focusing on *Ulva* are the Mak-Pak and the Mak-Pak Scale-Up (AWI 2023), the *Ulva* Farm project (Nordic Seafarm n.d.) and the SeaWheat COST Action (SeaWheat, n.d.). The SeaWheat COST Action (CA20106), is a project that joins together several researchers and small and medium-size enterprises (SMEs) from several different countries inside and outside of Europe. The aim is to make *Ulva* "a model for innovative mariculture" and "tomorrow's «wheat of the sea»". The *Ulva* Farm project aims to develop offshore cultivation of *Ulva* in Europe. The Mak-Pak and the Mak-Pak Scale-up used *Ulva* to produce biodegradable seaweed-based food packaging (Lomartire et al., 2022; AWI, 2023) and explored how to use the complete biomass instead of using extract compounds (e.g., *ulvan*), thus benefiting from *Ulva*'s original structure and composition (e.g., antioxidants that increase food' shelf-life). Additionally, the Mak-Pak Scale-Up project aimed to scale up the different production steps, from the cultivation of *Ulva* to the production of food packaging. The research in this project associated with *Ulva* cultivation is presented in this dissertation, which aimed to apply the results from strain selection and optimization of *Ulva* strains in a large-scale land-based recirculating aquaculture system (more details in **Objectives**).

1.2.5. Cultivation methods

Ulva cultivation occurs mostly limited to nearshore and onshore areas. In these areas, cultivation can be done in indoor or outdoor facilities (Buchholz et al., 2012; Sebök et al., 2019; Califano et al., 2020) and can be associated with the aquaculture of other organisms in systems such as the IMTA. Semi-closed onshore facilities such as raceways and flowthrough systems ease the process of seaweed cultivation as they allow for the adjustment of some cultivation conditions (Ed-Idoko, 2021). The right adjustments can represent the increase in efficiency of the system, by reducing costs and/or guaranteeing the quality of the seaweed (Zertuche-González et al., 2021). To this day, the method recognized as the most profitable in terms of achieving higher yields of biomass is the

Table 1.1 – List of exemplary companies producing seaweed-based bioplastics and other products around the world. The groups/species of macroalgae were determined based on the information and images provided on the companies' websites.

Company's name	Country	Groups/species of macroalgae	Products	Use of the biomass (complete/extraction)	Website
Algopack	France	*Sargassum* sp. and *Laminaria* sp.	100 % rigid seaweed packaging; Pellets of plastic and algae blend; Mugs	Unclear	https://www.algopack.com/
Biopac	Indonesia	Unclear	Bags; sachets; Hang tags; Ink	Extraction	https://biopac.id/
Biotic	Israel	Unclear	Biodegradable PHBV polymers	Extraction	https://www.biotic-labs.com/
B'zeos	Norway	Brown macroalgae	Packaging	Extraction	https://www.bzeos.com/
Carbonwave	United States of America, Mexico and Puerto Rico	*Sargassum* sp.	Fertilizer; Cosmetic emulsifier; Fabrics (e.g., leathers); Bioplastic	Extraction	https://carbonwave.com/
Eranova	France	Green macroalgae	Resins that can be used for the development of bio packaging	Extraction	https://eranovabioplastics.com/
Evoware	Indonesia	Unclear	Ello Jello Cup; Seaweed-based packaging	Extraction	https://rethink-plastic.com/home/
FlexSea	United Kingdom	Red macroalgae	Packaging for dry food; Cardboard packaging windows; Refill pouches for cosmetics; Protect textiles (e.g., substitutes of Polybags)	Extraction	https://flex-sea.com/
IJO	Indonesia	Brown and Red macroalgae	Bioplastic; Straws	Extraction	https://ijonesia.com/
Kelpi	United Kingdom	Brown macroalgae	Bioplastic packaging; Packaging coating	Extraction	https://www.kelpi.net/
Kelpy	Australia	Brown and Green macroalgae	Biopackaging	Extraction	https://www.kelpy.co/
Loliware	United States of America	Brown and Red macroalgae	Straws	Extraction	https://www.loliware.com/

Table 1.1 (*cont.*)

Company's name	Country	Groups/species of macroalgae	Products	Use of the biomass (complete/extraction)	Website
Neuro Pack	South Korea	Unclear	Trays; Bio-PE film	Extraction	https://neuro-pack.com/
Noriware	Switzerland	Red macroalgae	Biofilm for packaging	Extraction	https://www.noriware.com/
Notpla	United Kingdom	Brown macroalgae	Food containers; Laundry sachets; Zero waste paper; Gel pods; Dry food sachets; Food Oil Pipette; Bath Oil Sachets; Rigid Cutlery; Rigid Packaging	Extraction	https://www.notpla.com/
PlantSea	United Kingdom	Unclear	Water soluble biofilm; Packaging	Extraction	https://www.plantsea.co.uk/
Searo	United Kingdom	Brown or Red macroalgae	Seaweed-based natural polymer	Extraction	https://searo.co/
Sway	United States of America	Red and Brown macroalgae	Seaweed resin; Biofilm; Polybags; Package windows; Customized packaging	Extraction	https://swaythefuture.com/
Uluu	Australia	*Gracilaria* sp.	Rigid products (e.g., packaging, electronics, furniture and car interiors); Fabrics (as an alternative to polyester textiles)	Extraction	https://www.uluu.com.au/
Zerocircle	India	Brown macroalgae	Coating; Paper; Pellets	Extraction	https://www.zerocircle.in/

onshore cultivation associated with IMTA (Bolton et al., 2008; Zertuche-González et al., 2021). Nevertheless, the strong dependency on seawater limits the cultivation of *Ulva* (and any other seaweed) to onshore or nearshore areas, as the facilities require constant new and clean seawater. Moreover, the development of these systems is associated with high costs of construction and maintenance (Huguenin, 1976; Lüning & Pang, 2003; Titlyanov & Titlyanova, 2010, Ladner et al., 2018). For this reason, other ways for *Ulva* cultivation are being explored, such as offshore cultivation (Buck & Buchholz, 2005; Grote & Buck, 2017; Buck et al., 2018; Steinhagen et al., 2021; Maar et al., 2023; Zollmann et al., 2023).

One other known method already established for the aquaculture of fish and shrimp (van Khoi & Fotedar, 2011; Qiu et al., 2022) is the RAS, a closed land-based system that does not require a flow-through system, therefore reducing its dependency on seawater (Malone, 2013; Ed-Idoko, 2021). This eco-friendly system uses mechanical and biological filters to treat the water, reducing effluents that can be released into the environment (Ed-Idoko, 2021). This way, the water recirculates in the system instead of being constantly exchanged. This method reduces water consumption, enables the precise control of diseases, and opens the possibilities for the construction of this system in areas where it was not possible before (Ed-Idoko, 2021).

One other step that can be added to the cultivation in a system like RAS, is the use of artificial seawater (ASW) instead of natural seawater (NSW). NSW can vary in its properties depending on when and where it is collected, which adds a degree of uncertainty and risk to the system (e.g., the presence of toxins and heavy metals). These variations can cause a reduction in productivity and quality of the biomass, therefore reducing the profitability of the system (Losordo et al., 2004; Zhang et al., 2017, 2020; Ed-Idoko, 2021). Using ASW can remove the need for access to NSW and guarantee the quality of the water in the system (Allen & Nelson, 1910).

Seaweeds, including *Ulva*, have only been cultivated in RAS systems when used as biofilters to remove nutrients and keep the water from the tanks of the fish, prawns, shrimp, and abalone clean (Deviller et al., 2004; Cahill et al., 2010; van Khoi & Fotedar, 2011; Bambaranda et al., 2019). However, despite the positive results from such experiments and the clear potential of the RAS for cultivation purposes, to this day, there has never been a RAS system developed with the main purpose of cultivating *Ulva* species, except two Photobioreactors tested in Germany. A summary of the known cultivation systems tested for *Ulva* is shown in **Table 1.2**.

Table 1.2 – Compilation of the published aquaculture systems working with *Ulva* species in RAS or similar systems. Works were divided by the type of cultivation system (monoculture or IMTA), water flow (the technique used for moving the water around the system, with a special focus on RAS), species of *Ulva* cultivated, and water type (artificial or natural). The overview column consists of a short explanation of the system, providing the names of the species cultivated in the same system as *Ulva*. Systems with a minimum number of 2 different species were considered IMTA. Only systems working with more than 20 L of water per tank were considered for this work. Flow-through systems were deliberately excluded. Legend: a - species taxonomically debated, currently accepted as *U. fenestrata* (Hughey et al. 2019); b - includes species at the time identified as belonging to the genus Enteromorpha.

Cultivation system	Water flow	Species	Water type	Overview	Location	Reference
Monoculture	**RAS/Closed Photobioreactor**	*U. intestinalis*	Artificial	Water and salinity levels were adjusted once a month.	Thuringia, Germany	Schmitz & Kraft, 2021
		U. lactuca[a]		Ring-shaped culture vessels. Flow pumps were used to move the seaweed around the vessel. No replacement of culture medium during the experiment (7 days).	Germany	Sebök et al., 2019
	Semi-closed RAS	*U. ohnoi*	Natural	Seawater quality was maintained by mechanical filters and UV radiation. ~80% of the total pond volume was discarded once.	Baja California, Mexico	Revilla-Lovano et al., 2021
IMTA	**RAS + Flow-through**	*U. lactuca*[a]	Natural	Only 25% of recirculating water was used. 75 % of water was originated from the flowthrough system with Abalone (*Haliotis midae* Linnaeus).	Irvine and Johnson (I & J), Cape Cultured Abalone Pty, Ltd farm, South Africa	Nobre et al., 2009, 2022; Robertson-Andersson et al., 2008
				10 % exchange of seawater in the Atlantic salmon (*Salmo salar* Linnaeus) tanks. Water flows from fish tanks to the seawater tanks and back to the fish tanks.	Tasmania, Australia	Qiu et al., 2022
	Semi-closed RAS	*U. lactuca*[a]	Natural	Fresh water enters abalone (*H. midae*) pond, effluent from abalone pond flows to the gilthead sea bream (*Sparus aurata* Linnaeus) pond. Effluent from fish pond flows to biofilter shallow ponds with *Ulva*.	Unclear	Schuenhoff et al., 2003

Table 1.2 (*cont.*)

Cultivation system	Water flow	Species	Water type	Overview	Location	Reference
IMTA	**Semi-closed RAS**	*U. ohnoi*	Natural	Water from the seaweed tanks flows by gravity to a water reservoir (sump), water is pumped back to the tanks after being filtered. Wastewater from fish (*Lates calcarifer* Bloch) was daily drained to the sump of the seaweed system to add nutrients and compensate for water losses (e.g., evaporation).	Townsville, Australia	Mata et al., 2016
	RAS	*U. lactuca*	Unclear	Water pumped from the waste collection tank to the seaweed tank and then to the Prawns (*Penaeus latisulcatus* Kishinouye, 1896) tank, going back to the waste collection tank.	Bentley, Australia	van Khoi & Fotedar, 2011
				Water pumped from the abalone (*Haliotis iris* Gmelin) culture tank to the filtration tank with *Ulva*.	Dunedin, New Zealand	Cahill et al., 2010
		U. ohnoi		Effluent water from the fish (*Solea senegalensis* Kaup) tank flows to a buffer tank (with nitrifying biofilter), most of the water is sent back to the fish tank. A portion of the water is sent to the seaweed tank. The water from the seaweed tank is sent back to the buffer tank.	Castelldefels, Spain	Oca et al., 2019
		Ulva sp [b]		*Ulva* cultivated in a High-Rate Algae Pond (HRAP) used as a biofilter for the water from the sea bass (*Dicentrarchus labrax* Linnaeus) cultivated in RAS. Water adjustments were made twice a week in the fish ponds.	France	Deviller et al., 2004
				Water flowed from the seaweed tanks to the sea bass (*D. labrax*) tanks and was pumped back to the seaweed tanks. Once a week water was renewed by half.	Athens, Greece	Chatzoglou et al., 2020
			Natural	Water flowed from the *S. aurata* tanks directly to the seaweed tanks, then through filters, several pumps and skimmer, and then was pumped back to the fish tanks. Water was exchanged in the fish tanks when ammonia levels exceeded 1.0 mg L^{-1}.	Olhão, Portugal	Schol, 2022; Angelo et al., 2022

1.2.6. Cultivation bottlenecks and seeding techniques

Independent of the method used for *Ulva* cultivation, several key moments during production need to be achieved to guarantee the success of the system (e.g., high growth rates, consistent seedling material, and control over reproduction; Carl et al., 2014a, b; Gao, 2016; Steinhagen et al., 2022a, b). While limitations in the cultivation can vary depending on the specificity of each situation, some are known throughout the *Ulva* community and are considered major bottlenecks even when cultivating on different scales (e.g., laboratory or large scale). One of the known bottlenecks is the spontaneous loss of biomass, which can cause drastic changes in production and reduce the profitability of a system (Ryther et al., 1984; Bolton et al., 2008). While there is no clear definition of what "spontaneous loss of biomass" means, this event is usually associated with *Ulva* reproduction as *Ulva* dies after releasing spores and gametes (Ryther et al., 1984; Bolton et al., 2008; Obolski et al., 2022). Another bottleneck is guaranteeing the seeding material for constant production of *Ulva* throughout the year. To this day, two methods have been used to obtain seeding material. The first depends on the control over the life cycle and is usually associated with a nursery/hatchery system (Carl et al., 2014a; Steinhagen et al., 2021), while the second depends on vegetative fragments of *Ulva* that can be used as propagules to initiate cultivation (Gupta et al., 2018).

When dependent on the *Ulva*'s life cycle, one must have it completely under control. Otherwise, if *Ulva* becomes fertile during cultivation, the biomass is lost. This means, knowing when and how *Ulva* becomes fertile, knowing how to inhibit reproduction when it's not required or induce it in an optimized way to get as many seedlings as possible (Bolton et al., 2008; Carl et al., 2014b; Gao et al., 2017a, b; Balar et al., 2020; Steinhagen et al., 2022a, b). However, as mentioned before, *Ulva*'s life cycle is complex and varies from species to species, making it difficult to control (See **Reproductive biology)**.

By constantly growing *Ulva* vegetatively, while there is no need to control the life cycle, there is a need to keep one-quarter of the entire harvested material behind to use as seedling material for the next cultivation period (Radulovich et al., 2015). This means that there is a large amount of biomass being produced that cannot be sold or processed further. Moreover, constantly growing the same *Ulva* vegetatively can lead to a reduction in genetic variability, reducing the adaptability of the seaweed to the environment and making it more susceptible to diseases, causing the reduction of the seaweed's quality over time (Liu et al., 2012). Another option would be to restock the tanks with wild

biomass and use it for seeding (Bolton et al., 2008). This represents a dependency on the coast, where the process of re-stocking is limited to the occurrence of *Ulva* in the wild, which can vary seasonally.

A new technique has been explored to overcome the limitations of the methods presented prior. This new technique depends on the isolation of protoplasts from the *Ulva* thalli. Protoplasts are cells from which the cell wall was removed and that can regenerate into new individuals afterward. This technique has been developed and used multiple times with higher plants (e.g., *Arabidopsis thaliana* (L.) Heynh.; Dovzhenko et al., 2003; Davey et al., 2005, Sangra et al., 2019) but it has also been explored for microalgae (Echeverri et al., 2019) and seaweeds (Reddy et al., 1989; Fujita & Saito, 1990; Dipakkore et al., 2005; Gupta et al., 2018; Avila-Peltroche et al., 2019). While there are other ways to produce protoplasts (e.g., mechanical removal of the cell wall; Fjeld, 1972) the most used technique requires the use of enzymes that degrade the cell wall (Dipakkore et al., 2005; Gupta et al., 2018; Reddy & Seth, 2018; Avila-Peltroche et al., 2019).

This technique requires less biomass than the vegetative seeding method and does not need control over the life cycle as it only requires healthy pieces of thalli (Gupta et al., 2018). Several attempts at the isolation of protoplasts from *Ulva* can be found in the literature with its associated success (Reddy et al., 1989; Gupta et al., 2018; Reddy & Seth, 2018). However, despite several successful attempts, there are still limitations that have not allowed this technique to overcome the seeding techniques used nowadays. The reasons are twofold: 1) the protocol is long, complicated, and not reproducible and 2) the costs of the laboratory equipment and enzyme solutions required are too high to make this technique profitable in large-scale cultivations (Reddy et al., 2006; Gupta et al., 2018).

1.3. Objetives

As shown in this work, the interest in *Ulva* keeps increasing and the research for new ways for its cultivation increases as well. Facts such as *Ulva*'s plasticity, bioremediation potential (Mhatre et al., 2019), and the occurrence of fast-growing strains (e.g., green tide forming *Ulva*) suggest that *Ulva* is a good candidate to test in a RAS. However, there are several limitations to its cultivation and scale-up. The isomorphic and interchangeable morphology and difficult taxonomy make it difficult to distinguish between *Ulva* species. The plasticity of the genus causes wide variations of interesting biochemical compounds, limiting the control over biomass quality, while the reproduction cycle can limit the biomass quantity. Therefore, the strain selection and optimization of *Ulva* is a beneficial step to take before cultivation as it can increase the profitability of the system (Jagtap & Meena, 2022). The present dissertation has the goal of selecting and optimizing species and strains of *Ulva* for cultivation in a land-based recirculating aquaculture system with artificial seawater. The following **Fig. 1.4** summarizes the work presented in this dissertation, separated by the three main steps taken to achieve successful cultivation.

1.4. Research questions

Based on the background provided previously, three research questions were established to contribute to the goals of this dissertation. These questions were developed to include the entire cultivation process, from the seedling to the harvesting moment, and guarantee the understanding and possible optimization of those steps together with the optimization of the *Ulva* strains.

*Research question regarding **Strain Selection (Chapter 3)***

Can abiotic factors such as salinity be used as a tool for strain selection and optimization of *Ulva*?

To address this question, three species (four strains) of *Ulva* from the NE-Atlantic and Mediterranean (*U. lacinulata* – two geographical strains, *U. linza* and *U. flexuosa*) were cultivated at salinities ranging from 10 to 30 PSU for three weeks. The fresh weight and area size of the samples allowed for the determination of the Relative Growth Rates (RGR) of each species in each treatment. The experiment was performed in two moments of the seaweeds' development, as germlings and as adults. From the data

collected, optimal salinity conditions were determined for each species at each moment of their development. New samples of NE-Atlantic strains were then cultivated for 10 days under four salinity conditions to evaluate the influence of salinity on the variation of antioxidant activity (using the ABTS radical decolourization assay).

Hypothesis:

- Different species of *Ulva* will have different salinity preferences and achieve higher relative growth rates at the preferred salinity conditions, due to the known plasticity of the *Ulva* species.
- Salinity preference can vary at different stages of development (e.g., germlings or adults).
- Salinity can be used as a stressor and increase antioxidant activity in *Ulva*, therefore optimizing the quality of the biomass.

*Research question regarding **Strain Optimization (Chapter 4)***
Can the biomass quality of *Ulva* be optimized (increase antioxidant activity) before harvesting by increasing light irradiance?

To answer this question, one experiment was performed. The experiment compared the antioxidant activity (AA) of the two strains of *U. lacinulata* (NE-Atlantic and Mediterranean) after being cultivated under saturated irradiance (185 µmol photons m^{-2} s^{-1}) for five days while the light dose was kept the same as in the control group (light dose in both treatments: 4 µmol photons m^{-2} s^{-1}; Light irradiance in the control group: 70 µmol photons m^{-2} s^{-1}). Samples were collected at different times to evaluate the variation of AA (using the ABTS radical decolourisation assay). During this experiment, the photosynthetic performance (using a Pulse Amplitude Modulated fluorometer) was measured as well, to evaluate the photosynthetic differences between the two strains. The data collected during these experiments were crucial for the selection of a good candidate for further cultivation.

Hypothesis:

- The same optimization method can cause similar responses in two different strains of *U. lacinulata*, with both strains increasing their antioxidant activity.

- The saturating irradiance has an impact on the photosynthetic efficiency of the two strains.
- The photosynthetic efficiency is similar between the strains.

Research question regarding ***Overcoming cultivation bottlenecks (Chapter 5)***
How can *Ulva*'s degradation be controlled, and constant production guaranteed?

After determining which candidates to explore further in the land-based system, limitations arose that would limit their production. While presenting high growth rates, the NE-Atlantic *U. lacinulata* strain often went into spontaneous biomass loss. Moreover, this species never became fertile during the time it was cultivated under laboratory conditions, despite several attempts to induce reproduction. To understand these limitations and overcome them, an experiment was set up to follow the process of *Ulva* degradation in two different *Ulva* species, the NE-Atlantic *U. lacinulata*, the previously cultivated and selected strain for use in a large-scale facility and *U. compressa*, that had recently been collected from the wild. The selection of such different species was based on the microscopic observations of protoplast-like cells, occurring in both, during degradation or reproduction events. During such events, several *Ulva* pieces were collected, and their fresh weight was measured once a week for four weeks. The RGR's of the different pieces and biomass loss were calculated. The water, in which the pieces were being cultivated, was filtered once a week for four weeks. Both the seaweed samples and the water samples were observed under the optical and fluorescent microscope throughout the experiment and Calcofluor White (CWF) was used to determine the presence of protoplasts in both species. Protoplast yields were determined by counting the number of cells collected. Later, a known number of protoplasts was inoculated into several Petri dishes, and their regeneration was observed over five weeks. At the end of the five weeks, the number of new *Ulva* individuals in each Petri dish was counted and the protoplast regeneration rates were determined. With the observations and data collected during this work, it was possible to understand the process behind biomass loss and, for the first time, observe the natural occurrence of protoplasts in *Ulva* spp..

Hypothesis:

- Protoplasts occur naturally in *Ulva* species.

- Degradation is the cause of protoplast production.
- Protoplasts can be used as seeding material.

The work presented in this thesis was developed under the scope of the Mak-Pak Scale-Up Project. The strain selection experiments focused on the cultivation conditions available at the partner's land-based system (JoMaa-Spirulina, Germany) and optimization experiments focused on the qualities required for seaweed-based food packaging (developed by the Bremerhaven Hochschule, Germany, as a partner of this project; **Fig. 1.4**). This work will offer insight into how different abiotic factors (salinity and light irradiance) can be used for strain selection and optimization. Both *Ulva*'s natural development (germling and adult stage) and the cultivation process (seeding requirements and harvesting moment) were considered throughout this work to guarantee the feasibility of the main goals. Moreover, it will be shown how bottlenecks were faced and overcome, by following and analysing a recurring event of degradation, one of the major limitations in *Ulva* cultivation. The results of this work will be beneficial for the establishment of new and optimized ways of *Ulva* cultivation and for the understanding of underlying mechanisms that cause the spontaneous loss of biomass.

Figure 1.4 – Graphical representation of the aims of this work and the main key steps for *Ulva* cultivation: Strain selection, strain optimization, and overcoming bottlenecks for cultivation. The publications presented in this work (**Chapter III** and **Chapter IV**) compared *Ulva* species and strains from two geographical locations (North-East Atlantic and Mediterranean Sea) to select the ones that could be cultivated for the specific purpose and under the specific conditions required in the MAK-PAK Scale-Up project. [Graphical design created in Canva (Canva, n.d.)].

1.5. Publications and declaration of own contribution

Publication I (Chapter 3)

Isabel Cardoso, Anneke Meiβner, Anja Sawicki, Inka Bartsch, Klaus-Ulrich Valentin, Sophie Steinhagen Bela H. Buck, Laurie C. Hofmann (2023). **Salinity as a tool for strain selection in recirculating land-based production of *Ulva* spp. from germlings to adults.** *J Appl Phycol 35*, 1971–1986. https://doi.org/10.1007/s10811-023-02960-x

The experimental design was conceptualised by I. Cardoso, A. Meiβner, A. Sawicki and L. C. Hofmann. I. Cardoso drafted the manuscript and L. C. Hofmann supervised the studies. The studies were carried out by I. Cardoso, A. Meiβner, A. Sawicki who collected and analysed the data. S. Steinhagen conducted the molecular identification of the strains. L. C. Hofmann, I. Bartsch, K. Valentin, and S. Steinhagen provided technical and scientific supervision. B. H. Buck and I. Bartsch provided lab facilities and administrative support. B. H. Buck and L. C. Hofmann obtained funding for this project. The manuscript was critically revised by L. C. Hofmann, B. H. Buck, I. Bartsch, K. Valentin, and S. Steinhagen. All authors approved the manuscript for publication.

Personal contribution in % in the totality of the work (up to 100% for each category):

Conceptualisation and experimental design:	ca. 70 %
Experimental work and acquisition of the data:	ca. 60 %
Data analysis and interpretation:	ca. 90 %
Figures and tables:	ca. 100 %
Drafting the manuscript:	ca. 80 %

Publication II (Chapter 4)

Isabel Cardoso, Anneke Meiβner and Laurie C. Hofmann (2024). **The effect of irradiance versus light dose on the antioxidant activity of two strains of *Ulva lacinulata***. *Bot Mar*. https://doi.org/10.1515/bot-2023-0064

The experimental design was conceptualised by I. Cardoso, A. Meiβner, and L. C. Hofmann. I. Cardoso, A. Meiβner drafted the manuscript. The studied were supervised by L. C. Hofmann and I. Cardoso. A. Meiβner, collected and analysed the data. L. C. Hofmann and I. Cardoso provided technical and scientific supervision. L. C. Hofmann provided lab facilities and administrative support and obtained funding for this project. The manuscript was critically

revised by L. C. Hofmann. The authors accepted responsibility for the entire content of this manuscript and approved its submission.

Personal contribution in % in the totality of the work:

Conceptualisation and experimental design:	ca. 30 %
Experimental work and acquisition of the data:	ca. 10 %
Data analysis and interpretation:	ca. 80 %
Figures and tables:	ca. 100 %
Drafting the manuscript:	ca. 90 %

Publication III (Chapter 5) – in preparation for submission to New Phytologist

Isabel Cardoso, Inka Bartsch, Klaus-Ulrich Valentin, Sophie Steinhagen, Bela H. Buck, Laurie C. Hofmann (n.d.). **Naturally occurring protoplasts in Ulva spp. reveal a previously underestimated proliferation process in an ecologically and economically relevant seaweed.**

L. C. Hofmann and I. Cardoso conceptualised and designed the studies. I. Cardoso drafted the manuscript. L. C. Hofmann supervised the studies. I. Cardoso carried out the studies, collected and analysed the data. S. Steinhagen conducted the molecular identification of the strains used in this manuscript. L. C. Hofmann, B. H. Buck, I. Bartsch, S. Steinhagen and K. Valentin provided technical and scientific supervision. B. H. Buck and I. Bartsch provided lab facilities. B. H. Buck and L. C. Hofmann provided administrative support and obtained funding for this project. The manuscript was critically revised by L. C. Hofmann, B. H. Buck, I. Bartsch, K. Valentin, and S. Steinhagen. All authors approved the manuscript for publication.

Personal contribution in % in the totality of the work:

Conceptualisation and experimental design:	ca. 90 %
Experimental work and acquisition of the data:	ca. 90 %
Data analysis and interpretation:	ca. 90 %
Figures and tables:	ca. 90 %
Drafting the manuscript:	ca. 90 %

2. Methodology

The following sections outline all the experiments developed under the scope of this dissertation, to select and optimize strains of *Ulva* and improve culture conditions and seeding methods. Experiments that have been described in the publications associated with this work will be mentioned briefly to provide a complete overview of the entire three-year process. A detailed description of experimental approaches that have not been included in the publications will be provided.

2.1. Collection and cultivation of the biomass

Several *Ulva* species reported in this dissertation were analysed for different purposes. Most species have been molecularly identified by project partners from the University of Gothenburg, Sweden. The species that have been molecularly identified will be reported further based on the results of the molecular identification with the complete Latin name and following the current nomenclature (Hayden et al., 2003; Steinhagen et al., 2019a; Hughey et al., 2022). The species that have not been molecularly identified will be reported as "*Ulva*-1", "*Ulva*-2", and "*Ulva*-3". The species that hereafter will be referred to as *Ulva flexuosa* was molecularly identified as *Ulva flexuosa*/*californica* complex (Steinhagen et al., 2019b). The identification of this species is discussed further in **Publication I**.

The *Ulva* species used in this work were collected from different geographical areas in Europe. *Ulva lacinulata* (in this work referred to as NE-Atlantic *U. lacinulata*) and *Ulva linza* were collected in Óbidos Lagoon, Portugal in 2021. *U. flexuosa* and *U. lacinulata* (in this work referred to as Mediterranean *U. lacinulata*) were collected from Thessaloniki Bay, Greece, in 1986 and 2017, respectively. *Ulva*-1 and *Ulva*-2 were collected in Clonakilty estuary, Ireland in 2022. *Ulva*-3 was collected in Helgoland, Germany in 2022 and 2023 and *Ulva compressa* was collected in Dorum-Neufeld, Wurster North Sea coast, Germany in 2023 (**Table 9.1**, in **Appendix**). All the species, except the Greek ones, were collected to be used in this work's experiments, by the author or members of the Marine Aquaculture group at the Alfred Wegener Institute (AWI), Bremerhaven, Germany. The Greek species were collected by partners from the Fisheries Research Institute (Hellenic Agricultural Organization-DEMETER – ELGO-DEMETER) who kindly shared them, as unialgal cultures.

The Nagoya Protocol was followed to guarantee that each country's regulations were respected when *Ulva* species needed to be transported from the country of origin (Portugal and Greece) to AWI. To comply with the regulations from the Greek authorities, a report was submitted by the end of January 2024 detailing the findings regarding the two Greek *Ulva* species. Only the *Ulva* species collected in Ireland were strictly used in the University of Ireland, Galway, Ireland, during a Short-Term Scientific Mission (STSM) of three months, financed by the SeaWheat COST Action, CA20106, "SeaWheat *Ulva*: Tomorrow's 'Wheat Of The Sea', A Model For An

Innovative Mariculture" (SeaWheat, n.d.). Therefore, no legal measures needed to be taken into consideration.

Apart from the Greek species that were presented as unialgal cultures and had been under culture conditions for several years, all other *Ulva* species were thoroughly cleaned once they arrived in the laboratory, by rinsing them with natural seawater (NSW) abundantly to remove epiphytes and other debris.

All species used at AWI were placed under the following pre-established culture conditions suited for seaweed cultivation: 15 ˚C, ~ 30 PSU, ~ 60-90 µmol photons m^{-2} s^{-1}, 16:8 h light:dark photoperiod. During the first year (2021), cultivation was done in NSW. The water was filtered with a polypropylene water filter (mesh size of approx. 5 µm; EF-Filter, Netherlands) and pasteurized for 4 h at 99 °C with 100 % humidity (Palux, Germany). Afterwards, seaweeds started to be cultivated in artificial seawater (ASW) prepared by mixing approx. 360 g of salt Seequasal-Salz (Seequasal Salz Production and Trade GmbH, Germany) in 10 L of de-ionized water. The ASW was pasteurized under the previously mentioned conditions. Light intensity was adjusted several times during the work presented in this dissertation based on the results of experiments and to guarantee the appropriate cultivation conditions. Before each experiment, periods of acclimatization were provided, if necessary. During the first weeks of cultivation, seaweeds were provided with 0.5 mL L^{-1} of germanium dioxide (GeO_2) once a week, to eliminate contamination by diatoms (Shea & Chopin, 2007; Rautenberger, 2024). Once seaweeds grew and became dominant in the cultivation vessels, GeO_2 stopped being provided.

The macroalgae were cultivated in 5 L bottles, with aeration supplied to each culture vessel through compressed air via tubes connected with 0.2 µm air filters (Chromafil A-20/25, Macherey-Nagel GmbH & Co. KG, Germany). NSW exchanges were performed weekly and nutrients were added once or twice a week, depending on the nutrient source used. The first two species in the laboratory scale cultivation system (from Portugal) were provided with 56 µL L^{-1} of the commercial fertilizer Blaukorn (14% total nitrogen, 6% nitrate, 8% ammonium, 5.5% water soluble phosphate; COMPO SANA, Germany), added twice a week. However, after six months, the nutrient source for cultivation of all the *Ulva* species was changed to 10 mL L^{-1} of half-strength PES added once a week.

2.2. Strain selection

The following experiments were conducted to select robust *Ulva* strains that could grow under similar conditions to the ones in the large-scale facility from the Mak-Pak Scale-Up Project partner in Rockstedt, Germany. The experiments considered the high temperatures in the system and the costs of using salt to produce ASW. The relative growth rates (RGRs) of each *Ulva* species and strain were considered to select the ones that presented higher growth rates under high temperatures and low salinities.

2.2.1. Temperature as a tool for strain selection

Three species of *Ulva* (*Ulva linza*, *Ulva flexuosa*, and two strains of *Ulva lacinulata*), from temperate climates, were grown under a gradient of temperature conditions (10, 15, 20 and 25 ˚ C) for three weeks. Samples of healthy and non-fertile pieces of *Ulva* were selected at the beginning of the experiment and placed in 1 L beakers (n = 3). The initial fresh weight used was 0.83 ± 0.02 g of *U. flexuosa*, 0.34 ± 0.01 g of NE-Atlantic *U. lacinulata* and *U. linza,* or 0.26 ± 0.01 g of the Mediterranean *U. lacinulata*. The beakers were placed in water baths, each at a different temperature. Four immersion circulators (one per treatment; Huber, Germany) were used to keep the temperature in the water baths constant for the entirety of the experiment (**Fig. 2.1**). Aeration was provided as mentioned previously and NSW and nutrient (PES) exchange was done once a week. The fresh weight of the biomass from each beaker was measured to the nearest 0.1 mg increment (Sartorius, Germany) once a week until the end of the experiment. Before fresh weight measurements were conducted, biomass samples were gently dried with absorbent paper three times to remove the excess water. RGRs were calculated based on the fresh weights collected each week following Eq. (1):

Figure 2.1 – *Ulva flexuosa* growing in a water bath at 20 ˚C during the temperature experiment.

$$RGR\ \% = \frac{\ln Wf - \ln W0}{tf - t0}\ x\ 100 \quad (1)$$

2.2.2.Salinity as a tool for strain selection

Similar to the temperature experiment, the same *Ulva* species and strains were cultivated under different salinity treatments (10, 15, 20, and 30 PSU). The experiment was divided into two parts: 1) cultivation of adult pieces of *Ulva* under the different salinity treatments (**Fig. 2.2**); and 2) cultivation of germlings from each *Ulva* species and strain cultivated under the same salinity treatments. During the experiments, ASW was used. The adult thalli were provided with the commercial fertilizer Bla-ukorn, (COMPO SANA,

Figure. 2.2 – Salinity experiment with adult and germling material of the two *U. lacinulata* strains. **(A)** adult pieces of Mediterranean *U. lacinulata* growing at 30 PSU during the salinity experiment; **(B)** germling of NE-Atlantic *U. lacinulata* being measured while growing at 30 PSU during the salinity experiment (photo by Anja Sawicki, used with permission).

Germany), while the germlings were supplemented with PES. Details about the experimental setup, and the reasons behind the use of different nutrient sources can be found in **Publication I (Cardoso et al., 2023)**.

2.3. Strain optimization

The following experiments were executed to test different abiotic factors as strain optimization methods to apply before harvesting. The quality of the biomass was evaluated by the antioxidant activity (AA) levels of the strains, an important quality in food packaging material (e.g., antioxidants; El-Baky et al., 2009; El Fayoumy et al., 2022; Gomaa et al., 2022). The strains selected during the strain selection experiments were tested under different salinity (NE-Atlantic) and irradiance treatments (NE-Atlantic and Mediterranean *U. lacinulata)*, and their AA and photosynthetic efficiency were analysed.

2.3.1.Salinity as a tool for strain optimization

The NE-Atlantic *U. lacinulata* was placed for ten days under the same salinity conditions tested previously (10, 15, 20, and 30 PSU). *Ulva* samples were collected at different time points during the 10-day experiment, and dried. AA was determined with the ABTS assay (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation decolourisation assay), following the work from Re et al. (1999) and the extraction protocol by Tretiak et al. (2021). There was no acclimatisation period before the experiment as the aim was to evaluate the quick reaction of the strain. The experimental design and the statistical analysis performed are detailed in **Publication I**.

2.3.2. Irradiance as a tool for strain optimization

The two strains of *Ulva lacinulata* (NE-Atlantic and Mediterranean) were placed under a saturating irradiance treatment (185 µmol photons m^{-2} s^{-1}) for five days. Samples in the control group were placed under the same irradiance used during cultivation (non-saturating conditions, 70 µmol photons m^{-2} s^{-1}). The daily light integral (light dose) was kept the same (4 mol photons m^{-2} s^{-1}) between the control group and the saturating treatment by adjusting the time of exposure to light. The samples exposed to the saturated treatment were illuminated for 6 hours per day, while the samples from the control group were illuminated for 16 hours per day.

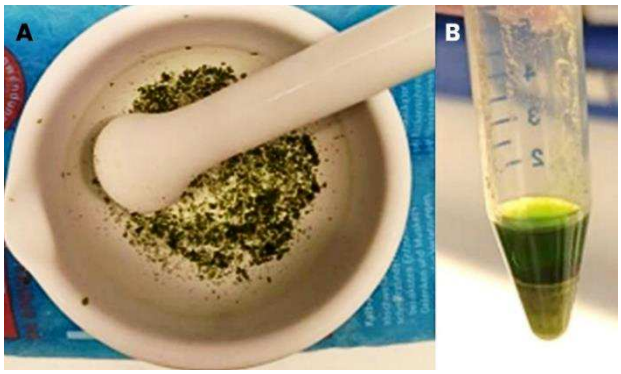

Figure 2.3 – Antioxidant extraction process. **(A)** maceration of dried *Ulva lacinulata* samples; **(B)** antioxidant extract in ethanol (photos by Anneke Meiβner, used with permission).

Ulva samples were collected at different time points and dried for the determination of their AA. AA was measured following the same methods reported for the salinity experiment (ABTS assay; **Fig. 2.3**). The photosynthetic activity of the different samples was monitored with a Pulse amplitude modulated (PAM) fluorometer (Imaging PAM, Heinz Walz GmbH, Germany) every two days. The details of this experiment can be found in **Publication II (Cardoso et al., 2024)**.

2.4. Optimization of culture conditions and seedling methods

The following experiments were developed to deepen the understanding about *Ulva* and its reproduction strategies and to tackle some of the limitations found in *Ulva* cultivation nowadays. From the several limitations known in *Ulva* cultivation, the works presented in this section of the dissertation aimed to find solutions for the difficult control of *Ulva*'s reproduction, and the occurrence of spontaneous biomass loss. To guarantee the successful cultivation of NE-Atlantic *U. lacinulata* in a large-scale land-based facility, protoplast isolation methods were tested, and the optimization of the successful method attempted. To understand the spontaneous biomass loss the degradation process observed in the NE-Atlantic *U. lacinulata* was followed. To understand how these two limitations impact *Ulva* cultivation, a survey was used to inquire SeaWheat COST Action members who cultivate or have cultivated *Ulva* spp..

2.4.1. Protoplast isolation from *Ulva* spp.

The protoplast isolation methods described in the literature are complex and expensive for large-scale purposes of obtaining seeding material (Gupta et al., 2018). Different methods were tested and adjusted during a three-month short-term scientific mission (STSM) at the University of Ireland, Galway, Ireland, with the supervision of members of the SeaWheat COST Action, the project that funded the STSM. The aim was to establish a less complex and cheaper method of protoplast isolation that can be used for large-scale systems.

Two Irish *Ulva* species (*Ulva*-1 and *Ulva*-2) were tested. Culture conditions were set at 14 ˚C, 34 PSU (ASW) with the light intensity at 100 µmol photons m^{-2} s^{-1} and a 12:12 light:dark photoperiod. The ASW was supplemented with 250 µL L^{-1} of F/2 medium (Guillard & Ryther, 1962; Guillard, 1975). The ASW and medium exchange was done once a week. Aeration was provided following what has been previously described.

The protoplast isolation experiment was separated into two steps: 1) eco-physiological experiments (light and salinity); and 2) protoplast isolation experiment. The eco-physiological experiments were designed to evaluate the impact of the light and salinity treatments on the cell wall thickness of the *Ulva* species tested. The initial protocol used in this work for protoplast isolation was created by compiling the information from several different works reported in the literature (Reddy et al., 2006; Gupta et al., 2018; Reddy & Seth, 2018). The final aim of these experiments was to use the eco-physiological treatments that resulted in a reduction in cell wall

thickness as pre-treatments to the protoplast isolation, thus reducing the need for expensive enzyme solutions that limit the use of this seeding obtention method for large-scale purposes.

In the eco-physiological experiments, samples from *Ulva*-1 (tubular morphology) were cut into small filaments with similar width and length (28.3 mm) while samples from *Ulva*-2 (blade morphology) were cut into 6 mm discs. The species were placed, separately, under each treatment in an orbital shaker (Stuart Obrital Shaker SL1, Cole- Parmer, United Kingdom) at 60 rpm (n = 6). In each Petri dish (with 50 mL of fresh medium), there were six discs/filaments (subsamples) of the same sample. A plastic structure in the shape of a star was created to separate each subsample inside each Petri dish (**Fig. 2.4**).

Two salinity treatments (10 and 40 PSU) and one light irradiance treatment (10 µmol photons m^{-2} s^{-1}) were tested and later compared to the results from the two controls (34 PSU and 100 µmol photons m^{-2} s^{-1}). Every day for five days, one disc/filament was collected from each Petri dish and placed in a 15 mL falcon tube with 4 % Paraformaldehyde in 0.2 M phosphate buffer. After 24h the samples were removed from the paraformaldehyde solution and placed in a 15 mL falcon tube with 0.2 M phosphate buffer. Samples were kept refrigerated (4 ˚C) for later microscopic analysis. The samples were rinsed with buffer solution and placed in a battery of ethanol solutions (40, 70, 95, 100, and 100 %) before microscopic analysis. Each sample was kept for 5 minutes in each ethanol solution and left for two hours in the second 100 % solution, to guarantee the removal of all pigments. The samples were rehydrated by rinsing them with distilled water and stained with safranin (Thermo Fisher Scientific Inc, USA). Once the samples were prepared for observation under the inverted fluorescence and phase contrast microscope (Olympus IX51, Olympus, Japan) photographs were taken at a magnification of 40x. The photographs were later used to measure the thickness of the cell walls of the thalli by using the ImageJ software (Rasband, 2024).

Figure 2.4 – Petri dishes used during eco-physiological experiments with different salinity treatments. In the middle of the Petri dishes was placed a white plastic structure to keep the discs and filaments separated while moving in the shaker during the 5-day experiment.

In the second part of the experiment, a polysaccharide-degrading enzyme mixture was prepared for protoplast isolation. The enzyme solution was prepared with a pre-cooled (at 4 °C) mixture of 50 % distilled water and 50 % ASW at 34 PSU. While working at 4 °C, 500 mg of Dextran sulphate, 1g of NaCl and 488 mg of 2-[NMorpholino] ethane sulfonic acid (MES) were added to make 100 mL of stock solution. The stock solution was centrifuged (5810r, Eppendorf, Germany) at 10,000 xg for 20 minutes at 4 °C. The clear supernatant was used for further

protoplast isolation. The pH of the stock solution was adjusted to 6.0. 200 mg of Cellulase Onozuka R-10 (Yakult Co. Ltd., Tokyo, Japan) and 1.457g of Mannitol were added to 10 mL of the stock solution and mixed to create a homogenous solution. A version of the enzyme solution tested included Macerozyme R-10 (Yakult Co. Ltd., Tokyo, Japan), as it has been previously described that Macerozyme R-10 increases the protoplasts yields in tubular forms of *Ulva* (Uppalapati & Fujita, 2002).

For isolation of the protoplasts, the *Ulva* thalli, from both species, were separated into 50 mg pieces, that were chopped finely (< 1mm) and rinsed 2-3 times in filtered ASW. The chopped pieces were transferred to multiwell plates (12 wells) with 2.5 mL of enzyme solution and incubated at 25 °C ± 1 °C on a rotary shaker (80 rpm) in the dark for 3 hours (n = 4). The cultures were observed periodically to check for protoplast release. After the incubation period, the mixtures of enzyme solution and *Ulva* thalli were filtered through a nylon mesh of pore size 30 µm to remove debris and undigested fragments. The filtered solution was centrifuged for 5 minutes at 120 xg at room temperature. The supernatant was discarded, and the pellet was resuspended in ASW at 34 PSU with F/2 medium. The presence of protoplasts was evaluated under the fluorescent microscope after staining subsamples of the resuspended pellet with 0.01 % of Calcofluor White (CFW; Merck KGaA, Darmstadt, Germany). When protoplasts were found, the solution was inoculated into glass Petri dishes (60 mm x 15 mm) with 10 mL of ASW and F/2 medium. The concentrations of protoplast solution inoculated varied during the experiment as most attempts were unsuccessful. The Petri dishes were placed in the dark for 24 hours at 25 °C ± 1 °C, in a rotary shaker (80 rpm).

Protoplast isolation was attempted several times until success was achieved. The changes in the protocol were documented. The following **Table 2.1** reports the variations to the original protocol that were tested until protoplasts were successfully isolated.

Once the protocol was established, a new experiment was set up to test if the low light pre-treatment would increase the protoplast yields. Samples of both *Ulva* species were placed under a pre-treatment of low light (10 µmol photons m^{-2} s^{-1}) for eight days.

Table 2.1 – Variations to the original protocol for protoplast isolation. Variations were made in different steps of the protocol. The alterations tested were not made in any specific order.

Isolation steps	**Biomass**	**Enzyme mixture**				**Incubation**	
	Weight (mg)	Distilled: ASW ratio	Cellulase: macerozyme ratio	Mixing mannitol	Volume (mL)	Time (h)	Temperature (˚C)
Variations to the protocol	100	1:1	1:1	Manual	1	3	25
	50	Only distilled water	Only Onozuka	Vortex	2,5	2	20
	30	-	-	-	5	1	-

The protoplasts were isolated following the corrected protocol for protoplast isolation and counted with an automated cell counter (Luna II, Logos Biosystems, South Korea). Protoplast yields were calculated based on the results provided by the automated cell counter (in cell mL^{-1}). The number of protoplasts in the entire protoplast suspension was calculated and then divided by the grams of biomass used for protoplast isolation.

2.4.2. Degradation event and naturally occurring protoplasts in *Ulva* spp.

The often occurring degradation of the NE-Atlantic *U. lacinulata* was a limitation to cultivating this strain. An experiment was designed to follow the process of degradation and understand the impact these events have on the reproduction and dispersion of new *Ulva* material.

Several pieces of this strain were observed for four weeks while degrading (**Fig. 2.5A**). Each week the fresh weight of the biomass was measured and at the end of the experiment, the RGRs of the degraded material were calculated. Additionally, protoplast-like cells found during degradation were collected weekly (**Fig. 2.5B**) and dyed with CFW to evaluate the presence/absence of cell walls. Protoplast yields were calculated based on the biomass at the beginning of the experiment and the biomass in the beakers each week. A subsample of the protoplasts collected was used in a germination experiment to assess the capacity of the protoplasts to regenerate and develop into new individuals.

To test if similar observations could be found in wild *Ulva* material, *Ulva compressa* was collected from the wild. This species was kept in NSW, under laboratory conditions for five days until the biomass became fertile and similar protoplast-like cells were found. The same experiment performed for *U. lacinulata* was done with *U. compressa*. Protoplast yields and germination rates of the two species were compared. A more detailed description of the experiments performed on the two *Ulva* species and the statistical analysis done can be found in **Publication III (Cardoso et al., n.d.)**.

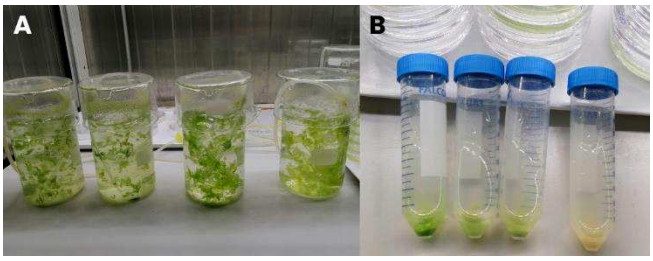

Figure 2.5 – Degradation experiment with NE-Atlantic *U. lacinulata*. **(A)** Four replicates used to follow the degradation process of *U. lacinulata* (n = 4); **(B)** protoplasts collected from each replicate beaker. Variation in protoplast concentration was responsible for the variation in colour in the falcon tubes (green solution represents higher protoplast concentration; pale solution represents low protoplast concentration).

2.4.3. Survey: Reproduction and degradation of *Ulva* - Bottlenecks for its large cultivation

A survey aimed at people with experience cultivating *Ulva* species was developed to understand some of the bottlenecks that *Ulva* cultivation presents nowadays and compare them to the limitations faced during the cultivation of the *Ulva* spp. presented in this dissertation. The survey

was developed in agreement with the SeaWheat COST Action (CA20106) that allowed its use during the Workshop "From *Ulva* Aquaculture to food and feed production – state-of-the-art, bottlenecks, risks, and gaps" that took place in 2023 in Lisbon, Portugal. Later the survey was shared by the SeaWheat COST Action with their members. In total, 36 participants answered the survey. The participants who did not reply coherently or answer most of the questions with "I don't know" were excluded.

The survey had a total of 33 questions divided into 3 topics: cultivation, degradation, and reproduction. Multiple-choice questions included several *Ulva* species names that are currently accepted taxonomically (Guiry & Guiry, 2024). Under each topic, participants could indicate the different species they worked with, and which species were molecularly identified. The scale of the cultivation (laboratory scale or large-scale cultivation) and whether the species were collected from the wild or kept under laboratory conditions for over 5 years were taken into consideration. The survey inquired about limitations found during *Ulva* cultivation, unexpected degradation, and the occurrence of new germlings without apparent fertility of the *Ulva* thalli.

2.5. Statistical analysis

Regression analysis was performed in R studio software (R Core Team, 2024) between the RGRs of each species and the different temperatures, to determine the optimal conditions to cultivate each of the *Ulva* strains. The non-linear correlations were represented by a second-degree polynomial function ($y = ax^2 + bx + c$). The coefficients were extracted using the "coef()" function, and the polynomial functions were used to calculate a model of RGR at each temperature (10 - 25 ˚C). From these data, the maximum RGR and the optimal temperature for cultivation of each species could be extracted.

Statistical analyses in the way of One-Way PERMANOVAs with pairwise comparison were performed to determine if cell wall thickness varied between the pre-treatments tested. Kruskal Wallis tests paired with Dunnes test were performed to compare the protoplast yields obtained by the two *Ulva* species and evaluate the effect of the eight-day light treatment. Regression analysis were performed as previously described. The non-linear correlations were represented by a second-degree polynomial function ($y = ax^2 + bx + c$) and a third-degree polynomial function ($y = ax^3 + bx^2 + cx + d$).

The survey raw data was transformed into percentages to simplify the analysis of the different questions. Different graphical representations of the data were used to clarify the results found (threemap chart, 100 % stacked columns, pie chart). A regression analysis was performed to evaluate the correlation between the number of participants who observed *Ulva* degradation and the number of participants who found germlings in their cultures without observing the biomass becoming fertile. A second-degree polynomial ($y = ax^2 + bx + c$) was the most accurate representation of the correlation found.

All the analyses and graphical representations of the results presented in this dissertation were produced with the R studio software (“PERMANOVA”, “Vegan” and “ggplot2” packages; R Core Team 2024), except if stated otherwise. The representation of the regression analysis performed were developed using the “geom_smooth” function with the method “lm” from the package “ggplot2”.

Publication I

Salinity as a tool for strain selection in recirculating land-based production of *Ulva* spp. from germlings to adults

Isabel Cardoso, Anneke Meiβner, Anja Sawicki, Inka Bartsch, Klaus-Ulrich Valentin, Sophie Steinhagen, Bela H. Buck, Laurie C. Hofmann

2023

Published in Journal of Applied Phycology, Collection Ulva 2022
https://doi.org/10.1007/s10811-023-02960-x

Journal of Applied Phycology
https://doi.org/10.1007/s10811-023-02960-x

RESEARCH

Salinity as a tool for strain selection in recirculating land-based production of *Ulva* spp. from germlings to adults

Isabel Cardoso[1,2] · Anneke Meißner[1,2] · Anja Sawicki[1,2] · Inka Bartsch[1] · Klaus-Ulrich Valentin[1] · Sophie Steinhagen[3] · Bela H. Buck[1,4] · Laurie C. Hofmann[1,4]

Received: 15 December 2022 / Revised: 24 March 2023 / Accepted: 27 March 2023

Abstract
The genus *Ulva* is globally distributed and has been thoroughly studied because of its functional biochemical composition, rapid growth rates and opportunistic features, and interest in *Ulva* cultivation is growing worldwide. In Europe, mostly near- and on-shore flow-through cultivation systems are used and land-based recirculating aquaculture systems (RAS) using fresh water or artificial seawater have not been developed for *Ulva*. While RAS provides quality control and can be located inland, maintenance costs are high. Using selected strains adapted to low-salinity could reduce seawater production costs and improve the economic feasibility. Therefore, our study assessed how salinity can be used as a tool for strain selection and optimization of functional traits. Growth rates and antioxidant activity of three species (four strains) of tubular and foliose *Ulva* from the NE-Atlantic and Mediterranean (foliose: *Ulva lacinulata* – two geographical strains, tubular: *Ulva linza* and *Ulva flexuosa*) were followed for three weeks at salinities ranging from 10 to 30 PSU. The tubular strains achieved optimal growth at a lower salinity than *U. lacinulata*. However, growth rates of both foliose strains were higher than of tubular strains, even at sub-optimal salinity. Therefore, *U. lacinulata* is a good candidate for RAS with artificial seawater, and the cost of salt can be reduced by up to 33.3% (20 PSU) without significantly reducing the growth rate of *U. lacinulata*. Higher antioxidant activity was achieved by reducing the salinity to 10 PSU for 10 days, suggesting that the functional traits of cultivated *Ulva lacinulata* can be optimized prior to harvest.

Keywords Antioxidant Activity · Artificial Seawater · Cultivation · Recirculating Aquaculture System (RAS) · Salinity · *Ulva* · Chlorophyceae

Introduction

The green macroalgal genus *Ulva* is widely known for its global distribution and its functional biochemical composition, including high protein content (Shuuluka et al. 2013; Rasyid 2017; Juul et al. 2021), presence of unique polysaccharides (Ganesan et al. 2018; Kaeffer et al. 1999; Li et al. 2018b; Olasehinde et al. 2019), and its use in biofiltration and biorefinery (van der Wal et al. 2013; Mhatre et al. 2019). Moreover, its antioxidant properties are important when exploring *Ulva* for different industries (e.g. pharmaceutical, food and food packaging industries) (Mo'o et al. 2020; Leyva-Porras et al. 2021; Lomartire et al. 2022). The genus *Ulva* is characterized by a high variation in protein content (Marsham et al. 2007) consisting of up to 39% of essential amino acids (Wong and Cheung 2001) and additionally, it is rich in polysaccharides (mostly ulvan) (Ortiz et al. 2006; Lahaye and Robic 2007; Peña-Rodríguez et al. 2011). In contrast, *Ulva* has a low lipid content (Ortiz et al. 2006; Yaich et al. 2011) but approx. 1/3 of total fatty acids are polyunsaturated (Taboada et al. 2010). Regarding ash content, the highest value reported was 52% DW (Foster and Hodgson 1998). Unfortunately, large variations in the biochemical composition of *Ulva* have been reported depending on season, geographical location, and the environment

✉ Isabel Cardoso
cardoso_isabel@outlook.com

1 Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Bremerhaven, Germany

2 University of Bremen, Bremen, Germany

3 Tjärnö Marine Laboratory, Department of Marine Sciences, University of Gothenburg, Strömstad, Sweden

4 Bremerhaven University of Applied Sciences, Bremerhaven, Germany

Published online: 29 May 2023

(Holdt and Kraan 2011; Toth et al. 2020). Furthermore, there are intra-specific differences in biochemical profiles between strains from "green tide" regions and other strains (Fort et al. 2019; 2021; Toth et al. 2020). Therefore, it is very difficult to accurately report a precise chemical profile for different *Ulva* species and strains.

This green macroalgal genus is also known for causing extensive "green tides" that can result in the impoverishment of ecosystems (fresh and marine ecosystems) as well as an impairment of local economies (e.g., tourism, fisheries) (Mineur et al. 2014; le Luherne et al. 2017; Rybak and Gąbka 2018; Cai et al. 2021). Additionally, the costs of cleaning are as high as 30.8 million US dollars (Charlier et al. 2008; Liu et al. 2013; Louis 2017; Song et al. 2022). While the biomass produced during "green tide" events is often used as fertilizer and has not yet been sufficiently valorised, the strains causing "green tides" usually have advantageous traits for the aquaculture of high-valuable crops (Charlier et al. 2007; Fort et al. 2020). High-quality *Ulva* biomass is recognized as a valuable food and feed, and the interest in *Ulva* cultivation has been growing worldwide (Fleurence et al. 1995; Lordan et al. 2011; Li et al. 2018a; McCauley et al. 2018; Dominguez and Loret 2019) with ongoing attempts to optimize and scale up its cultivation (Flodin and Whitfield 1999; Yildiz et al. 2012; Pereira 2016; Mantri et al. 2020).

Recent work has shown that cultivation of *Ulva fenestrata* Postels & Ruprecht in a sustainable large-scale offshore aquaculture is possible (Steinhagen et al. 2021). Nevertheless, in Europe, the most common *Ulva* cultivation methods have been limited to nearshore and on-shore production (with in- and outdoor cultivation) (Buchholz et al. 2012; Sebök et al. 2019; Califano et al. 2020; Steinhagen et al. 2021). Although on-shore *Ulva* cultivation is common, it is usually limited to close proximity to the coast, and there are types of land-based aquaculture systems that are still only being explored for fish and shrimp production, such as Recirculating Aquaculture Systems (RAS). Only a few studies have produced macroalgae in RAS to date, and so far, only in combination with fish aquaculture (Table S1).

RAS is a type of closed system where water is reconditioned and recirculated to the tank, in contrast to a flow-through system, which continuously pumps in new, unused seawater (Malone 2013; Ed-Idoko 2021). RAS is independent from location and distance from the coast because the water is biologically and mechanically cleaned in an operating treatment device connected to the cultivation tanks. Since there is de facto no wastewater, the ecological footprint is lower. Optimal conditions can be set for the cultivation of algae, invertebrates and fish at any time, which increases production efficiency, guarantees welfare, and allows the cultivation of non-native species, as there is no discharge of process water into the surrounding ecosystem (environment) and therefore no cross-contamination (Ed-Idoko 2021).

The biggest weakness of RAS is the high financial investment required, associated with the construction, operation, labour, and maintenance costs (e.g., artificial lighting and artificial seawater) (Lüning and Pang 2003; Mata et al. 2016; Sebök et al. 2019; Steinhagen et al. 2021). Furthermore, the carbon footprint of such facilities must still be evaluated (Bermejo et al. 2022). Nevertheless, Ladner et al. (2018) estimated that on-shore cultivation of *Ulva lactuca* Linnaeus would be less expensive than offshore cultivation at the end of a 5-year period. Additionally, some studies suggest that it is important to select the right type of RAS and the right species to produce in it to be profitable (Malone 2013; Ed-Idoko 2021). With this in mind, RAS has the potential for scaling-up macroalgae production, but solutions are required to reduce the cost and guarantee a low carbon footprint.

The RAS can use artificial seawater (ASW) instead of natural seawater, reducing the probability of drastic changes in the culture conditions (e.g., natural fluctuation of water quality that adds a degree of risk and uncertainty to the cultivation) (Losordo et al. 2004; Kuhn et al. 2013; Zhang et al. 2020; Ed-Idoko 2021). Using ASW guarantees the traceability and consistent quality of the water while reducing the risk of harmful compounds or organisms that might be present in natural seawater (e.g., microalgal spores, parasites, toxins, heavy metals) (Allen and Nelson 1910; Zhang et al. 2017). Furthermore, ASW can increase the strict control over the environmental parameters managed in a RAS, facilitating the optimization of high-value compounds in the macroalga biomass (e.g., polysaccharides, pigments, or antioxidants). Therefore, a RAS with ASW for seaweed cultivation could be a promising way to scale-up production of highly productive and high-value macroalga biomass with low risk and uncertainty.

Another step to optimize such a system is to select desirable strains adapted to the conditions of a RAS system. For example, strains adapted to lower salinities would reduce the production costs and the associated environmental impact of water disposal by reducing the salt concentration and the cost of water desalination treatments. Although RAS requires very little water renewal, water exchanges are occasionally necessary and water treatments for seawater desalinization, (e.g., reverse osmosis) are usually expensive and require high amounts of energy (Sharrer et al. 2007; Liu 2013; Chang et al. 2022).

In order to counteract the high costs of a land-based RAS system at low-salinity, we chose the genus *Ulva* as a promising candidate for cultivation for its known phenotypic plasticity under broad ranges of environmental conditions (Hofmann et al. 2010; Bruhn et al. 2011; Yildiz et al. 2012; Carl et al. 2014; Mata et al. 2016; Rybak 2018; Fort et al. 2020; Mantri et al. 2020; Lawton et al. 2021; Zertuche-González

et al. 2021; van der Loos et al. 2022). Some species can even grow in freshwater environments at 0.5 PSU (Rybak 2018). Simultaneously, salinity stress can affect growth rates and induce oxidative stress and antioxidant defence in *Ulva* cells (Lu et al. 2006; Luo and Liu 2011). Therefore, our aim was twofold:

1) to select a strain well suited for cultivation in a large-scale land-based RAS at low salinity by investigating the influence of salinity on two tubular and one foliose *Ulva* species (the latter with two geographical strains) in two different life stages (germlings and adults), 2) to determine if salinity also can be used as a tool to optimize the functional traits of the biomass, in this case antioxidant activity (AA), before harvesting, as an extra-step for the optimization of the selected strain and the overall production.

Materials and methods

Biomass collection and cultivation

The salinity tolerance of species with different adult morphologies and potential intra-specific variances was evaluated by comparing, two foliose *Ulva lacinulata* (Kützing) Wittrock strains (NE-Atlantic and Mediterranean origins), a tubular strain of *Ulva linza* Linnaeus (NE-Atlantic origin), and a tubular strain of *Ulva flexuosa* Wulfen (Table S2).

The species and strains used here originated from two warm-temperate regions (Mediterranean Sea and NE-Atlantic) characterized by relatively high mean maximum summer temperatures of 27 and 24 °C, respectively (Pereira et al. 2009; Genitsaris et al. 2019) (Table S2).

The NE-Atlantic *U. lacinulata* and *U. linza* were collected in the Óbidos Lagoon, Portugal, in January 2021. The material was transported to the laboratory where it was rinsed several times with fresh running seawater and thoroughly cleaned to remove epiphytic organisms. For transportation from Portugal to Germany, the material was placed between sheets of absorbent paper damped with seawater and was kept cool (approx. 6 °C) and dark for two days until it was transported to the Alfred Wegener Institute (AWI) in Germany. The Mediterranean species/strains were unialgal clones taken from the AWI culture collection (*Ulva flexuosa*: AWI culture number 1262; *Ulva lacinulata:* AWI culture number 1290) and originally isolated in 1986 and 1987 by S. Orfanidis (Fisheries Research Institute (ELGO-DIMITRA), Kavala, Greece in Thessaloniki Bay.

Upon arrival the NE-Atlantic material was acclimated in 5 L glass bottles with natural seawater at 30 PSU (±2 PSU) (Refractometer, Atago, Japan) with aeration. The natural seawater used was filtered with a polypropylene water filter with a mesh size of approx. 5 µm (EF-Filter, Netherlands) and pasteurized for 4 h at 99 °C. The temperature-controlled cooling chamber was kept at 15 °C (±1 °C) and an irradiance of 70 µmol photons m^{-2} s^{-1} (measured in the air) with a 16:8 h light:dark photoperiod (LD). The seawater was supplemented with the commercial fertilizer Blaukorn (14% total nitrogen, 6% nitrate, 8% ammonium, 5.5% water soluble phosphate) (COMPO SANA, Germany) at a concentration of 55.5 µL L^{-1}. The commercial fertilizer was used to demonstrate the feasibility of growing *Ulva* spp. at low cost. These conditions were kept until the start of the experiments.

The unialgal clones from the Mediterranean species had been maintained as stock cultures in 25 mL glass bottles at 10 °C in a temperature-controlled cooling chamber with < 5 µmol photons m^{-2} s^{-1}, 16:8 h light:dark photoperiod (LD) in filtered and pasteurized natural seawater supplemented with half-strength Provasoli in a concentration of 10 mL L^{-1} (PES; Provasoli 1968; modifications: HEPES-buffer instead of TRIS, double concentration of Na_2glycerophosphate; iodine enrichment following Tatewaki 1966). The medium was replaced once per year.

Before the start of the experiment a pre-cultivation step was necessary to grow sufficient biomass for the experiments. During the pre-cultivation phase all experimental material was placed in culture conditions of 15°C, 100 µmol photons m^{-2} s^{-1} in a 16:8 LD photoperiod in filtered and pasteurized artificial seawater (30 ± 2 PSU) (Seequasal-Salz, Seequasal Salz Production and Trade GmbH, Germany) with the addition of full Provasoli medium (PES). The medium was exchanged once per week. Nutrient analyses (SEAL Analytical, UK) were performed to compare the artificial seawater (ASW) used in the experiments and the natural seawater (NSW) used in the original cultures. The differences in nutrient concentration were statistically negligible. In the ASW the ammonium concentration was 0.014 ± 0.002 mg L^{-1} and the nitrite concentration was 0.010 ± 0.001 mg L^{-1}, whereas in the NSW, the ammonium concentration was 0.010 ± 0.005 mg L^{-1} and the nitrite concentration was 0.038 ± 0.003 mg L^{-1} (mean ± standard error). In both water types, phosphate, and nitrate were below the detection level.

Before the experiments, all material was acclimated to 15°C, 100 µmol photons m^{-2} s^{-1} in a 16:8 LD photoperiod in pasteurized seawater (30 ± 2 PSU) enriched with the commercial fertilizer Blaukorn (COMPO SANA, Germany) at a concentration of 55.5 µL L^{-1}.

Molecular identification of species using the plastid-encoded marker tufA

Total genomic DNA was extracted from silica-dried samples using the Invisorb Spin Plant Mini Kit (Stratec, Germany), following the manufacturer's protocol. The *tufA* gene was PCR amplified using the primers *tufGF4* (Saunders and Kucera 2010) and *tufAR* (Famà et al. 2002) following the protocol described by Steinhagen et al. (2019a). The *tufA*

gene was used for species identification of the *Ulva* strains used in this study. *TufA* has been evaluated by different studies as one of the best markers for species identification and delimitation (Saunders and Kucera 2010; Tran et al. 2022). As this study is not focusing on the systematic or taxonomic relationships within the genus *Ulva*, it was not necessary to include more marker genes. The PCR products were first assessed by gel electrophoresis and subsequently purified using the QIAquick PCR Purification Kit (Qiagen). Sanger sequencing of the purified amplicons was performed by Eurofins Genomics (Konstanz, Germany). Forward and reverse sequence reads were assembled in the DNA sequence analysis software Sequencher (version 4.1.4, Gene Codes Corporation). Using the BLAST function in GenBank, initial identifications using the specimens' *tuf*A sequences were made. To better resolve species identities, a set of peer-reviewed and annotated reference sequences downloaded from GenBank were used in subsequent phylogenetic analyses (data not shown). Host species were identified according to the latest taxonomic revisions by Hughey et al. (2022). All sequences are publicly available in GenBank (OP778143, OP778144, OP778145, OP778146).

Effect of salinity on the growth rate of adult Ulva

Since the main objective of this work was to select a strain to cultivate in a large-scale system, artificial seawater was enriched with the commercial fertilizer Blaukorn to model the conditions in a RAS, where the use of PES would not be economically feasible (Fig. S1).

Artificial seawater was prepared by mixing deionized water and salt to the pre-established concentrations of 10, 15, 20, and 30 PSU, to test how salinity influences the growth rates of different *Ulva* strains. Final salinity was always controlled with a Refractometer (Atago, Japan).

A uniform amount of fresh thalli (foliose species: 0.25 g ± 0.05 g fresh weight, tubular species: 0.5 g ± 0.05 g fresh weight) from each species and population were placed into 1 L glass beakers with salinities of 10, 15, 20 and 30 PSU (each condition n = 3) and cultivated over 3 weeks (irradiance of 100 µmol photons m^{-2} s^{-1} and 16:8 LD photoperiod). As the NE-Atlantic material was not clonal but came from the wild, several genetically different pieces of thalli were included in each beaker whilst for the unialgal Mediterranean material all thalli used for the experiment originated from the same clone. Previous experiments showed that the added concentration of fertilizer was taken up within 2 days (data not shown), therefore, water was changed once a week and 55.5 µL L^{-1} of the fertilizer was added twice a week. The average fresh:dry weight ratio of foliose NE-Atlantic *U. lacinulata* and Mediterranean *U. lacinulata* was 5.58 and 5.08, respectively (data not shown), while the tubular species *U. flexuosa* and *U. linza* had fresh:dry weight ratios of 8.38 and 10.33, respectively (data not shown). Therefore, the amount of biomass used for foliose and tubular species was adjusted for the experiments based on the fresh:dry weight ratios so that the resulting biomass:volume ratio of all material was 0.05 g of dry weight L^{-1} on average (data not shown).

The fresh weight was measured once a week by collecting the macroalgae and removing the excess water with absorbent paper three times before weighing the samples (Sartorius, Germany). Every time, each sample was weighed 3 times in a row and the means were used for further data analysis.

We calculated the relative growth rate (RGR) via Eq. (1):

$$RGR\ \% = \frac{ln\ W_f - ln\ W_0}{t_f - t_0} \times 100 \quad (1)$$

where W_f is the fresh weight at the end of the experiment, W_0 is the fresh weight at the beginning of the experiment, and t_f and t_0 are the time, in days at the end and the start of the growth period, respectively.

Effect of salinity on the growth rate of Ulva germlings

In this experiment, we expected that germlings would be more sensitive to suboptimal salinity and nutrient conditions and, to guarantee their survival, we used PES medium rather than commercial fertilizer, which resulted in good growth rates in previous studies.

Germlings from the four *Ulva* strains were obtained from fertile adult material. The NE-Atlantic germlings originated from the wild material, while the germlings from the Mediterranean species were progenies of the used clones. While working with *U. lacinulata,* controlled induction of reproduction was unsuccessful and reproduction events were scarce. The tubular species (*U. linza* and *U. flexuosa*) however, could be induced to reproduce by exposition to low temperatures (4 °C) for two hours and returning them back to the regular culture conditions at 15 °C. Three days after this treatment, thalli became reproductive. The resulting germlings were kept in dense cultures and low light conditions (30 µmol photons m^{-2} s^{-1} in 16:8 LD photoperiod) to ensure their slow development until the start of the experiment. During this period the culture medium was changed every two weeks. For the experiment, germlings of similar age (approx. 2 months) were selected from all the species. As *U. lacinulata* did not respond to the induction methods, two experiments were conducted at different times. The first experiment was conducted with *U. linza*, *U. flexuosa* and NE-Atlantic *U. lacinulata* while the experiment with germlings of the Mediterranean *U. lacinulata* was conducted later. For all of the germlings, to ensure their slow

development until the start of the experiment, the material was kept in dense cultures and low light conditions (30 µmol photons·m^{-2} ·s^{-1} in 16:8 LD photoperiod) with culture medium changes every two weeks.

At the start of the experiment, 3 individual germlings per species were placed into separate multi-well plates with 12 wells (35 mm diameter, 16.5 mL volume) and subjected to the same salinity treatments as above (n = 3 per species). In this experiment the artificial seawater was enriched with half-strength Provasoli medium. As we were insecure whether germlings may suffer from the commercial fertilizer we did not replicate the conditions of large-scale production, instead we created the conditions of a small scale nursery.

Because germlings were too small to be weighed, the wells were photographed each week and the germlings' area was measured with Image J (Rasband 2021). RGR was calculated as above, but the weight was substituted by the total surface area of the germlings.

Effect of salinity on the antioxidant activity of adult Ulva lacinulata

Based on the results of the first two experiments, the NE-Atlantic *U. lacinulata* was selected as the preferable strain for cultivation in lower salinity. In order to improve the food quality of the biomass, this strain was tested for its capacity to increase antioxidant activity in lower salinities as well.

To evaluate the antioxidant activity (AA) response of *U. lacinulata* (NE-Atlantic) to salinity stress, six discs (2 cm diameter) with a total fresh weight of approx. 1.8 g were placed in each replicate 1-L beaker (1 disc for each sampling time) (n = 3). This sampling regime facilitated the collection of sub-samples (at different time points) while guaranteeing the minimum amount of biomass required for antioxidant extraction. The macroalgal material was then subjected to the same 4 salinity treatments (10, 15, 20, and 30 PSU) for ten days at 15 °C, 100 µmol photons m^{-2} s^{-1}, and 16:8 LD photoperiod as above. The AA was measured during a short period to avoid a decrease in RGR (as observed after 2 weeks with the adult material). Moreover, based on the results from the first experiment, and for the purpose of cultivating *Ulva* at low salinity, the material was acclimated at 20 PSU for several weeks before the start of the experiment. For antioxidant analysis, one subsample (one disc) with 0.3 ± 0.05 g of macroalga was collected from each replicate at 0 h, 3 h, 24 h (day 1), 120 h (day 5), 192 h (day 8), and 240 h (day 10). Because of the practical industrial use and low energy input required, the material was rinsed with deionized water and oven-dried at 30˚C for 48 h. This drying method is used for producing macroalgae-based packaging from the biomass produced in this study, and recent studies have shown that oven drying at low temperatures (e.g., 30˚C) guarantees a higher holding capacity and the extraction of antioxidant compounds (Silva et al. 2019; Hassanzadeh et al. 2022). We were more interested in evaluating the functional antioxidant activity after processing under realistic and cost-effective conditions than the potentially more precise, but less practical, method of freeze-drying.

The AA was determined by the ABTS radical cation decolourisation assay (Re et al. 1999). The ABTS (Hoffmann—LaRoche AG, Switzerland) was diluted in distilled water to a concentration of 7 mM and the potassium persulfate ($K_2S_2O_8$ Honeywell International Inc., USA) was diluted to a concentration of 2.45 mM. The solutions were then mixed and incubated at room temperature for 16 h in the dark. Meanwhile, macroalgae extracts were obtained by grinding 0.06 ± 0.005 g of dried macroalgae with 0.3 g of sand in a mortar on ice (SiO_2 Merck, Germany) until obtaining a fine paste. During the grinding process, 600 µL 70% ethanol (EtOH) was added to the paste, and another 600 µL 70% ethanol (EtOH) was added at the end to wash the paste into a centrifugation tube. The centrifugation tubes with the extracts were incubated in a shaking water bath (45 ˚C, 130 rpm) for 6 h before centrifugation (4 °C and 3628 rpm) for 10 min. Afterward, the supernatant was transferred to a new centrifugation tube and the pellet was mixed with 1.2 mL EtOH. All samples were incubated for one more hour in the water bath (as described before). The tubes were centrifuged a second time (with previous settings) after which the supernatants from the first and second extractions were mixed, and the pellets discarded.

The AA was determined according to the protocol of Re et al. (1999). The ABTS solution was diluted with ethanol to absorption of 0.7 at 734 nm. The AA was measured by adding 20 µL of each extract to a 96 well-plate, followed by 280 µL of diluted ABTS solution. In addition, a positive control (20 µL of Trolox solution (100 µg mL^{-1} in ethanol)) and negative control (ethanol) were added to each well plate. The well plates were incubated in the dark for 8 min at room temperature, after which the absorption of the samples at 734 nm was analyzed with a microplate reader (Infinite 200 Microplate Reader, Tecan Trading AG, Switzerland). Blank wells were measured with each reading to eliminate the absorption of the plate. A Trolox standard curve was created by measuring the absorption of different Trolox concentrations (0—100 µg mL^{-1}) in ethanol after being mixed with ABTS. The Trolox calibration curve was then used to calculate the AA of the samples in Trolox equivalents TE in µg mL^{-1}.

Statistical analysis

Permutational multivariate analyses of variance (PERMANOVA) were performed to guarantee the robustness of the statistical results from the experiments with a low

number of replicates. This low number of replicates was due to the difficulty of working with strains of *U. lacinulata* and its unpredictable degradation and reproduction patterns.

For the RGR of germlings and adult *Ulva*, two-way PERMANOVAs (9999 permutations) were performed (one for each experiment) to evaluate the interaction between strains and salinity and between morphology and salinity. Additionally, one-way PERMANOVAs (9999 permutations), for each species were performed to compare the effect of the different salinity treatments on growth. The one-way PERMANOVAs were associated with a pairwise comparison (with Bonferroni correction).

All the data were analyzed with the software R studio ("PERMANOVA" and "Vegan" package) (R Core Team 2021). Because the Bonferroni correction was made during the statistical analyses, the results of the analyses were given in adjusted p-values, and therefore, statistically significant results were considered when $p < 0.05$.

Regression analyses were performed between RGR and salinity to obtain the optimal salinity for growth for each species as adults and germlings and to calculate the maximum RGR based on the model regression. When the relationship between RGR and salinity was linear, a simple linear regression was conducted using the "lm()" function in R. If the relationship was non-linear, different degrees of polynomial equations (second, third, fourth and fifth degree) were tested using the function "lm()" to determine the best fit. In most cases, a second-degree polynomial function ($y = aX^2 + bX + c$) provided the best non-linear fit. Once the best fit was determined, the coefficients of the fit were extracted using the "coef()" function, and the polynomial function was used to calculate a model of RGR at each salinity between 10 and 30 PSU. From these data, the maximum RGR could be extracted as well as the corresponding optimal salinity.

To assess the impact of salinity on AA of adult *U. lacinulata* over time, linear regressions were conducted using the "geom_smooth" function (package ggplot2) with the method "lm" and the grey areas show the range of the confidence intervals.

Results

Molecular identification of species using tufA gene sequence

The molecular identification showed that the species of *Ulva* collected in the NE-Atlantic were *U. lacinulata* and *U. linza*, while the species collected in the Mediterranean were identified as *U. lacinulata* and *U. californica/flexuosa* complex (hereafter reported as "*U. flexuosa*") (Table S2).

Foliose material of the NE-Atlantic and the Mediterranean (AWI stock culture no 1290) were identified as *U. lacinulata* as sequences were > 99% identical with the type of *U. lacinulata* (Hughey et al. 2022). Tubular material of the NE-Atlantic was identified as *U. linza* as sequences were > 99% identical with several specimens previously identified as *U. linza* (e.g., JN029337; MH475449). Tubular material of AWI stock culture no 1262 from the Mediterranean Sea was identified as belonging to the *U. flexuosa*-complex as sequences showed > 98% similarity with sequences identified as such species. As there is an unclear taxonomic status of several of the GenBank entries we refer to these specimens as a complex (Steinhagen et al. 2019a). As this study was not intended to elaborate on phylogenetic or systematic relations, and since sequences of respective type material are absent, we cannot clearly delimit such individuals to a distinct species and therefore this material is referred to as belonging to the wider *U. flexuosa*-complex in the scope of this study.

Effect of salinity on the growth rate of adult Ulva spp.

In general, the two-way PERMANOVA did not show a significant interaction between salinity and species ($p > 0.05$). However, one-way PERMANOVAs performed for each species showed significant results.

The two foliose strains of *U. lacinulata* had significantly higher growth rates than the tubular species *U. linza* and *U. flexuosa* ($p < 0.01$) (Fig. 1, Table S3). The RGR of *U. flexuosa* was the same in all salinity treatments (Fig. 1a, Table S3) while the other strains showed significant variations in RGR under the different salinity treatments (Fig. 1, Table S3). When comparing the mean RGR of each species at the lowest salinity tested (10 PSU) the tubular species (*U. linza* and *U. flexuosa*) had low RGRs of 2.3% day^{-1} and 0.9% day^{-1}, respectively (Fig. 1a-b) while the foliose strains of *U. lacinulata* showed two–sixfold higher mean RGRs of 5.6% day^{-1} and 6.2% day^{-1}, respectively (Fig. 1c-d). The optimal salinity range for growth in the foliose strains was between 20 and 30 PSU ($p < 0.05$). The lowest growth rates for these strains were observed at 10 PSU, although growth rates were not always significantly different from the other treatments (Table S3).

As *U. flexuosa* did not show a trend in growth along the salinity gradient, regression analysis was only performed for the other three strains (Fig. 1b-d). The optimal salinity for growth of adult *U. linza* was 21 PSU with a maximum RGR of 5.5% day^{-1}. The Mediterranean and NE-Atlantic strains of adult *U. lacinulata* would grow optimally at 28 PSU with a maximum RGR of 15 and 16.9% day^{-1}, respectively.

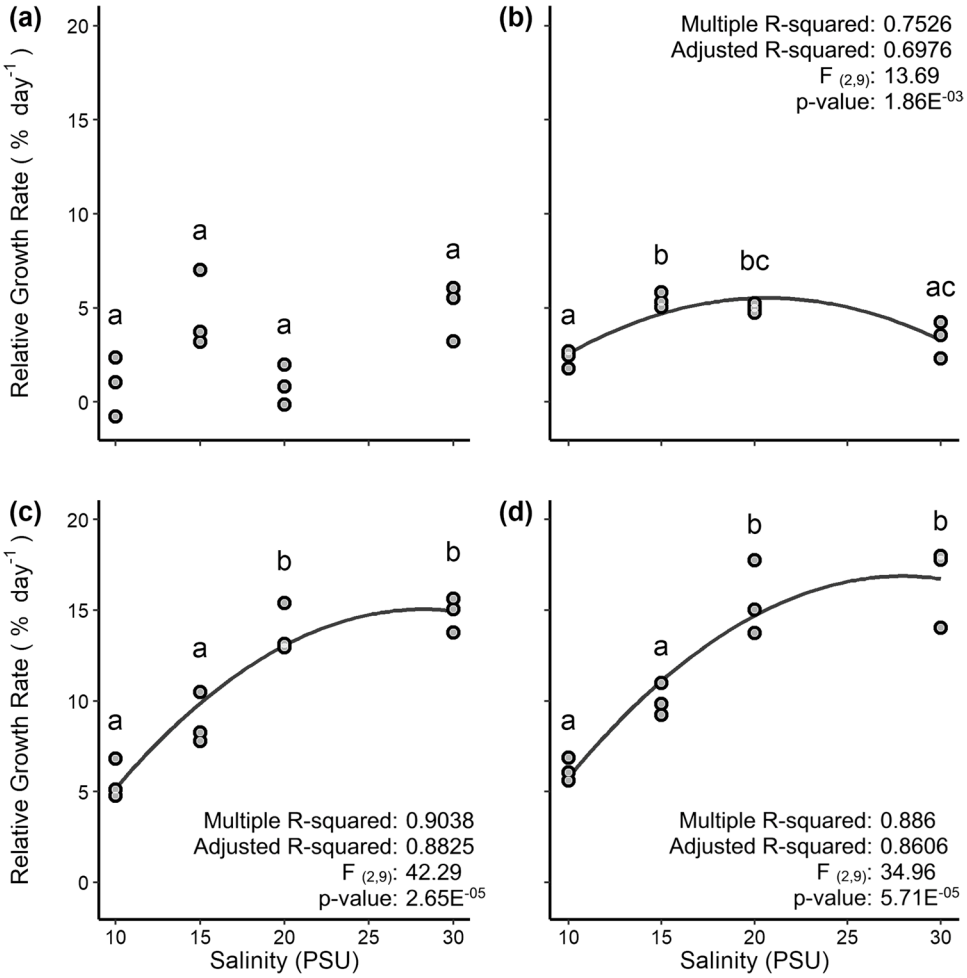

Fig. 1 Relative growth rate (RGR % day $^{-1}$) of adult *Ulva* spp. after 2 weeks of exposure to different salinity conditions (n = 3). Regression analysis performed for three of the four strains. *U. flexuosa* did not present a clear trend. (**a**) *U. flexuosa*, (**b**) *U. linza*, (**c**) *U. lacinulata* (Mediterranean), (**d**) *U. lacinulata* (NE-Atlantic). One-Way PERMANOVA and pairwise comparison between salinity treatments (with Bonferroni correction); statistically significant differences between treatments are represented by different lower case letters

Effect of salinity on the growth rate of Ulva spp. germlings

Overall, the germlings from the tubular species had significantly higher growth rates than the foliose strains ($p < 0.01$). The two-way PERMANOVA reported a significant interaction between salinity and species ($p < 0.05$) that can be seen between 20 and 30 PSU, where both strains of *U. lacinulata* showed an increase in RGR while *U. linza* showed a decrease. Germlings from the two tubular species did not show a significant growth response to salinity (Fig. 2a-b). *Ulva flexuosa* germlings showed a non-significant trend and the mean RGRs ranged between 7.1 and 13.1% day^{-1}, with highest RGR at 30 PSU (Fig. 2a). The mean RGR of *U. linza* germlings ranged from 14.7% day^{-1} and 15.0% day^{-1} in the salinity treatments with a non-significant reduction of RGR at 30 PSU (14.7% day^{-1}) (Fig. 2b).

In both the Mediterranean and NE-Atlantic foliose strains of *U. lacinulata*, the germling RGR was significantly higher in 30 PSU compared to lower salinities ($p < 0.05$) (Fig. 2c-d, Table S4). The Mediterranean strain presented mean RGRs ranging from 3.7% day^{-1} to 9.0% day^{-1} growing similarly high at 30 and 20 PSU (9% day^{-1}) and significantly lower at 10 compared to 30 PSU while growth rate at 15 PSU was the same as in 10 and 20 PSU ($p > 0.05$) (Fig. 2c). At the highest salinity (30 PSU) the NE-Atlantic *U. lacinulata* had the highest RGR which was significantly different from all other treatments. At 20 PSU the RGR of this strain was significantly different from the 10 PSU and the 30 PSU treatments (Fig. 2d, Table S4). The mean RGRs for the foliose NE-Atlantic strain varied between 4.6% day^{-1} and 13.9% day^{-1}.

Ulva flexuosa and *U. linza* germlings did not show significant differences in growth over the salinity gradient and regression analysis was thus only performed for the Mediterranean and NE-Atlantic strains of *U. lacinulata*,. The model results revealan optimal salinity for growth at 30 PSU with a maximum RGR of 8.4 and 14% day^{-1}, respectively (Fig. 2c-d). This is in accordance with the mean averages obtained during the experiment, which also showed that the maximum RGR for both strains was at 30 PSU (Fig. 3).

The lowest and highest RGRs obtained for each species at each life stage (as germlings and adults) are summarized in Fig. 3. In three out of the four strains, the maximum RGR for both germlings and adults was at 30 PSU, while the

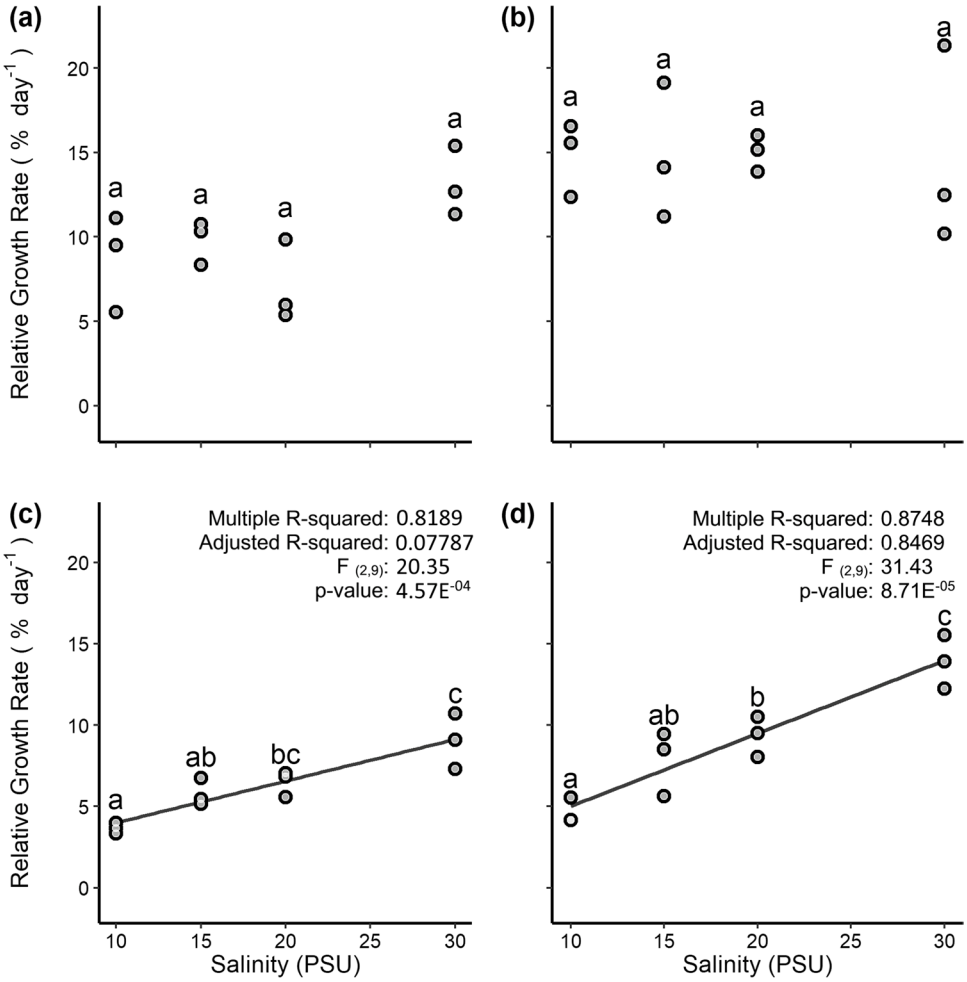

Fig. 2 Relative growth rate (RGR % day $^{-1}$) of germlings of *Ulva* spp. after 3 weeks of exposure to different salinity conditions (n = 3). Regression analysis performed for two of the four species. *U. flexuosa* did not present a clear trend. (**a**) *U. flexuosa*, (**b**) *U. linza*, (**c**) *U. lacinulata* (Mediterranean), (**d**) *U. lacinulata* (NE-Atlantic). One-Way PERMANOVA and pairwise comparison between salinity treatments (with Bonferroni correction); statistically significant differences between treatments are represented by different lowercase letters

minimum RGR was observed at 10 PSU (for both strains of *U. lacinulata*) and 20 PSU (for *U. flexuosa*). *Ulva linza* was the exception with the maximum RGR at 20 PSU (15% day^{-1}) and a minimum at 30 PSU (14.7% day^{-1}) for the germlings and a maximum RGR at 15 PSU (5.4% day^{-1}) and a minimum at 10 PSU (2.3% day^{-1}) for the adults. When comparing the two *U. lacinulata* strains, the NE-Atlantic strain grew best at 30 PSU with 13.9% day^{-1} compared to the highest RGR of 9.0% day^{-1} for the Mediterranean strain in the same treatment. The NE-Atlantic *U. lacinulata* reported a minimum RGR at 10 PSU of 4.6% day^{-1} while the Mediterranean strain reported only a minimum RGR of 3.7% day^{-1} (Fig. 3).

Effect of salinity on the antioxidant activity of adult Ulva lacinulata (NE-Atlantic)

Over time, a decrease in AA took place at 15 and 20 PSU treatments, showing a statistically significant inverse linear relationship between these treatments and AA ($p < 0.05$ and $p < 0.01$, respectively). The AA content stayed the same over time and on a high level at 10 and 30 PSU ($p_{10} = 0.74$, $p_{30} = 0.93$) (Fig. 4).

In the lowest salinity treatment (10 PSU), the results show a slight increase in AA over time; (Fig. 4). In contrast, the AA remained constant at 30 PSU. After 120 h the algae exposed to the 10 PSU treatment had 23%, 27% and 2% higher AA than the algae grown at 15, 20 and 30 PSU, respectively. After 240 h the algae exposed to 10 PSU had 42%, 146% and 44% higher AA than the algae grown at 15, 20 and 30 PSU, respectively.

Discussion

Recirculating aquaculture systems potentially offer an innovative method for cultivating high-quality macroalgae on a large-scale, but it is essential to consider the weaknesses and try to overcome them. Using salinity as a tool for strain selection and biomass optimization, we aimed to show that reducing the costs of production was possible and optimization of certain functional traits could be achieved.

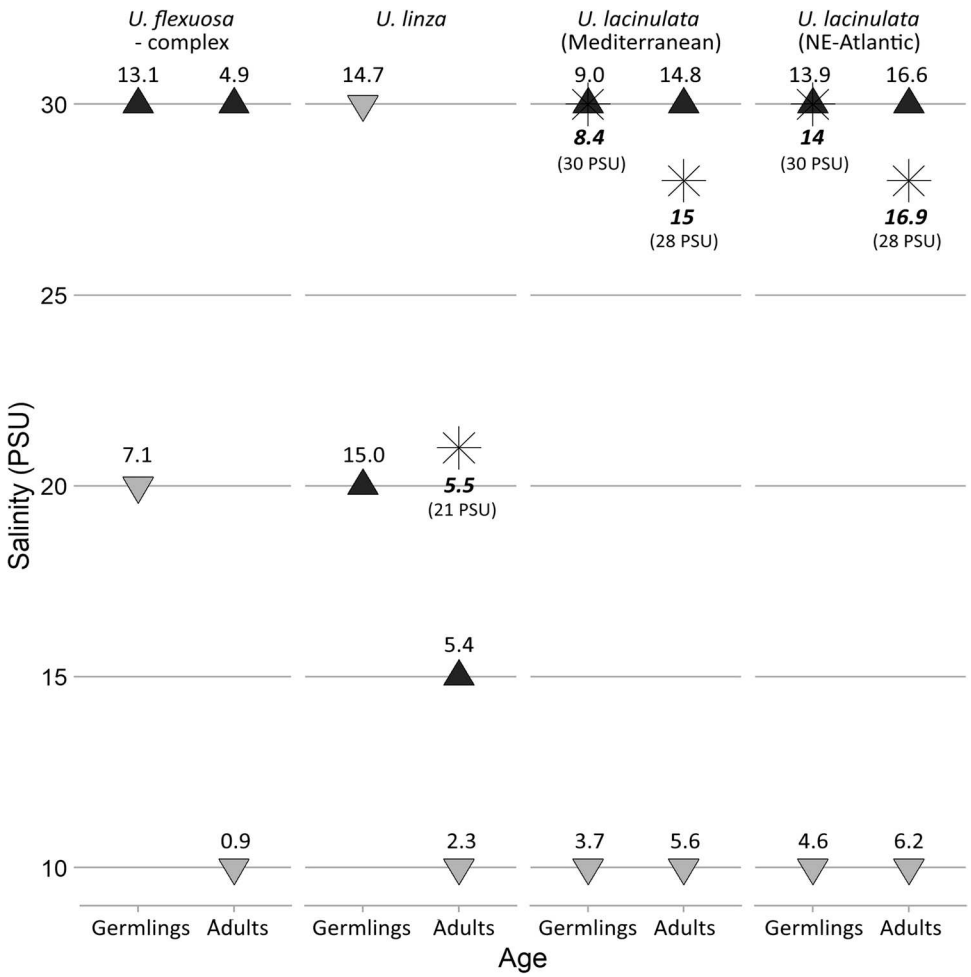

Fig. 3 Salinity treatments in which the lowest and highest RGR were measured in adults and germlings (n = 3). Results are shown as the mean of the replicates. Dark grey triangle: maximum RGR observed during the experiments; light grey upside down triangle: minimum RGR observed during the experiments; black star: the maximum RGR of each species when grown at the optimal salinity (based on the regression model). The values of the RGRs in each treatment are indicated above each point. Results from the regression analysis indicating the RGRs when seaweeds are grown at their optimal salinity are indicated in bold and italic below each point

As a first step for strain selection, in this work we chose warm-temperate strains adapted to a wide range of temperatures to reduce costs of temperature regulation in land-based systems, and adaptation to high maximum summer temperatures reduces the energy required for cooling the RAS during extreme heat waves in summer. The experiments reported in this work are the second and third steps for strain selection and optimization.

The results showed that the adult material of both strains of foliose *U. lacinulata* grew fastest in higher salinities and had the highest growth rates throughout the different treatments compared with the tubular strains. However, among the four strains tested, tubular *U. linza* was the species that performed best at low salinity. Nevertheless, its RGR did not reach 7% of daily biomass increase (fresh weight) that is necessary for large-scale production (Huguenin 1976). Therefore, based on our first observations, its potential for large-scale cultivation is limited. At optimal salinity (21 PSU) this species would only achieve a RGR of 5.5% day $^{-1}$. Alternatively, *U. lacinulata* proved to be a good candidate as the production of the two strains always exceeded the 7% threshold at 15 PSU (half of the highest salinity tested). Based on the regression analysis performed, the lowest salinity possible for cultivation (without crossing the 7% threshold) is 12 PSU. The reduction in salinity from 30 to 12 PSU would amount to a reduction in the salt cost of 60%. However, these results should be taken with caution because the 7% threshold was determined in 1976 and the current threshold necessary for a profitable return may be higher. However, for optimal RGRs, both strains of *U. lacinulata* should be cultivated between 20 and 30 PSU.

Still, the results of this work are limited to two weeks of growth in the different treatments. After 3 weeks a slight decline in growth was observed (Fig. S2). This decline might be associated with a nutrient limitation in the beakers caused by the increase in biomass. Based on our previous experiments, a minimum of 3 weeks is required to estimate how *Ulva* is impacted by the changes in the cultivation conditions. Therefore, further work needs to be carried out to understand the long-term impact that the respective treatments might have during extended cultivation periods. The biomass increase should be taken into consideration during the experiment so adjustments on the nutrient concentration and the vessel sizes can be made.

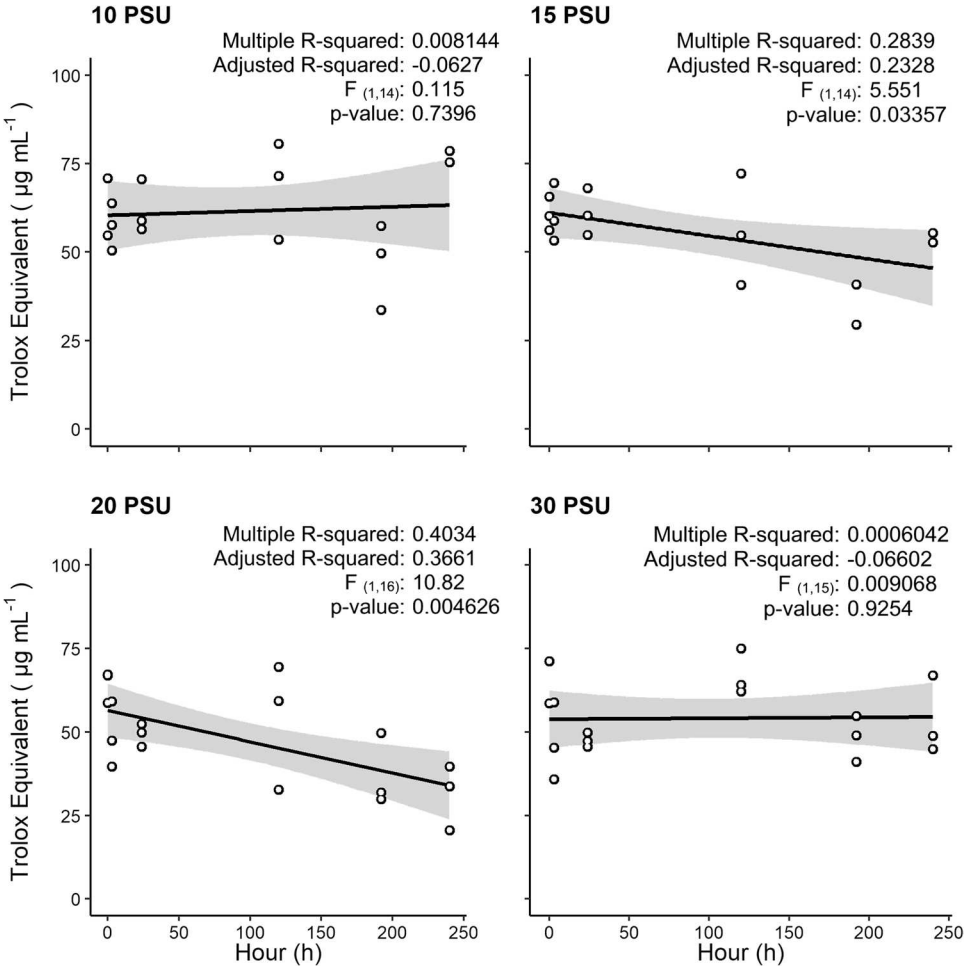

Fig. 4 Antioxidant activity based on the antioxidant concentration (Trolox Equivalent in µg mL^{-1}) of adult *U. lacinulata* (NE-Atlantic) under different salinity conditions over 10 days (samples taken at 0 h, 3 h, 24 h, 120, 192 h, and 240 h) (n = 3). Linear regressions were performed for each salinity treatment. Grey areas show the range of the confidence intervals. Each point represents a replicate. Treatment at 20 PSU considered as control

It is also important to mention that the species *U. lacinulata* presents a challenge for industrial-scale production. This species grew mostly vegetatively during this work and it was difficult to induce sexual reproduction. Therefore, guaranteeing new material and genetic variability can be a challenge and the control of the initial stages of development (e.g., germlings) might not be applicable. Furthermore, the possibility of hybridization experiments and strain optimization becomes more difficult because they often depend on sexual reproduction. Before considering the implementation of species as *U. lacinulata* (without a controlled reproduction cycle) in a large-scale system, the development of new methods should be considered, for example, economically feasible protoplast isolation (Reddy et al. 1992; Gupta et al. 2018).

Under nursery conditions, germlings of the four *Ulva* strains showed a different response to salinity compared to those of their adult counterparts. During germling development, salinity conditions were not as important as during the adult stage. This suggests that the germlings have a broader salinity tolerance than the adults and that salinity does not play an important role on germling development, especially in the germlings of the tubular species.

Germlings of tubular species showed higher RGR than the germlings of the foliose strains (*U. lacinulata*). Thus tubular species are a good candidate for cultivation with short harvesting intervals and may lead to a fast production with high turnover. Based on our results *U. linza* should be grown as a germling at 15 PSU and be transferred to 21 PSU at its adult stage. One example of an already established cultivation based on a tubular species of *Ulva* is the wild collection of gut weed (*Ulva intestinalis* Linnaeus) that represented 63.6% of the world's wild cultivation of *Ulva* spp. in 2019 (FAO 2021). For a long time, *U. intestinalis* has been collected and cultured for local consumption in Malaysia, the Philippines and Indonesia. At the same time, other tubular species such as *U. compressa*, *U. flexuosa* and *U. prolifera* have been reported to be widely used throughout the world as food, feed, fertilizer, and medicine demonstrating the economic interest in cultivating tubular species of *Ulva* (Prud'homme van Reine and Trono 2001).

For seedling and nursery purposes, the rapid development of new generations to guarantee the re-seeding process of the tanks and continuous production is essential. For that reason, tubular species should be considered as a good candidate at

this moment of their development (as germlings). Despite the rapid growth of the tubular species it should be mentioned that at 30 PSU the germlings of the NE-Atlantic strain of *U. lacinulata* had a similar growth rate to the ones of the tubular species therefore being a good candidate as well. Consistent with our results, another study has shown that temperature and light proved to be more important factors than salinity (and even nutrients) to promote growth in germlings of the tubular species *U. intestinalis* (Kim et al. 2021). In our work, both temperature and light were assumed to be optimal. The temperature setting was defined based on the average temperature registered in the natural environment of the species (Table S2) while the light setting was determined by the literature of work performed with several *Ulva* spp. (Fortes and Lüning 1980; Toth et al. 2020; Wang et al. 2020).

Concerning the different morphologies, in this work we showed that tubular and foliose strains had distinctively different growth rates (both as germlings and adults), even though, as germlings, the morphology is identical between species. This suggests that the tolerance for lower salinity environments is already present in early stages of the germlings' development, and it is not dependent on the current morphology, at least not at the germling stage. Therefore, despite similar morphologies during the germling stage, germlings from tubular species thrive in low salinity, while germlings originating from foliose species show lower RGRs. Nevertheless, *Ulva* is known for its capacity to change between different morphologies. This has been observed and studied both under laboratory conditions (Provasoli and Pintner 1980; Matsuo et al. 2005; Spoerner et al. 2012; Wichard 2015; Wichard et al. 2015) and under natural conditions in New England, the German North Sea, and the Baltic Sea (Hofmann et al 2010; Steinhagen et al. 2019b). Tan et al. (1999) also discovered the presence of foliose *Ulva compressa* Linnaeus (usually found in its tubular form) in brackish water in Scotland. Moreover, similar specimens were found in the Wadden Sea in areas with a salinity range between 30 and 33.5 PSU and in environments with drastic changes in temperature and salinity (e.g., basins and drain channels). Tubular specimens, however, were rare in such conditions (Steinhagen et al. 2019b). In Steinhagen et al. (2019b) it was suggested that the foliose morphotype of *U. compressa* was not as limited by salinity as its tubular morphotype. In another work, it was suggested that the reduced RGR of germlings of *Ulva fasciata* Delile (a foliose species) in low salinities could be related to a reduced cell viability. This reduced cell viability is unlikely to occur in cells from species that are known to live and strive in low salinity environments (e.g., tubular species) (Chen and Zou 2015).

In accordance with previous in situ observations, our results suggest that the tubular species grow better at low salinities than the foliose species (Rybak 2018). In situ, foliose species are not present in fresh-water (< 0.5 PSU) or oligohaline habitats (0.5–5 PSU), but tubular species reside in habitats ranging from < 0.5 PSU to 50 PSU (Rybak 2018). In contrast, foliose *Ulva* species are mostly present in areas with salinities ranging from 18 to 40 PSU (Rybak 2018). Similarly as reported here, optimum salinity for growth and photosynthetic activity ranged between 20 and 35 PSU for foliose species and between 10 and 32 PSU for tubular species in other laboratory studies (Choi et al. 2010; Chen and Zou 2015; Xiao et al. 2016; Li et al. 2017; Bastos et al. 2019; Bews et al. 2021; Kim et al. 2021).

Because of the similarities between *Ulva* species and the fact that their tubular and foliose morphology can change depending on the environment (Hofmann et al 2010; Steinhagen et al. 2019b), morphological identification can result in incorrectly identified species and wrong conclusions if species identification is not supported by molecular identification (Steinhagen et al. 2019b).

The response of different life phases of *Ulva* to salinity differs to other macroalgae groups. Germlings of brown macroalgae of the genus *Alaria esculenta* (Linnaeus) Greville, *Undaria pinnatifida* (Harvey) Suringar and *Saccharina latissima* (Linnaeus) C.E.Lane, C.Mayes, Druehl & G.W.Saunders were more sensitive to changes in salinity than adults (Fredersdorf et al. 2009; Peteiro and Sánchez 2012). In contrast to other green and brown algae such as *Caulerpa sertularioides* (S. G. Gmelin) M. Howe, *Caulerpa brachypus* Harvey and *U. pinnatifida* (van Ginneken 2018), *Ulva* (and its germlings) has one of the highest reported salinity tolerances by its ability to change the K^+, Na^+, and Cl^- in response to salinity variations. This tolerance might also be associated with the antioxidant defence mechanisms present in *Ulva* (van Ginneken 2018).

Similarly as important as growth rates, the biochemical bouquet of *Ulva* at time of harvest might be important to increase the quality of the product (Lu et al. 2006; Luo and Liu 2011). The antioxidant activity experiment with the NE-Atlantic strain of *U. lacinulata* in the different salinity treatments showed that a reduction in salinity can cause an increase in AA in adult *U. lacinulata*. Over a period of 10 days, at 10 and 30 PSU the AA concentration kept stable and on a high level (increasing slightly at 10 PSU) while it decreased at 15 and 20 PSU.

After being acclimated for several weeks at 20 PSU, the NE-Atlantic strain of *U. lacinulata* showed a reduction in AA when exposed to the same salinity during the 10 days of the experiment. The high RGR and the reduction in AA suggest that this salinity treatment was not stressful to this strain (Lu et al. 2006; Luo and Liu 2011).

For this experiment the number of measures taken in the beginning was higher (0, 3, and 24 h) to examine the immediate reaction of the alga to the treatments. Daily variation has been described in brown macroalgae, suggesting that shorter intervals between samples could guarantee more accurate

data and accurate comparisons between samples from different days (Abdala-Díaz et al. 2006; Connan et al. 2007). As we always measured AA at the same time from day 1 onwards, we avoided potential variance due to diurnal changes. Future work should consider taking measures in short intervals (e.g., every few hours) to detect daily variations and the simultaneous use of multiple methods for AA measurements (Chakraborty and Paulraj 2010; Magnusson et al. 2015).

Our work corroborates previous studies suggesting that *Ulva* is a promising candidate for on-shore productions in general, including both RAS and integrated multi-trophic aquaculture (IMTA) systems (Cohen and Neori 1991; Neori et al. 2003; Cahill et al. 2010; Ladner et al. 2018). Considering the similarities between the two systems, selecting strains for production in a low salinity RAS system might also be beneficial for IMTA production at low salinity.

Conclusion

Although we showed that *U. linza* grew best at low salinity of 15 PSU, we would suggest *U. lacinulata* as a good candidate for a land-based recirculating system with artificial seawater. Although the optimal growth takes place at 28 PSU, even a reduction of artificial seawater by 2 PSU would reduce costs by 6,7%. An additional cost reduction by 33.3% could be achieved if using 20 PSU as growth was not significantly reduced in *U. lacinulata* and did not create antioxidative stress. To achieve an optimized cultivation of this strain, the best conditions for its growth should be at 30 PSU (for germlings) and at 28 PSU (for adults). A 60% reduction on salinity costs is possible until 12 PSU without crossing the 7% threshold of necessary daily biomass increase. However, these results should be taken with caution because the 7% threshold was determined in 1976 and the current threshold necessary for a profitable return may be higher. Additionally, higher antioxidant activity can be achieved by reducing the salinity to 10 PSU for 10 days, suggesting that the functional traits of cultivated *Ulva* spp. can be optimized prior to harvest.

Despite lower growth rates in general, the tubular species *U. linza* showed optimal growth rates at 15 PSU and 21 PSU as germlings and adults, respectively, and could also be a good candidate for low salinity systems with a more regular harvesting period (for higher turnover).

In future work *U. lacinulata* should be tested in a large-scale setting to validate our findings. Different abiotic factors such as temperature and light intensity should be tested to further increase quality and productivity. Hybridization experiments with *U. lacinulata* could potentially guarantee the development of a highly productive and trustworthy strain, considering the different strains exhibited different growth rates in our study. However, considering the difficulty found in inducing sexual reproduction in this species, the development of new and economically feasible methods of inducing and controlling reproduction must be developed (e.g., protoplast isolation) before further hybridization experiments can be tested.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10811-023-02960-x.

Acknowledgements The authors would like to thank Sabine Strieben, Claudia Daniel, and Andreas Wagner for laboratory supplies and technical support, and Dr. Sotiris Orfanidis, Greece for providing the two Mediterranean *Ulva* strains used in this work. Additionally, we thank Rebecca Aepfler for her support in complying with the regulations of the Nagoya Protocol.

Authors contributions IC, AM, AS and LCH conceptualized and designed the studies, IC drafted the manuscript, LCH supervised the studies, IC, AM, and AS carried out the studies, collected and analyzed the data. SST conducted the molecular identification of the strains used in this manuscript. LCH, IB, KV, and SST provided technical and scientific supervision, BB & IB provided lab facilities and administrative support, BB & LCH obtained funding for this project, and LCH, BB, IB, KV, and SST critically revised the manuscript. All authors approved the manuscript for publication.

Funding Open Access funding enabled and organized by Projekt DEAL. This research was supported within the framework of the MakPak Scale-Up Project by funds from the Federal Ministry of Food and Agriculture (BMEL) based on a decision of the parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support program (Funding Reference Number 281DL02B20). The authors furthermore thank the Formas-funded project 'A manual for the use of sustainable marine resources' (Grant no. 2022–00331) for financial support.

Data availability The raw data used in this work is submitted in PANGAEA and cited in the supplementary material (Cardoso et al. 2023a, 2023b, 2023c, 2023d, 2023e).

The molecular sequences of the species presented in this work are deposited in GenBank with the accession numbers: OP778143 (NE-Atlantic *Ulva lacinulata*), OP778144 (Mediterranean *Ulva lacinulata*), OP778145 (NE-Atlantic *Ulva linza*), OP778146 (Mediterranean *Ulva flexuosa*-complex).

Declarations

Nagoya Protocol We have written confirmation by the *Instituto da Conservação da Natureza e das Florestas (ICNF)* in its function as ABS National Focal Point as well as Competent National Authority that although Portugal is party to the Nagoya Protocol no national legislation nor any regulatory requirements drawing from the Nagoya Protocol for access to genetic resources in mainland Portugal exist presently. As the samples for this project were collected in mainland Portugal, there are no applicable prior informed consent requirements. The Greek *Ulva* material was isolated in 1986 (AWI culture number 1262) and in 1967 (AWI culture number 1290). Hence, the samples were taken before the Nagoya Protocol came into force in 2014. Although Regulation EU-No. 511/2014 does not apply accordingly, we complied with our due diligence by asking the Greek National Focal Point about national ABS permit requirements and were granted access with a research permit for flora (RECALL/Δ ΡΔ/12548/797) issued by Ministry of The Environment & Energy- GDD & DP—Forest Protection Directorate).

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

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Supplementary Information

Table S1 - List of published Recirculating aquaculture systems (RAS) working with seaweed (based and adapted from Ramli et al., 2020).

Species	**System**	**Overview**	**Indoor/ Outdoor**	**Reference**
Caulerpa lentillifera J. Agardh	Closed recirculating Integrated Multi-Trophic Aquaculture (IMTA) system (fish+macroalga)	*C. lentillifera* was used as an effective biofilter of aquaculture effluents	Outdoor	Bambaranda et al. 2019
Ulva lactuca Linnaeus [a]	Closed recirculating IMTA system (prawns+macroalga)	*U. lactuca* removed very efficiently the inorganic nutrients and dissolved inorganic nitrogen	Indoor	van Khoi and Fotedar 2011
Ulva lactuca [a] and *Undaria pinnatifida*	Recirculating IMTA system (fish+macroalga)	Macroalga kept ammonium at lower concentrations and can improve the efficiency and productivity of recirculating aquaculture	Indoor	Cahill et al. 2010
Ulva sp. [b] and *Cladophora* sp.	HRAP + Recirculating fish rearing system	The HRAP decreased the dissolved inorganic nitrogen and the phosphate concentration by 25% and 9%, respectively, over one year.	Outdoor HRAP	Deviller et al. 2004

[a] *- species taxonomically debated, currently accepted as U. fenestrata* (Hughey et al. 2019); [b] *- includes species at the time identified as belonging to the genus Enteromorpha*

Table S2 - Morphology and origin of the species collected for this work.

Species name	General morphology of the thallus	Origin		Abiotic Conditions In the collection sites				References
		Location	GPS coordinates	Temperature	Salinity	Light (daylength)	Irradiance -based on Global Horizontal Irradiation data	
U. flexuosa	tubular	Mediterranean Thessaloniki Bay, Greece	40°33'57.4"N 22°57'28.0"E	Max.: 27 °C Min.: 7°C	Max.: 38.5 PSU Min.: 35.5 PSU	Max.:15h05 Min.: 09h16	623.5 to 645 µmol photons m^{-2} s^{-1} 290 to 300 W m^{-2}	(Haritonidis 1978; Kambezidis 2021)
U. linza	tubular	NE-Atlantic Óbidos Lagoon, Portugal	39°23'41.5"N 9°12'48.9"W	Max.: 24 °C Min.: 6 °C	Max.: 35 PSU Min.: 25 PSU	Max.: 14h57 Min.: 09h24	392.69 µmol photons m^{-2} s^{-1}	(Cavaco et al. 2016; Mendes et al. 2021)

							1600 kWh m^{-2} (annually)	
U. lacinulata	foliose	Mediterranean Thessaloniki Bay, Greece	40°34'11.2"N 22°57'12.5"E	Max.: 27 °C Min.: 7°C	Max.: 38.5 PSU Min.: 35.5 PSU	Max.: 15h05 Min.: 09h16	623.5 to 645 µmol photons m^{-2} s^{-1} 290 to 300 W m^{-2}	(Haritonidis 1978; Kambezidis 2021)
U. lacinulata	foliose	NE-Atlantic Óbidos Lagoon, Portugal	39°23'41.5"N 9°12'48.9"W	Max.: 24 °C Min.: 6 °C	Max.: 35 PSU Min.: 25 PSU	Max.: 14h57 Min.: 09h24	392.69 µmol photons m^{-2} s^{-1} 1600 kWh m^{-2} (annually)	(Cavaco et al. 2016; Mendes et al. 2021)

Effect of Different Nutrient Media on the Growth Rate of Adult Ulva

Another experiment was run to compare the effect of the nutrients on the RGR of adult *Ulva* to guarantee the veracity of the statistical results from the salinity treatments. We compared the RGR (% day^{-1}) after three weeks of *Ulva* treated with Blaukorn fertilizer against the RGR of *Ulva* treated with half-strength Provasoli's. The statistics showed no significant differences between treatments in three of the four species. The exception is *U. linza* which presented a significant difference ($p = 0.024$)) with higher RGR while growing with ½ PES.

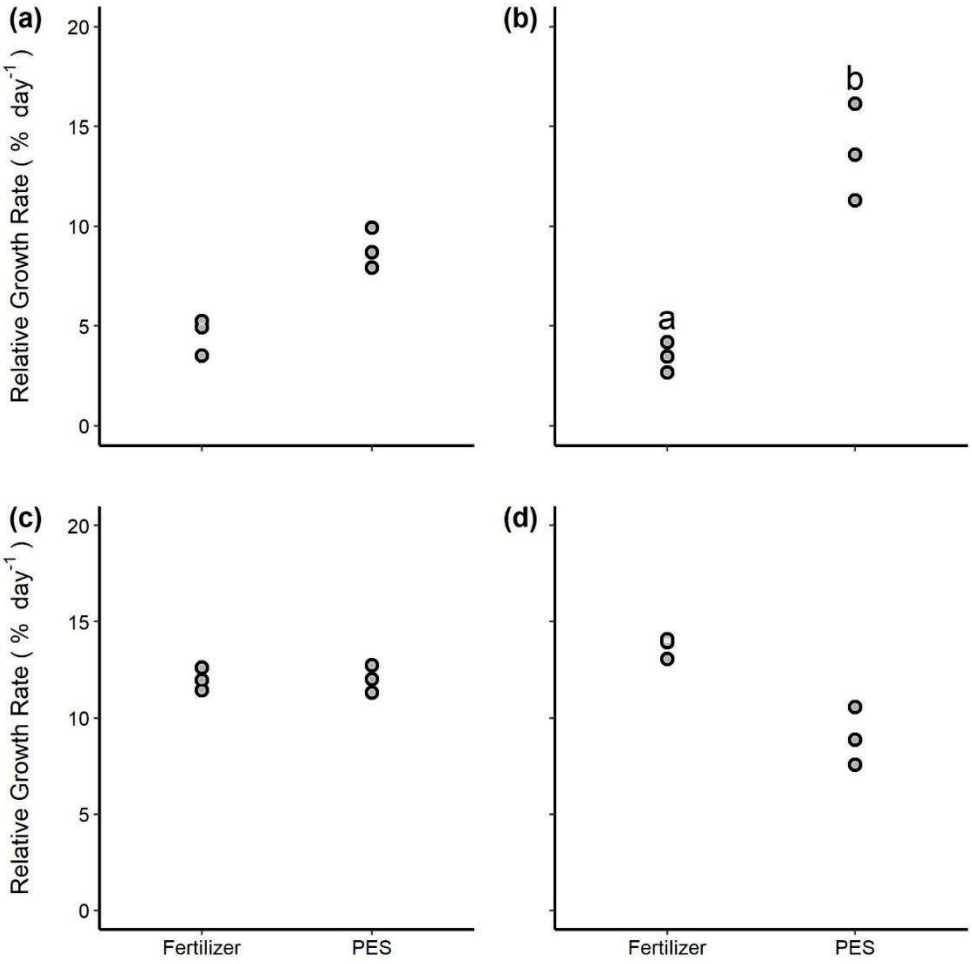

Fig. S1 Relative growth rate (RGR % day $^{-1}$) of adult *Ulva* spp. after exposure to different nutrients (n = 3). One-Way PERMANOVA and pairwise comparison between salinity treatments (with

Bonferroni correction). (a) *U. flexuosa*, (b) *U. linza*, (c) *U. lacinulata* (Mediterranean), (d) *U. lacinulata*_(NE-Atlantic). "Fertilizer" - commercial fertilizer + natural seawater. "PES" - 1/2 strength PES + artificial seawater. Statistically significant differences between treatments are represented by different letters

Statistical analysis from salinity experiment with adults of Ulva spp. after 2 weeks of exposure to different salinities

Table S3 Results of the One-Way PERMANOVA analysis of the effect of salinity on relative growth rates of adult Ulva spp. after 2 weeks of exposure (n = 3) and pairwise comparisons between salinity treatments. "Adj. p": adjusted p-value after Bonferroni correction. Statistically significant differences between treatments are presented in bold

	U. flexuosa		***U. linza***		***U. lacinulata* (Mediterranean)**		***U. lacinulata* (NE-Atlantic)**	
Contrast between treatments	F-exp $_{1,8}$	Adj. P	F-exp $_{1,8}$	Adj. P	F-exp $_{1,8}$	Adj. P	F-exp $_{1,8}$	Adj. P
10-15 PSU	4.18	0.10	20.56	**0.01**	5.34	0.08	4.26	0.12
10-20 PSU	0.00	1.00	15.63	**0.01**	33.97	**0.01**	25.21	**0.01**
10-30 PSU	4.87	0.13	2.41	0.45	42.65	**0.01**	31.46	**0.01**
15-20 PSU	4.18	0.17	0.34	1.00	12.37	**0.01**	8.74	**0.04**
15-30 PSU	0.03	1.00	8.89	**0.04**	17.81	**0.01**	12.57	**0.01**
20-30 PSU	4.87	0.16	5.76	0.10	0.49	1.00	0.35	1.00

Effect of Salinity on the Growth Rate of Adult Ulva – after 3 weeks

Between the second and the third week, the results suggest a small decline in the RGR while keeping a similar trend.

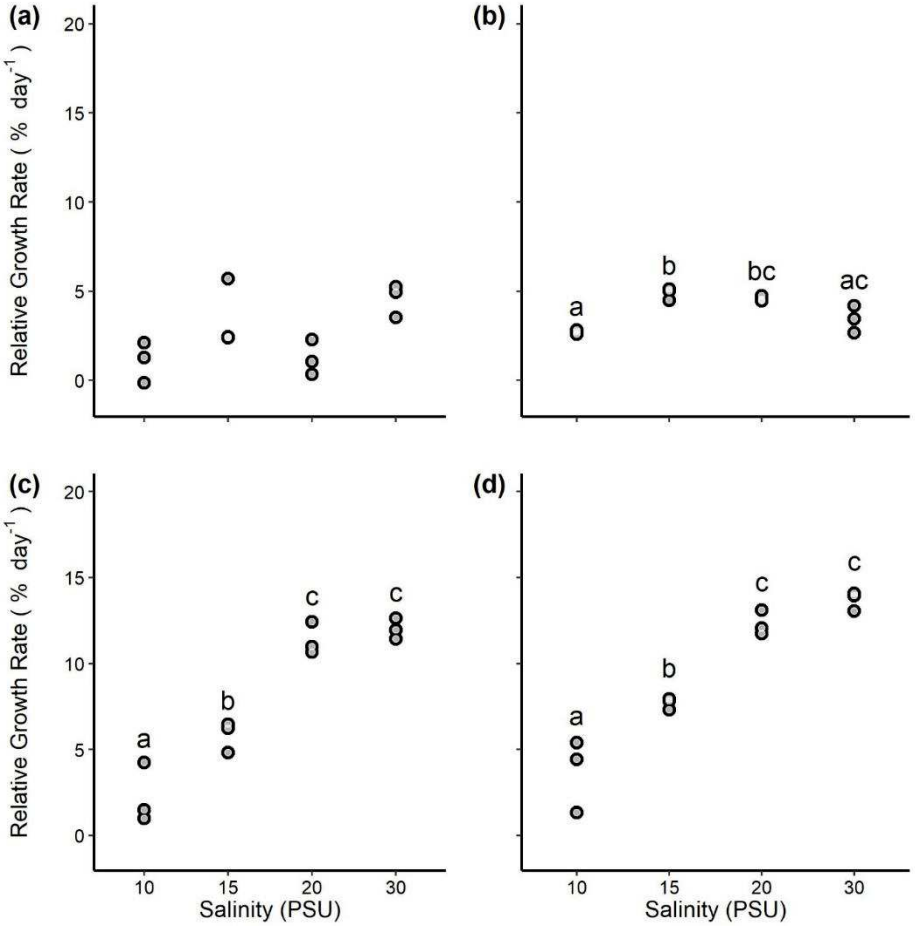

Fig. *S2* Relative growth rate (RGR % day $^{-1}$) of adult *Ulva* spp. after 3 weeks of exposure to different salinity conditions (n = 3). One-Way PERMANOVA and pairwise comparison between salinity treatments (with Bonferroni correction). (a) *U. flexuosa*, (b) *U. linza*, (c) *U. lacinulata* (Mediterranean), (d) *U. lacinulata* (NE-Atlantic). Statistically significant differences between treatments are represented by different letters

Statistical analysis from salinity experiment with germlings of Ulva spp. after 3 weeks of exposure to different salinities

Table S*4* Results of the One-Way PERMANOVA analysis of the effect of salinity on relative growth rates of germlings of *Ulva* spp. after 3 weeks of exposure (n = 3) and pairwise comparisons between salinity treatments. "Adj. p": adjusted p-value after Bonferroni correction. Statistically significant differences between treatments are presented in bold

	U. flexuosa		***U. linza***		***U. lacinulata* (Mediterranean)**		***U. lacinulata* (NE-Atlantic)**	
Contrast between treatments	F-exp $_{1,8}$	Adj. P	F-exp $_{1,8}$	Adj. P	F-exp $_{1,8}$	Adj. P	F-exp $_{1,8}$	Adj. P
10-15 PSU	0.17	1	0	1	3.12	0.25	3.59	0.19
10-20 PSU	0.42	1	0	1	5.46	**0.04**	7.62	**0.02**
10-30 PSU	2.92	0.24	0	1	19.78	**0.01**	29.23	**0.01**
15-20 PSU	1.13	0.89	0	1	0.33	1	0.75	1
15-30 PSU	1.67	0.62	0	1	7.19	**0.05**	12.33	**0.01**
20-30 PSU	5.54	0.10	0.01	1	4.45	0.10	7	**0.04**

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4. Strain optimization

Publication II

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

Isabel Cardoso, Anneke Meiβner, Laurie C. Hofmann

2024

Published in Botanica Marina, Special Issue Phylogeny and ecology of the green seaweed *Ulva,* https://doi.org/10.1515/bot-2023-0064

Research Article

Isabel Cardoso, Anneke Meißner* and Laurie C. Hofmann

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

https://doi.org/10.1515/bot-2023-0064
Received August 10, 2023; accepted February 6, 2024;
published online March 12, 2024

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

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

1 Introduction

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

Isabel Cardoso and Anneke Meißner share first authorship.

***Corresponding author: Anneke Meißner**, Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Bussestrasse 27, 27570, Bremerhaven, Germany; and University of Bremen, Bibliothekstrasse 1, 28359 Bremen, Germany, E-mail: anneke.meissner@web.de. https://orcid.org/0009-0007-4555-5981
Isabel Cardoso, Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Bussestrasse 27, 27570, Bremerhaven, Germany; and University of Bremen, Bibliothekstrasse 1, 28359 Bremen, Germany. https://orcid.org/0000-0002-8244-6465
Laurie C. Hofmann, Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Bussestrasse 27, 27570, Bremerhaven, Germany; and Bremerhaven University of Applied Sciences, An der Karlstadt 8, 27568, Bremerhaven, Germany. https://orcid.org/0000-0002-2307-3750

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

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

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

2 Materials and methods

2.1 Biomass collection

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

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

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

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

2.3 Experimental set-up

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

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

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

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

2.4 Determination of antioxidant activity

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

$$x = \frac{y - b}{a} \qquad (1)$$
$$\mathrm{TE} = \frac{A734\,\mathrm{nm} - b}{a}$$

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

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

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

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

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

2.5 Photosynthetic performance

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

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

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

2.6 Statistical analysis

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

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

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

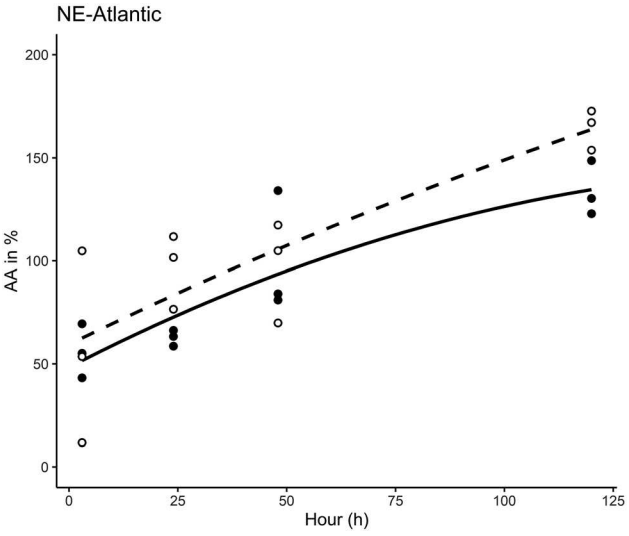

Figure 1: Antioxidant activity (% of initial) of the NE-Atlantic strain of *Ulva lacinulata* as a function of time. White circles: saturating light irradiance (SL) treatment (185 µmol photons m^{-2} s^{-1}); black circles: control (70 µmol photons m^{-2} s^{-1}). The broken line represents the regression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

3 Results

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

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

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

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

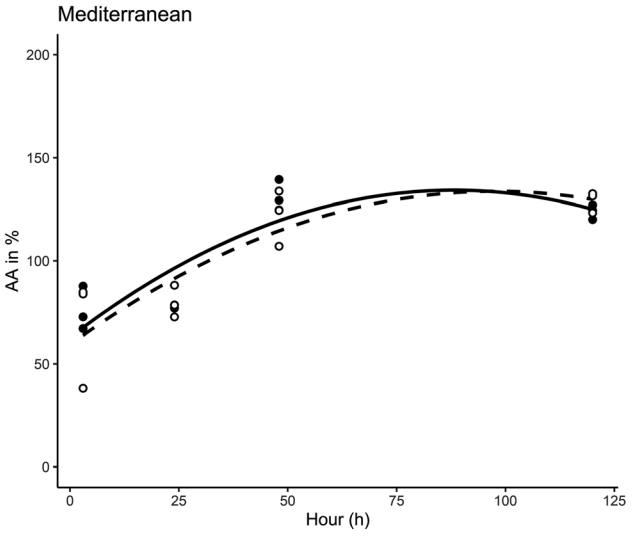

Figure 2: Antioxidant activity (% of initial) of the Mediterranean strain of *Ulva lacinulata* as a function of time. For other details see Figure 1.

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

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

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

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

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

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

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

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

SL, saturated light treatment; C, control.

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

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

SL, saturated light treatment; C, control.

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

4 Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

5 Conclusions

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

Acknowledgements: The authors would like to thank Prof. Dr. Bela H. Buck, Dr. Sophie Steinhagen, Dr. Klaus-Ulrich Valentin, Dr. Inka Bartsch for their support and helpful discussions. Moreover, we would like to thank Claudia Daniel, Andreas Wagner, Sabine Strieben, Ute Marx and Meeno Mathes for the laboratory supplies and technical support. We thank Dr. Sotiris Orfanidis, Greece for providing the Mediterranean *Ulva lacinulata* strain used in this work. Finally, we thank Rebecca Aepfler for her support in complying with the regulations of the Nagoya Protocol. Furthermore, the authors would like to acknowledge the COST Action Tomorrow's 'wheat of the sea': *Ulva*, a model for an innovative mariculture (SEAWHEAT), CA20106, supported by COST (European Cooperation in Science and Technology).

Research ethics: Not applicable.

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

Competing interests: The authors state no financial conflict of interest. Additionally, Isabel Cardoso and Laurie C. Hofmann have been collaborating with the guest editor of this special issue, Dr. Sophie Steinhagen, who is also part of the Thesis Advisory Committee of Isabel Cardoso's Ph.D. thesis. Therefore, this collaboration represents a conflict of interest. Anneke Meiβner states no conflict of interest.

Research funding: This research was supported within the framework of the Mak-Pak Scale-Up Project by funds from the Federal Ministry of Food and Agriculture (BMEL) based on a decision of the parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support program (Funding Reference Number 281DL02B20). We acknowledge support by the Open Access Publication Funds of Alfred-Wegener-Institut Helmholtz- Zentrum für Polar- und Meeresforschung.

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

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

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

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Supplementary Material: This article contains supplementary material (https://doi.org/10.1515/bot-2023-0064).

Bionotes

Anneke Meißner
Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Bussestrasse 27, 27570, Bremerhaven, Germany
University of Bremen, Bibliothekstrasse 1, 28359 Bremen, Germany
anneke.meissner@web.de
https://orcid.org/0009-0007-4555-5981

Anneke Meißner is a biology student at the University of Bremen, Germany. She did an internship and her Bachelor's thesis within the Mak-Pak Scale-Up project of the Marine Aquaculture working group at Alfred Wegener Institute Bremerhaven, Germany. After finishing an internship regarding taxonomy of brown crustose macroalgae at the Research Center in Biodiversity and Genetic Resources – Azores, Portugal, Anneke will be starting the Master's program in Biological Oceanography at GEOMAR Helmholtz Centre for Ocean Research Kiel, Germany.

Isabel Cardoso
Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Bussestrasse 27, 27570, Bremerhaven, Germany
University of Bremen, Bibliothekstrasse 1, 28359 Bremen, Germany
https://orcid.org/0000-0002-8244-6465

Isabel Cardoso is a PhD candidate of the Marine Aquaculture working group at the Alfred Wegener Institute and the Bremen University, Germany. Isabel's PhD project is under the scope of the MAK-PAK Scale-Up project and aims to investigate ways of strain selection and optimization of Ulva spp. for recirculating land-based aquaculture systems.

Laurie C. Hofmann
Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Bussestrasse 27, 27570, Bremerhaven, Germany
Bremerhaven University of Applied Sciences, An der Karlstadt 8, 27568, Bremerhaven, Germany
https://orcid.org/0000-0002-2307-3750

Laurie C. Hofmann is a marine biologist at Alfred Wegener Institute Helmholtz Center for Polar and Marine Research and professor of Applied Marine Biology at the Bremerhaven University of Applied Sciences. Her research interests include innovative uses, growth, optimization and sustainable aquaculture of macroalgae. Currently leading the strain selection, optimization and scaling-up of land-based cultivation of *Ulva* spp. for the development of food packaging within the Mak-Pak Scale-Up project funded by the German Federal Ministry of Food and Agriculture.

Supplementary Material

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

Isabel Cardoso et al.

DOI 10.1515/bot-2023-0064

Trolox calibration curve

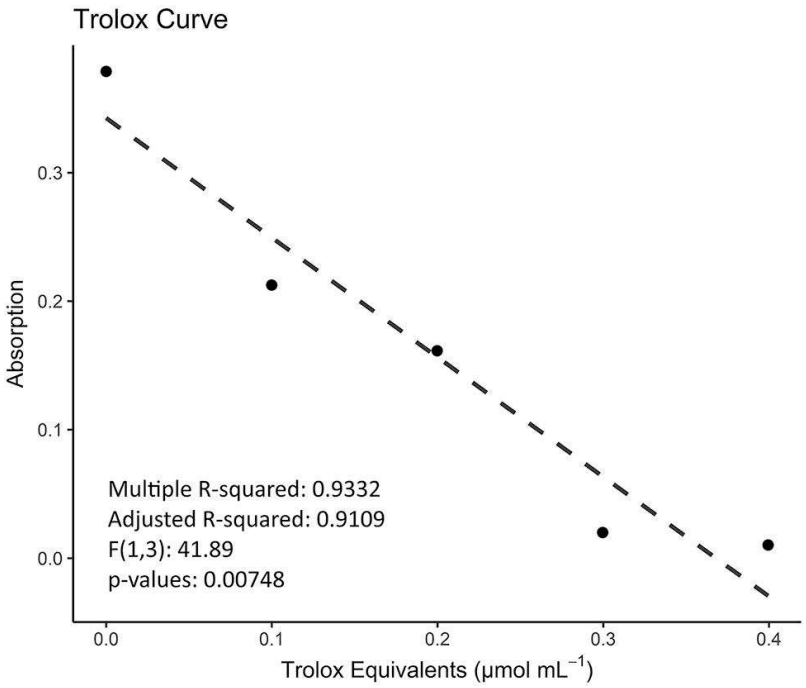

Figure S1: Absorption of different Trolox dilutions (0-0.4 µmol g^{-1} Dry Weight (DW); black dots). The Trolox concentration in µmol g^{-1} DW is depicted on the x-axis, the absorption on the y-axis. The broken line shows a linear regression line, which was created using the data points. The trend follows the formula: $y = -1.0028x + 0.357$. The adjusted R-squared value of the trend line is 0.9109.

Calculation of daily light integral (DLI)

The daily light integral (DLI) describes the amount of photons illuminating an area of one square meter throughout one day (Faust and Logan 2018). The aim was to keep the DLI similar in the control group and the group under saturated light, so only the irradiance and not the number of photons throughout the day would have an influence on the results. The following formula was used to calculate the DLI:

Equation A.1: $DLI = PPFD * time\ of\ illumination\ (h/d) * (3600/1.000.000)$

With PPFD describing the photosynthetic photon flux density (µmol photons m^{-2} s^{-1}). The factor 0.0036 was obtained through the conversion of µmol in mol and the conversion of one hour into seconds (Faust and Logan, 2018).

The DLI of the control group in air will therefore be:

DLI_{LL} = 70 $\mu mol\ photons\ m^{-2}s^{-1} * 16\ h/d * 0.0036$

= $\mathbf{4.03\ mol\ photons\ m^{-2}d^{-1}}$

The DLI of the saturated light treatment in air is:

DLI_{HL} = 185 $\mu mol\ photons\ m^{-2}s^{-1} * 6\ h/d * 0.0036$

= $\mathbf{4\ mol\ photons\ m^{-2}d^{-1}}$

Statistical information - AA regression analysis

Table S1: Statistical information from the regression analysis performed on the percentage of antioxidant activity throughout the experiment per each strain and treatment, as shown in Figures 1 and 2.

Strain	Treatment	Multiple R-squared	Adjusted R-squared	$F_{(2,9)}$	p-value
NE-Atlantic	SL	0.70	0.64	10.66	0.00423
	C	0.79	0.74	17.04	0.00087
Mediterranean	SL	0.74	0.68	12.81	0.0023
	C	0.75	0.69	13.43	0.00199

Antioxidant activity in Trolox Equivalents

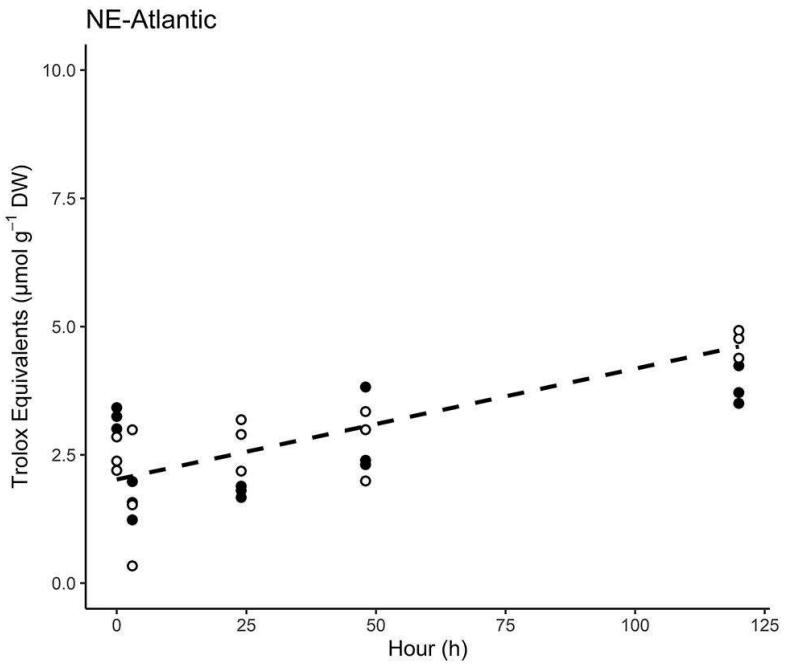

Figure S2: Antioxidant activity in Trolox Equivalents (µmol g^{-1} dry weight) of the NE-Atlantic strain of *Ulva lacinulata* as a function of time. Linear trend was found in the saturating irradiance treatment. For other details see Figure 1.

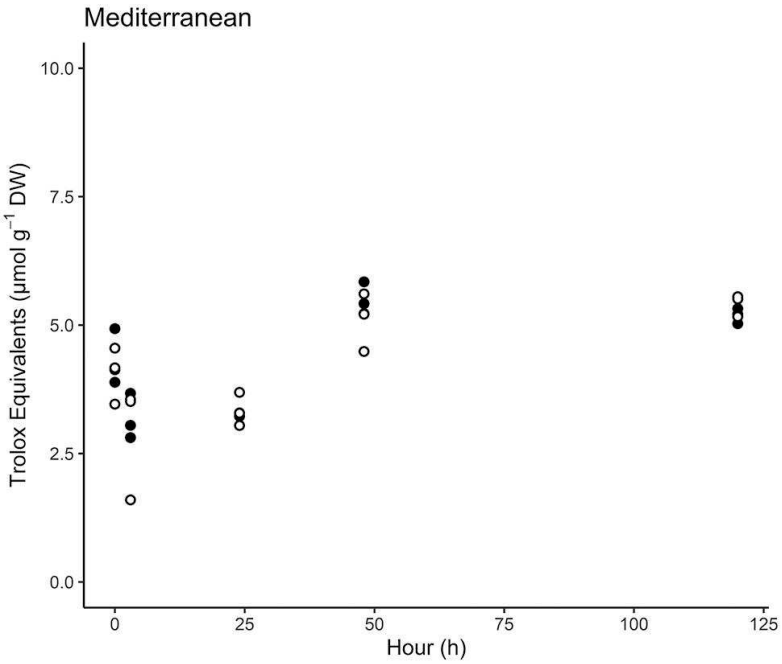

Figure S3: Antioxidant activity in Trolox Equivalent (µmol g^{-1} dry weight) of the Mediterranean strain of *Ulva lacinulata* as a function of time. For other details see Figure 1.

Table S2: Statistical information from the regression analysis performed on the antioxidant activity in Trolox Equivalent (µmol g^{-1} dry weight) throughout the experiment in the NE-Atlantic strain under the saturating irradiance treatment, as represented in Figure S2.

Strain	Treatment	Multiple R-squared	Adjusted R-squared	$F_{(1,13)}$	p-value
NE-Atlantic	SL	0.66	0.63	24.75	0.0002

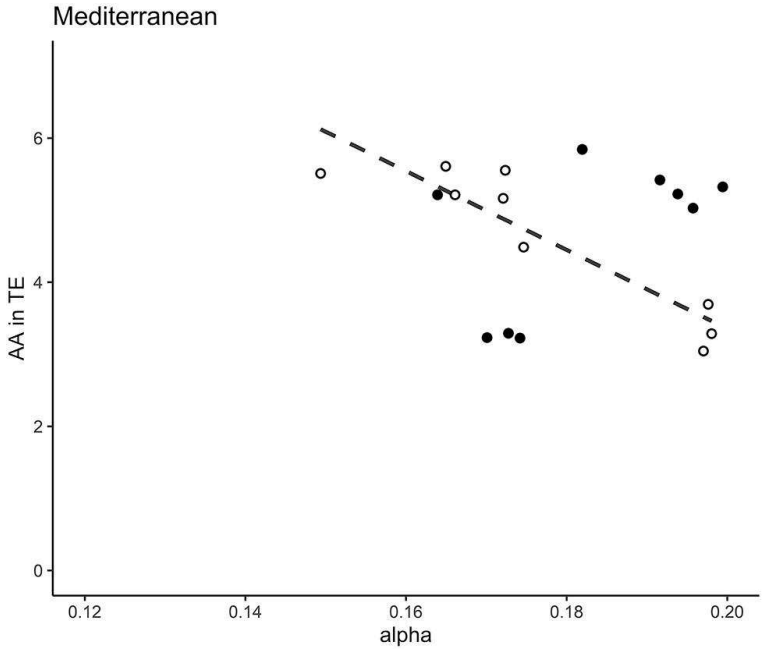

Figure S4: Antioxidant activity in Trolox Equivalent (µmol g^{-1} dry weight) of the Mediterranean strain of *Ulva lacinulata* under saturated irradiance conditions (SL; 185 µmol photons m^{-2} s^{-1}) and in the control treatment, as a function of alpha. The broken line represents the linear trend obtained by the regression analysis performed with the data from the SL treatment.

Table S3: Statistical information from the regression analysis performed between alpha values and antioxidant activity in Trolox Equivalents (µmol g^{-1} dry weight) throughout the experiment for the Mediterranean strain under the saturating irradiance treatment. Linear regressions were determined by the "lm()" function in R software, as represented in Figure S4.

Strain	Treatment	Multiple R-squared	Adjusted R-squared	$F_{(1,7)}$	p-value
Mediterranean	SL	0.83	0.81	34.97	0.0006

Supplementary Reference

Faust, J.E. and Logan, J. (2018). Daily light integral: a research review and high-resolution maps of the United States. HortScience 53: 1250–1257.

5. Overcoming cultivation bottlenecks

Publication III

Naturally occurring protoplasts in two Ulva spp. reveal a previously underestimated proliferation process in an ecologically and economically relevant seaweed

Isabel Cardoso, Inka Bartsch, Klaus-Ulrich Valentin, Sophie Steinhagen, Bela H. Buck, Laurie C. Hofmann

Publication in preparation to be submitted in New Phytologist journal

Naturally occurring protoplasts in two *Ulva* spp. reveal a previously underestimated proliferation process

Isabel Cardoso [1,2] *, Inka Bartsch[1], Klaus-Ulrich Valentin [1], Sophie Steinhagen [3], Bela H. Buck [1,4], Laurie C. Hofmann [1,4]

[1]Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Bremerhaven, Germany,
[2]University of Bremen, Germany,
[3]Tjärnö Marine Laboratory, Department of Marine Sciences, University of Gothenburg, Strömstad, Sweden
[4] Bremerhaven University of Applied Sciences, Bremerhaven, Germany,

Corresponding author: isabel.cardoso@awi.de

Authors ORCID
Isabel Cardoso: 0000-0002-8244-6465
Inka Bartsch: 0000-0001-7609-2149
Klaus-Ulrich Valentin: 0000-0001-7401-9423
Sophie Steinhagen: 0000-0001-8410-9932
Bela H. Buck: 0000-0001-7491-3273
Laurie Hofmann: 0000-0002-2307-3750

Abstract

Interest in *Ulva* cultivation has been growing worldwide and, with it, the attempts to overcome the associated bottlenecks. Developing effective seeding methods, controlling the reproductive cycle, avoiding the spontaneous loss of biomass, and reducing the costs of biomass production are some of the current challenges faced in *Ulva* cultivation. Loss of biomass after the release of gametes or spores during a reproductive event in *Ulva* spp. is a commonly observed and well-understood occurrence. However, the process of biomass degradation in the absence of reproduction, despite being known among *Ulva* specialists, still requires investigation. We designed an experiment to closely observe and describe the process of a degradation in two *Ulva* species. For four weeks, the process of degradation of *Ulva lacinulata* and *Ulva compressa* was followed. Microscopic observations using fluorescent dye revealed the natural occurrence of protoplasts in both species. Results from *Ulva lacinulata*, a non-sporulating species, showed that degrading biomass regenerated after 4 weeks, and the highest protoplasts yields were observed when biomass began regrowing. In the sporulating *Ulva compressa* natural protoplasts were also found, during a reproduction event. The total protoplast yields were 4.26 x 10^8 (*U. lacinulata*) and 5.54 x 10^5 cells g^{-1} (*Ulva compressa*). Respectively, 2 and 4 % of the protoplasts regenerated. During germination, protoplasts of *Ulva lacinulata* displayed several morphologies. The protoplasts either developed into unattached discs, unattached germlings, or underwent gametogenesis after multiple cell divisions. Our results provide the first evidence of the natural production of protoplasts in *Ulva* spp. and characterize for the first time the biomass degradation in a non-sporulating *Ulva* species, closing an important knowledge gap in our understanding of the life-cycle of *Ulva* spp., which has significant implications for large-scale *Ulva* cultivation. Further investigations are needed to pinpoint the environmental or intrinsic triggers that cause this phenomenon and any possible relation our findings can have to the development of "green tides".

Keywords: Cell wall, Degradation, Gametogenesis, Lifecycle, Protoplasts, Reproduction, *Ulva*

Introduction

Interest in valorising *Ulva* biomass in industries such as medicine, agriculture, aquaculture, food and others (Kaeffer et al., 1999; Ganesan et al., 2018; Li et al., 2018; Olasehinde et al., 2019) has existed for many decades due to its interesting bioactive profile (e.g. *Ulva*n; Amin, 2020; Gomaa et al., 2022), potentially high protein content (Shuuluka et al., 2013; Rasyid 2017; Juul et al., 2021; Stedt et al., 2022), and bioremediation properties (Nielsen et al., 2012; Bews et al., 2021). More recently, this genus of green macroalgae has been investigated as a candidate for packaging production (Bosse & Hofmann, 2020; Minicante et al., 2022; ERANOVA, n.d.), either for biodegradable plastics (ERANOVA, n.d.) or for biodegradable and edible food packaging (Bosse & Hofmann, 2020). Nevertheless, valorisation of "green-tide" biomass, massive amounts of biomass that wash onshore in many coastal regions around the world (Van Alstyne et al., 2015; Bermejo et al., 2022), has proved difficult due to contamination issues (e.g. heavy metals, sand) and high costs (Rybak et al., 2012, Smetacek & Zingone, 2013, Mineur et al., 2014; le Luherne et al., 2017; Rybak & Gąbka, 2018; Cai et al., 2021). Alternatively, cultivation of *Ulva* can provide high-quality biomass with optimized functionality or traits (e.g. high protein, improved antioxidant activity; Lüning & Pang, 2003; Ridler et al., 2007; Titlyanov & Titlyanova, 2010; Steinhagen et al., 2022a, 2023b). Nevertheless, cultivation methods still face many challenges, including effective seeding methods, controlling the reproductive cycle, avoiding the spontaneous loss of biomass, and reducing the costs of production (Huguenin, 1976; Bolton et al., 2008; Hiraoka & Oka, 2008; Carl et al., 2014; Gao, 2016; Steinhagen et al., 2022a).

Vegetative propagation of *Ulva* is a relatively simple technique for producing *Ulva* biomass with a low investment because it does not require direct control of the reproduction and life cycle. It depends on the use of vegetative fragments as clonal material, which, in theory, guarantees the constant production of biomass (if kept vegetative) and finding the carrying capacity and optimal stocking density for each system is essential (Zertuche-González et al., 2021). However, clonal material tends to lose quality after some time, and inoculation with new biomass is required from time to time (Hurtado et al., 2013). Moreover, if a disease breaks out in such a system, or if there's an abrupt change in the culture's conditions, the risk of losing all biomass is very high (Gachon et al., 2010; Wang et al., 2010). In addition, this method requires that one-fourth of the entire biomass produced stays in the system as the initial input material for the next production period (Radulovich et al., 2015).

On the other hand, seeding tanks or ropes with spores or gametes requires very extensive knowledge and control of the reproduction of *Ulva* (Bolton et al., 2008; Hiraoka & Oka, 2008; Carl et al., 2014; Steinhagen et al., 2022b). Controlling reproduction can be challenging, as molecules that regulate *Ulva*'s life cycle and reproduction are clade specific (Alsufyani et al., 2014), and the triggers to induce or inhibit reproduction can vary between species and strains (Brawley & Johnson, 1992; Lüning et al., 2008; Balar & Mantri, 2020). This can be a limiting factor for cultivation, because during reproduction, *Ulva* is not growing and is, in fact, losing biomass because of sporulation that leads to the disintegration of the reproductive thalli (Ryther et al., 1984; Bolton et al., 2008). Nevertheless, nursery/hatchery systems can be used to guarantee the zoospore and gamete formation and development before being placed in the main cultivation system or in the field (Carl et al., 2014; Gupta et al., 2018; Praeger et al., 2019; ALGAplus, 2023). When working with meiotically divided zoospores or mated gametes, this method allows for more genetic variability and provides seeding material on demand, in

controlled concentrations, therefore guaranteeing high quality and yields (Rößner et al., 2014; Gupta et al., 2018; Praeger et al., 2019; Boderskov et al., 2023). However, it increases the cost of the overall production and requires the use of a sporulating species and a reliable method for inducing sporulation, which may include variations in salinity, dehydration, segmentation, temperature shock, light colour, and intensity, or utilizing sporulating inhibitors (Carl et al., 2014; Gao et al., 2017b; Schwoerbel et al., 2019; Mantri et al., 2011). It also requires several steps for scaling-up the cultivation from nursery scale to large scale, which represents the use of a lot of space and manpower. After each harvest, the tank will need to be inoculated with new *Ulva* (Radulovich et al., 2015) from the nursery, which requires carefully timing the induction of reproduction and the scaling-up process.

An alternative method that has been proposed for the cultivation of *Ulva* spp. in order to avoid the bottlenecks of vegetative propagation and control over the life cycle, is the isolation of protoplasts. This method has been commonly applied to higher plants (e.g., *Arabidopsis thaliana*) (Dovzhenko et al., 2003; Davey et al., 2005; Sangra et al., 2019), but also to seaweeds, including *Ulva* spp. (Fujita & Saito, 1990; Dipakkore et al., 2005; Reddy et al., 2006; Gupta et al., 2011; Gupta et al., 2018; Reddy & Seth, 2018; Wu et al., 2018; Avila-Peltroche et al., 2019; Avila-Peltroche et al., 2022). In theory, this method allows for each cell in a small piece of the thalli to regenerate as a new individual. This technique requires little initial biomass compared to the vegetative cultivation method (Gupta et al., 2018) and does not require a profound understanding of the life cycle. However, this technique is considered expensive for large-scale purposes, since it requires the use of costly laboratory equipment, facilities, and enzyme solutions (Gupta et al., 2018).

Regardless of the cultivation method used (vegetative, seeding with gametes or spores, or protoplast isolation), spontaneous loss of biomass due to reproductive or degradation events is a common challenge for large-scale production (Ryther et al., 1984; Bolton et al., 2008; Obolski et al., 2022). While the loss of biomass after the release of gametes or spores during a reproductive event in *Ulva* spp. is a commonly observed and well-understood occurrence (Bolton et al., 2008; Bruhn et al., 2011; Gao et al., 2017c; Steinhagen et al., 2022a), the process of biomass degradation in the absence of reproduction is poorly understood. Such degradation events are often not reported in the literature but are well-known among specialists in *Ulva* cultivation. Personal communication with members of the SeaWheat COST Action (CA20106) – «*Ulva*: Tomorrow's "Wheat of the sea", a model for an innovative mariculture» reported having observed degradation of their *Ulva* cultures (data not shown) at some point during cultivation and in half of the cases, the entire cultivation was lost despite the controlled growing conditions. To this day, and to the best of our knowledge, the terms "degradation", "disintegration", "fragmentation" and "loss of biomass" during cultivation have only been used in the literature in the context of a reproduction event (i.e. the release of spores or gametes; Ryther et al., 1984; Bolton et al., 2008). Poor culture conditions and the age of the material have also been reported as causes of degradation and loss of biomass (Carl et al., 2014; Obolski et al., 2022), but most likely because they can both be triggers for sporulation, thereby causing the associated loss of biomass.

During a period of two years, where culture conditions were controlled and optimized (Cardoso et al., 2023, 2024), some of the species did not show any indications of reproduction (maturation of the thalli was never observed and gametes and spores were never found) but degradation would occur often and new germlings would be found in the cultures. We hypothesized that the typical thalli maturation process and subsequent release of swarmers (gametes or spores) is not required for *Ulva*'s reproduction. We further hypothesized that separate events involving the degradation of biomass without the maturation of thalli can result in the production of germlings and new biomass, as well. To date, these events have been closely observed and described once,

by Bonneau (1978) when working with *Ulva lactuca*. Bonneau (1978) reported *U. lactuca* developing asexual generations for over two years, while swarmer release was infrequent, and induction of swarmer formation was unsuccessful.
Considering the several processes that can impact the loss of biomass and degradation of *Ulva*, and how devastating these events can be in large-scale cultivation systems, we designed an experiment to closely observe and describe the process of degradation in *Ulva* spp. and the events that follow. Previous observations of protoplast-like cells in our *U. lacinulata* (Kützing) Wittrock cultures lead us to hypothesize that degradation events in this, apparent, non-sporulating species were associated with protoplast production. In addition, based on previous observations of a local *Ulva* sp., we hypothesized that the production of protoplasts during degradation events was not unique to lab-grown cultures, but may also be observed in wild specimens.
In this work, we describe and define a degradation event, and distinguish it from sexual reproduction events. We show evidence from *Ulva lacinulata*, a non-sporulating species, that degradation of biomass is associated with the natural production of protoplasts and quantify natural protoplast production and germination rates. We also describe in detail the morphological development of regenerated and germinated natural protoplasts. Furthermore, we show evidence that protoplasts can also be produced during sporulation events in a sporulating *Ulva* sp. We discuss how our findings close an important knowledge gap in our understanding of the life cycle of the non-sporulating *U. lacinulata*, and their implications for overcoming some of the currently existing bottlenecks in large-scale production of this species.

Material and Methods

Biomass collection

The *Ulva lacinulata* (Kützing) Wittrock from the NE-Atlantic was collected in January 2021, in Lagoa de Óbidos, Portugal (39°23'41.5"N 9°12'48.9"W) where temperature and salinity vary between 6 and 24 ˚ C (during the curse of a year) and 25 and 35 PSU, respectively (Cavaco et al., 2016; Mendes et al., 2021). *Ulva compressa* Linnaeus from the North Sea was collected in June 2023 in Dorum (Wurster North Sea coast, Lower Saxony), Germany (53°44'30.8"N 8°30'52.4"E) where temperatures vary, within one year, between 5.1 and 17.6 C (Sea temperature, 2023) and salinity is around 30 PSU (Frohse et al., 2008; Klein et al., 2021). Both species were cleaned by rinsing the seaweed by running natural seawater several times to eliminate epiphytes and small organisms on the surface of the blades.

Molecular analysis

Due to the considerable phenotypic variability observed in various species of the *Ulva* genus, accurate identification of the utilized biomass often requires the application of molecular identification methods, such as DNA barcoding. The molecular identification of the here applied strains followed the description in Steinhagen et al., (2023a).
Ulva lacinulata had been previously identified (accession number: OP778143) by using the plastic-encoded marker *tuf*a (Cardoso et al., 2023). Following the same method, *Ulva compressa* was identified for this work. Additionally, molecular identification was performed in the F1 generation of individuals developed from *U. lacinulata* (OP778143) protoplasts obtained at the end of the regeneration experiment described in this work with the purpose of confirming its origin.

Pre-cultivation

Ulva compressa was collected a month before the start of the experiment. As the purpose of the experiment with this species was to assess the natural occurrence of protoplasts, *Ulva compressa* was grown in 5 L glass cultivation bottles filled with pasteurized natural seawater at 30 PSU ± 2 PSU (Refractometer, Atago, Japan). The bottles were placed in a climate-controlled chamber set to 15°C and were illuminated on a 16:8 light/dark cycle with an intensity of ~ 80 µmol photons m^{-2} s^{-1} (measured in air; LI-250A, Li-cor, Germany). Nutrient supply was provided by supplementing the seawater with half-strength Provasoli´s culture medium in a concentration of 10 mL L^{-1}, as it had resulted in good growth rates in previous works with *U. lacinulata* (data not shown; PES; Provasoli, 1968; modifications: HEPES-buffer instead of TRIS, double concentration of Na_2glycerophosphate; iodine enrichment following Tatewaki (1966)). The medium in each bottle was changed every week and all bottles were aerated with compressed air via tubes.

Ulva lacinulata had been cultivated under laboratory conditions since 2021 and it was cultivated for 25 months before the experiment reported in this work. As *U. lacinulata* had been previously tested for its cultivation in a recirculating aquaculture system (RAS) with artificial seawater (Cardoso et al., 2023, 2024), this strain was grown in 5 L glass cultivation bottles filled with pasteurized artificial seawater (Seequasal-Salz, Seequasal Salz Production and Trade GmbH, Germany) at 30 PSU ± 2 PSU (Refractometer, Atago, Japan). Except for the water used, all the culture conditions used for this species were identical to the previously reported for *U. compressa*.

Experimental Design

To observe and describe the degradation event in *U. lacinulata*, pieces of degrading thalli were examined under a microscope (Olympus CKX41, Olympus, Japan) and photographed for later analysis. The morphology of the *Ulva* thalli and its cells (including the characterization of their structures and organization) were described based on the work of Maggs et al. (2007) related to *U. compressa* and *U. rigida* C. Agardh.

During a degradation event, intact, green, and non-bleaching pieces of the thalli were collected and separated into four 1 L beakers. In each beaker was placed 0.18 ± 0.005 g of fresh-weight of biomass. The pieces of *Ulva* were placed in scientific absorbent paper and gently squeezed three times to remove the excess water before fresh weight measurements. Each beaker was filled with pasteurized artificial seawater enriched with half-strength Provasoli's, as described during the pre-cultivation period. All the remaining cultivation conditions were kept as described previously. Water samples were collected from the beakers on days 0, 9, 16, 23 and 30. On the same days, the totality of the fresh biomass in each beaker was weighted as previously described. The weekly fresh weight data was used to calculate the relative growth rate (RGR) of the original pieces of thalli.

The water samples collected from each beaker were filtered through a 30 µm mesh (PluriStrainer®, Pluriselect, Germany), following a similar process as described in the literature for protoplast isolation (Gupta et al., 2018; Reddy & Seth, 2018). Each beaker was rinsed twice with artificial seawater to guarantee the removal of possible floating cells as well as the mesh where smaller pieces of the thallus would get collected. The water was filtered and collected in 50 mL falcon tubes for further analysis.

A similar experiment was designed for the *Ulva compressa*. In this case, there were signs of reproduction (normal discoloration of the thallus margins as described in the literature and observation of swarmer release). But microscopic analysis showed the presence of protoplast-like cells in the thalli and the water, mixed with swarmers from fertile pieces of the thalli. 0.18 ± 0.003 g FW of healthy and non-fertile biomass was added to six separate 1-L beakers.

Pasteurized natural seawater was used instead of artificial seawater. The experiment was conducted for three weeks and samples were taken on days 0, 5, 12 and 19. Because germlings were found in the beakers after 2 weeks of the experiment, during the process of water filtration and fresh weight measurements, the weight of the germlings was measured separately from the original pieces of the *Ulva* placed in each beaker. The data reported in the results section of this work only refers to the fresh weight of the original biomass.

Protoplast confirmation and yield quantification

During the previous experiment, samples of the degraded thalli and the water in the beakers were collected for confirmation of protoplasts. The cells were stained with 0.01 % calcofluor white (CFW), following the process reported by Reddy et al. (1989). The presence or absence of a cell wall was confirmed through observations under a fluorescent microscope (Filter set 02, Axioplan 2 imaging, Zeiss, Germany). CFW binds with the cell-wall's cellulose (Albani, 2011), therefore fluorescing when the cell wall is present. This process followed multiple works related to protoplast isolation in *Ulva* species (Reddy & Fujita, 1991; Reddy & Seth, 2018, Avila-Peltroche et al., 2022).

The water samples collected from each beaker, weekly, were centrifuged at 124 x g for 5 minutes (Eppendorf Centrifuge 5810-R, Germany) to concentrate the approx. 1 L protoplast solution into 12.5 mL. The supernatant was discarded between each centrifugation step and, after the last centrifugation, the pellet was resuspended in new pasteurized artificial seawater enriched with Provasoli's medium until a volume of 12.5 mL. Subsamples of the resuspended pellet of each sample were placed in disposable hemacytometers (Neubauer Improved, C-Chip, NanoEnTek Inc., Korea) to count the number of *Ulva* protoplast-like cells. Per beaker, 10 counts were performed, in the case of *U. lacinulata,* and 14 counts were performed for the *U. compressa*. Cell counting was performed by counting the 4 larger squares (top right, top left, bottom right and bottom left) in the hemacytometer. Protoplast concentration (in cell mL^{-1}) was calculated by the product between the known number of cells counted in the hemacytometer and the multiplication factor (10^4), divided by the number of squares counted (4). The timing for complete cell wall degradation is unknown and the timing for cell wall regeneration in *Ulva* protoplasts usually takes around three days (Gupta et al., 2018; Gupta & Reddy, 2018). During cell counting, it was assumed that protoplast-like cells could be produced continuously throughout the week, and they would be found at the time of counting in different stages of degradation or regeneration (thus, fluorescing partially or totally when dyed with CFW). To guarantee that all protoplast-like cells were considered, all the cells that resembled a protoplast were counted.

Protoplast yields (in cells g^{-1} of biomass) were calculated by dividing the concentration of protoplasts by the weight of the biomass measured the week prior. It was assumed that the biomass weighed one week before, was responsible for the protoplast production found in the current week. The average of the protoplast yields was used to estimate the protoplast yields produced by the measured weight of fresh biomass in a period of one week. The total protoplast yields produced each week were calculated by adding the protoplast yields found in each beaker during the same week. At the end of the experiment, the protoplast yields of each week were totalled to calculate the total amount of protoplasts per gram obtained during the experiment.

Protoplast Regeneration and Germination

Known volumes from the protoplast solutions obtained in each beaker were placed in Petri dishes with pasteurized seawater (30 PSU). The dishes were placed in a culture chamber with no aeration or shaking following (Gupta et al., 2018), under the same cultivation conditions as

described for each parental *Ulva* species. The determination of the volumes used differed between the two species.
For the *U. lacinulata* samples, a defined number of protoplasts (Table 1) was added to each Petri dish (90 mm diameter), based on the protoplast concentration calculated previously. Ten Petri dishes were filled with 50 mL of pasteurized artificial seawater with an added volume of the protoplast solution (Table 1).
For the *Ulva compressa* samples, the protoplast yields were lower than in the experiment with *U. lacinulata*. Per beaker, 10 smaller Petri dishes (60 mm diameter) were filled with 15 mL of pasteurized natural seawater. As previously mentioned, *U. compressa* was showing signs of fertility, and despite non-fertile pieces being selected for the experiment, the selected pieces eventually became fertile, and swarmers were observed in the protoplast solutions after filtration of the water. This could cause a density issue during the regeneration experiment, as this would represent a higher number of individuals (swarmers + protoplasts) being inoculated in each Petri dish. For this reason, to remove the density factor caused by the high number of reproductive cells, the same volume of protoplast solution (which included protoplasts and swarmers) was inoculated into each Petri dish (300 µL; Table 1). This meant that different Petri dishes were inoculated with different numbers of protoplasts, as the protoplast yields varied between replicates (Table 1). The number of *U. compressa*'s protoplasts inoculated in each Petri dish was calculated based on the protoplast yields that had been determined previously.

The regeneration of the protoplasts and further germination were followed by repeated microscope and binocular (Olympus SZX10, Olympus, Japan) observations for five (*U. lacinulata*) and 4 (*U. compressa*) weeks. At the end of the experiments, a binocular was used to count the number of visible germinated *Ulva* individuals and to calculate the germination rate. The same observations allowed the report of the different morphologies found at this stage of protoplast germination. The different morphologies observed at the end of the experiment were described as unattached discs (UD), unattached germlings (UG), and attached germlings (AG) based on morphology and the presence or absence of rhizoids.

Table 1 – Determined number of protoplast-like cells added to each Petri dish at the beginning of the germination experiment.

Species	**Volume of water in Petri dishes**	**Beaker**	**Protoplast-like cells concentration (cell per mL)**	**Volume of cell solution added (µL)**	**Number of cells per Petri dish**
U. lacinulata	50 mL	1	2.25×10^4	63.8	1.44×10^3
		2	1.26×10^5	11.4	1.43×10^3
		3	5.43×10^4	26.4	1.43×10^3
		4	5.75×10^3	250	1.44×10^3
North Sea *Ulva*	15 mL	1	1,07E+03	300 µL	3.21×10^2
		2	3,57E+02		1.07×10^2
		3	< 1,79E+02		$< 5.36 \times 10^1$
		4	1,25E+03		1.61×10^2
		5	5,36E+02		1.61×10^2
		6	1,79E+02		5.36×10^1

Statistical analysis
Regression analyses were performed to evaluate the RGR as a function of time (in weeks). Because the weekly measurements of the fresh weight of the original *U. lacinulata* thalli, showed strong variations between replicates (beakers), the regression analyses were performed for each

replicate separately. Because the relationship between time and RGR was non-linear, different degrees of polynomial equations (second, third, fourth and fifth degree) were tested using the function "lm()" to determine the best fit. A two-degree polynomial function ($y = ax^2 + bx + c$) provided the best non-linear fit when correlations where found. Regression analyses were performed to evaluate the variations of protoplast yields throughout the experiment with *U. lacinulata*. Linear functions were considered the best fit to report the correlations found between time and protoplast yields. The statistical data associated with each figure can be found in Table S1.

All the figures presented in this work reporting the results of the regression analyses were created by the "geom_smooth" function (package "Ggplot2") in the R studio software (R Core Team 2024). The 100 % stacked columns were created by R studio software (R Core Team 2024) using the function "geom_col" (package "Ggplot2").

Results

Protoplasts Production and Biomass change

Non-sporulating species

After observing *U. lacinulata*'s behaviour over two years and following the degradation process of the two *Ulva* species presented in this work, it was possible to define the concept of "degradation event". A "degradation event" was defined as the degradation, or disintegration, of the *Ulva* thallus combined with a loss of colour (from bright green to pale yellow-green and, in the end, white) in the absence of gametogenesis/sporogenesis. The event can be recognized at an early stage when the thalli become fragile, and the seawater becomes milky/cloudy. By the naked eye, a strong accumulation of debris becomes visible at the seawater surface where foam may also develop, indicating an accumulation of organic compounds and cell particles (Fig. 1). During a "degradation event", the surface view of the thallus showed heterogenous cell organization (Fig. 2a and b) that differed from the typical cell organization in *Ulva lacinulata* (Fig. 2C). During degradation, the thallus was divided into small pale cells and larger green cells. The small pale cells maintained their polygonal/square shape and were filled with granules, similar to starch granules reported in *Ulva ohnoi* (Prabhu et al., 2019). In these cells, the lack of green colour hindered the identification of cell structures such as pyrenoids or chloroplasts. The larger green cells presented a spherical form, with a clear parietal plastid in a cup-shape form with a visible chloroplast that yielded its bright green colour (Fig. 2). In some cases, in these larger cells pyrenoids were easily observed. Granules of starch were seldom found in these cells. These cells, with an average size of 39.3 ± 3.87 µm (diameter, number of cells measured = 20, se = 0.97) resembling protoplasts, were later detached, and released from the thalli. In these degraded pieces of thalli, no sign of sporogenesis or gametogenesis was ever observed. Microscopic fluorescent analysis showed the total or partial absence of cell walls in individual *Ulva* cells collected from the water and in pieces of the degraded *Ulva* thalli, confirming the degradation of the cell wall and the presence of protoplasts (Fig. 3).

The end of a degradation event can be identified once the water becomes clear and small thallus fragments are no longer found in the water column. In this work, the degradation event was considered to be terminated after 4 weeks. No changes in the culture conditions were made during the four weeks besides the weekly total water exchange and supplementation with nutrients.

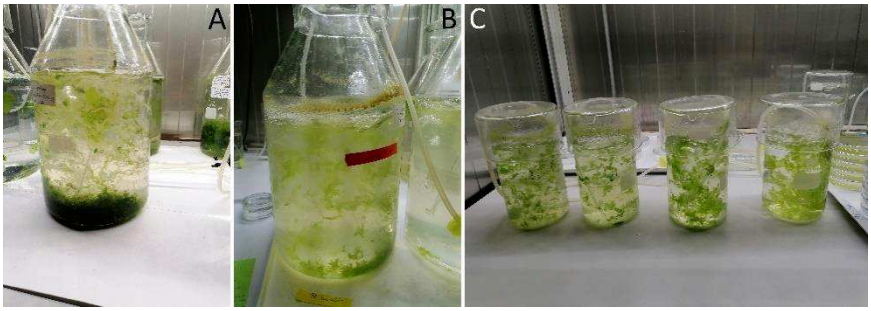

Fig. 1 – Fragmentation of *Ulva lacinulata* thalli during degradation observed in our cultures and at the beginning of the degradation experiment. A and B: 5 L bottles with *Ulva lacinulata* during "degradation events". Accumulation of debris and small pieces of *Ulva* are visible at the top of the bottles. C: Fragments of *U. lacinulata* undergoing degradation at the start of the experiment.

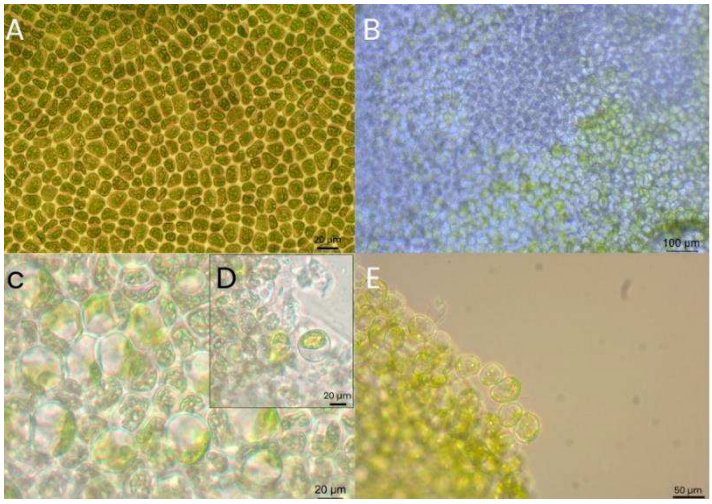

Fig. 2 – Microscopic observations of the thalli of *U. lacinulata* going through the degradation process (a and b show different pieces of *Ulva* at different magnifications). A: piece of NE-Atlantic *U. lacinulata* thalli as an example of the typical morphology this strain presents when not degrading. B: thalli heterogeneity. The pale cells without colour suggest the poor health of thalli. C: similar heterogeneity in the thalli between the same two cell morphologies large spherical green cells, with parietal chloroplasts, are visible in between smaller pale cells. D: spherical cell detaching from the thallus. E: piece of *Ulva* with only spherical green cells with some detaching from the main thallus. Different magnification shows how widespread in the thalli the heterogeneity of the cell morphology was during degradation. The larger green cells became protoplasts while the remaining cells died.

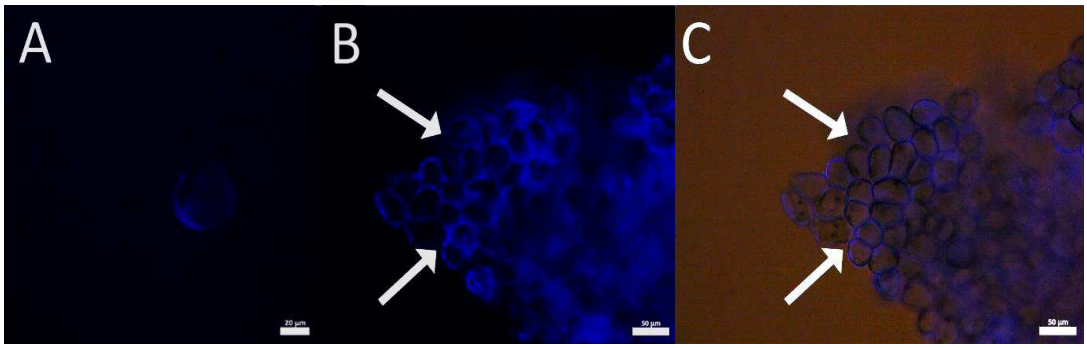

Fig. 3 – Fluorescent microscopy. *U. lacinulata* cells dyed with calcofluor white (CFW). A: barely visible, single cell with no fluorescence (scale: 20 µm) reflecting the fluorescence of other thalli pieces in the same sample); B and C: same picture with different fluorescence levels for comparison (C has a mixture of halogen light and fluorescent light) showing only partial fluorescence of the thalli and partial absence of cell wall (areas with no fluorescence), scale: 50 µm.

At the end of the experiment, more biomass (5.8 g FW) was found than the original amount placed in each beaker (0.716 g FW) at the beginning of the experiment. The biomass grew eightfold during the degradation event (Fig. 4). *Ulva lacinulata*, reported high RGRs, after the 4-week experiment, averaging 12.3 ± 3.3 % day^{-1} (standard error= 1.6), and a maximum RGR of 15.3 % day^{-1}. Non-linear trends were found with the RGRs decreasing mid-experiment. The strongest decrease in RGR was observed between the 1st and the 3rd week, where RGRs were close to 0 % or negative (Fig. 5, B1 and B3). Negative growth rates were observed in two replicates (B1 and B3), 25 % of the original biomass was lost. By the third and fourth week, the biomass recovered, and growth resumed (RGR increased). In some cases, by the end of the experiment, the biomass exceeded the initial RGRs (Fig. 5, Table S1). One replicate (B4) did not show a clear tendency, and the RGRs reported from the different weeks did not show a strong variation between weeks.

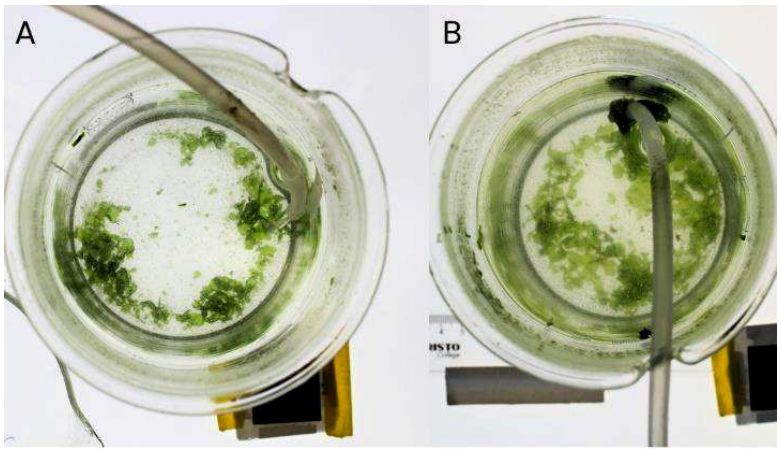

Fig. 4 – NE-Atlantic *U. lacinulata*'s biomass growth (same replicate, B2) between the 3rd and 4th week. Scale: 1 cm.

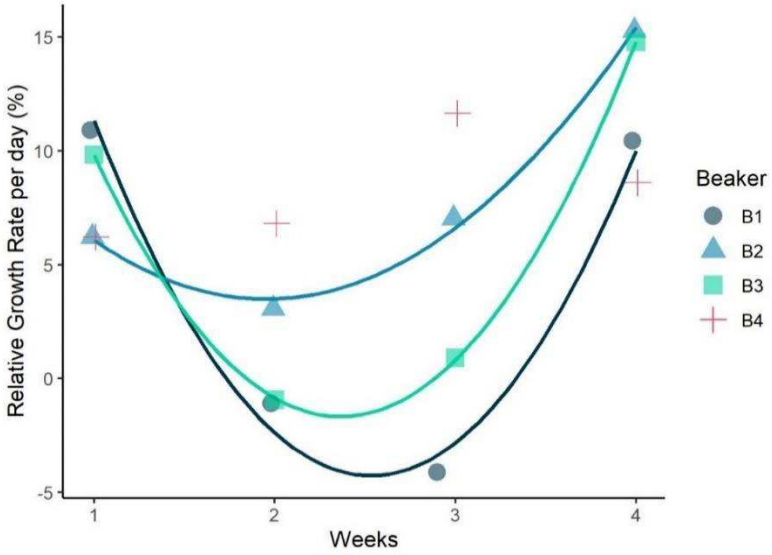

Fig. 5 – Relative growth rate of the original biomass of *U. lacinulata* samples in each beaker throughout the 4 weeks of the experiment. Lines represent the non-linear correlations found in RGR as a function of time (weeks) in each replicate (beaker). B4 did not show a statistically significant trend.

Assuming the biomass weight every week was responsible for protoplast production, the average production of protoplasts collected from the *U. lacinulata* after 4 weeks was 3.63 ± 0.295 x 10^6 cell g^{-1} (average ± standard error, se; n = 160). The highest protoplast yield observed was during the third week of the experiment with 14.0 ± 0.936 x 10^6 cell g^{-1} (average ± se, n = 10), and the lowest registered yield was reported in the last week with only 41 ± 5.29 x 10^4 cell g^{-1} (average ± se, n = 10). Assuming the totality of the original weight (0.716 g) placed in each beaker at the beginning of the experiment was responsible for the production of the totality of protoplasts produced over 4 weeks, the total protoplasts collected were 4.26 x 10^8 cell g^{-1} (n = 160).
Protoplast yields varied considerably between sub-replicates and beakers throughout the experiment with *U. lacinulata*, resulting in high variations in the data. An increase in protoplast yield over time was observed in two replicates (Fig. 6, Table S1), resulting in an average of 13.7 ± 0.894 x 10^6 and 8.75 ± 0.773 x 10^6 cell g^{-1} (average ± se, n = 10), respectively, by the end of the experiment. These were the same replicates in which the original biomass showed negative RGRs or RGRs close to zero (B1 and B3; Fig. 5).

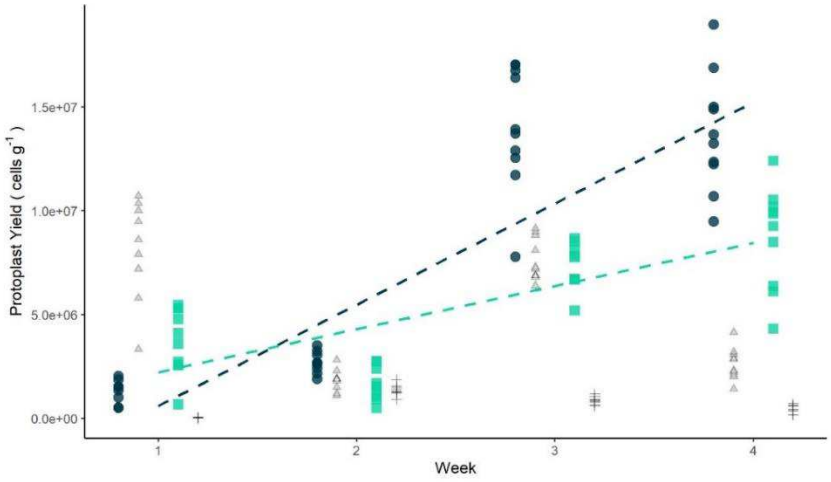

Fig. 6 – Protoplast yield (cells g^{-1}) as a function throughout the four-week experiment. The lines represent the positive linear correlation between the weeks and the number of protoplasts produced in beakers B1 and B3. Dark blue circles represent the estimated protoplast yields in B1, and green squares represent the estimated protoplast yields in B3. Grey triangles represent the estimated protoplast yields in B2 and black crosses represent the estimated protoplast yields in B4. B2 and B4 did not present a clear correlation throughout the four weeks.

Sporulating *Ulva* species

Cell heterogeneity was observed in pieces of thalli of *Ulva compressa*. Microscopic analysis enabled the comparison between pieces with typical cellular organization (Fig. 7A) and pieces or areas of the thalli where spherical, enlarged bright green cells (similar to the ones reported in *U. lacinulata*; Fig. 2) could be found (Fig. 7B-D). These bright green cells, with similar parietal chloroplasts, had an average size of 20.5 ± 2.62 µm (diameter, number of cells measured = 74, se = 0.30). Some of these cells were found close to the thalli but already detached (Fig. 7C-D). In this case, mature areas of the thalli were visible and *Ulva* swarmers were observed swimming inside of the cells and being released from the thalli. In between fertile cells, bright green spherical cells were found (Fig. 6B-C). Fluorescent microscopic analysis showed the total or partial absence of cell walls in individual *Ulva* cells and pieces of the degraded *Ulva* thalli (Fig. 8).

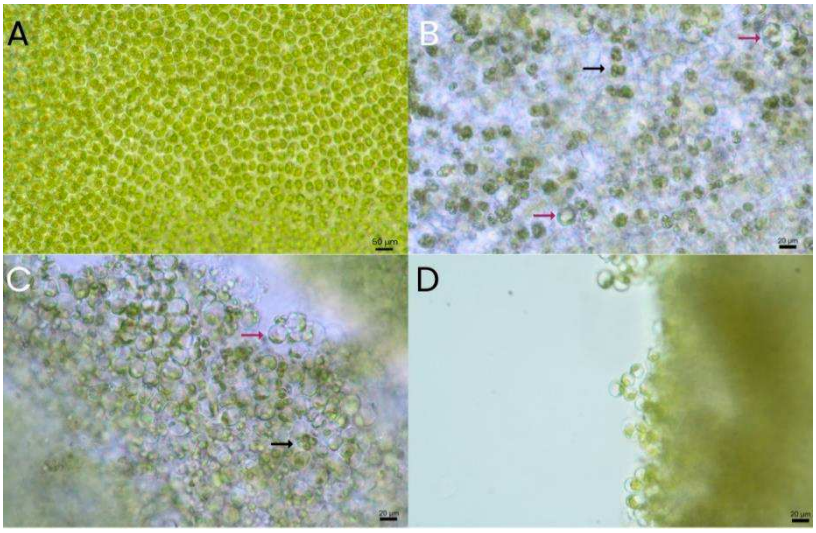

Fig. 7 – Microscopic observations of the thalli of North Sea *Ulva* simultaneously producing swarmers and protoplasts. A: non-fertile, healthy piece of *Ulva compressa* thalli collected when the reproduction event was first observed; B: mature area from the same piece of North Sea *Ulva* thalli, three types of cells can be distinguished: cells full with swarmers, empty cells, and larger and rounder cells (protoplasts-like cells); C: centre area of the same piece of the thalli, protoplast-like cells mixed with zoospores and/or gametes released by the same blade; D: the edge of the same piece of thalli showing detached round *Ulva* cells (considered protoplast-like cells); The larger green cells became protoplasts while the remaining cells went through sporo/gametogenesis. Legend: red arrows: protplast-lice cells; black arrows: swarmers.

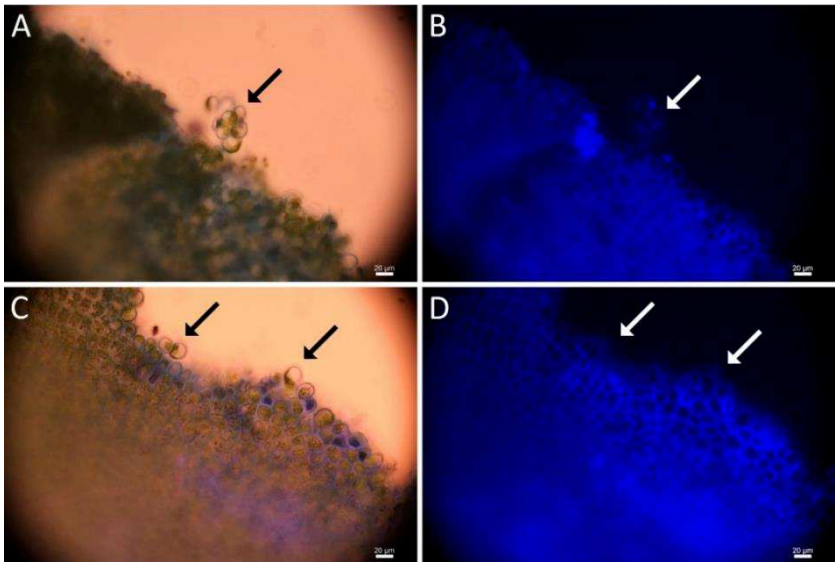

Fig. 8 - Fluorescent microscopy. Thalli from North Sea *Ulva* dyed with CFW. a and b: same picture with different conjugations of fluorescence and halogen light. a: fluorescent and halogen light; b: fluorescent light without halogen light (absence of cell wall in the cells from the extremity of the thalli). c and d: same picture with different conjugations of fluorescence and halogen light. c: fluorescent and halogen light; d: fluorescent light without halogen light (visible absence of cell wall in cells released from the thalli).

The results from the experiment with the *Ulva compressa* showed an overall tendency for biomass loss with close to zero or negative RGRs. After three weeks, 27 % of the original biomass had been lost, and after four weeks the original biomass was completely lost (except one replicate). This resulted in low or non-existent fresh weight measurements and only allowed for the collection of data at three time points. For those reasons, regression analysis could not be performed. Positive RGRs were observed in the 1st and the 2nd week (1.2 ± 2.18 and 4.70 ± 2.68 % day^{-1}, respectively; average ± se; n = 6). After the second week, the reported RGRs were close to zero or negative (-16.8 ± 9.61 % day^{-1}; average ± se; n = 6). Only replicate B5 registered a continuous increase in RGR during this period reaching a RGR of 10.3 % by the end of the third week.

Protoplast yields were low during the experiment with *Ulva compressa*. Protoplasts were found during the second and third weeks, the yields were considered too low to be reliable. Because of the low RGRs reported during those weeks, the protoplast yields per gram of fresh weight were

considered inaccurate. Only the protoplast yields from the first week were used to compare between the two species. After the first week, an average of 6.59 ± 2.33 x 10^3 cell g^{-1} (average ± se, n = 84) was collected from all six replicates. The total protoplasts produced by 1,07 g during the first week was 5.54 x 10^5 cell g^{-1}.

Protoplast regeneration, germination, and resulting morphologies

Ulva individuals developed into four distinct morphologies: microscopic spherical cell masses, unattached discs (UD), unattached germlings (UG), and attached germlings (AG) (Fig. 9). Before the regeneration and germination experiment a portion of the spherical cell masses became fertile (Fig. S1 in the Supplementary material) and did not develop further. Protoplasts, as single cells, sometimes aggregated together and developed attached to each other, but each cell developed into a separate individual (Fig. 10). Only the UD, UG, and AG morphologies were found once the individuals grew to a macroscopical size. Two of the morphologies, UD and UG were visible within 2 to 4 weeks of protoplast isolation. The AG morphology was only visible at the start of the 5th week. During the 5th week, the AG became more numerous than the two other morphologies in the Petri dish and exceeded the initial number of protoplasts added to the Petri dish. For this reason, we did not consider the AG morphology when calculating germination rates. The source of these attached germlings is discussed below.

The germination rate of *U. lacinulata* protoplasts was calculated based on the visible germinated individuals (UD and UG) found in each Petri dish after 5 weeks of cultivation. Samples inoculated from the replicates B2, B3, and B4 were used to determine the germination rates as the Petri dishes from B1 were found contaminated after one week of the germination experiment. The germination rates varied between 0.8 % and 3 % and the average of the total germination was 2 %.

The germinated individuals were counted and separated by morphology. UDs and UGs were visible to the naked eye one week before the end of the experiment. However, in the last week (5th week), discoloration from green to yellowish-brown and finally to white was observed in a portion of the individuals with the UD morphology (Fig. 11). At the same time, AG became visible as well (Fig 12). Closer observation indicated that the UDs had become fertile (Fig. 11D, E). While the green discs under the microscope presented a clear cell organization similar to what has been reported for *Ulva lacinulata* (Fig. 11B, C), the change in colour to brown was an indication of the maturity of the discs (Fig. 1). This was confirmed by the presence of swarmers inside the cells as well as empty cells, suggesting the previous release of swarmers (Fig. 1E). Swarmers were observed moving inside of the cells (Fig. S1). Phototactic techniques determined these swarmers to be gametes, as they swam towards the light. After the release of the gametes, the discs became white and showed only empty cells, as is typical for *Ulva* tissue that becomes fertile.

All of the gametes released by the unattached discs (Fig. 12A, B) developed into single-branched germlings attached to the bottom of the Petri dish with a rhizoid (Fig. 12C, D). This development confirmed that the attached germlings did not develop directly from the protoplast cells, but rather developed from gametes released by cell masses and floating disks, also explaining why they appeared later than the unattached discs and germlings.

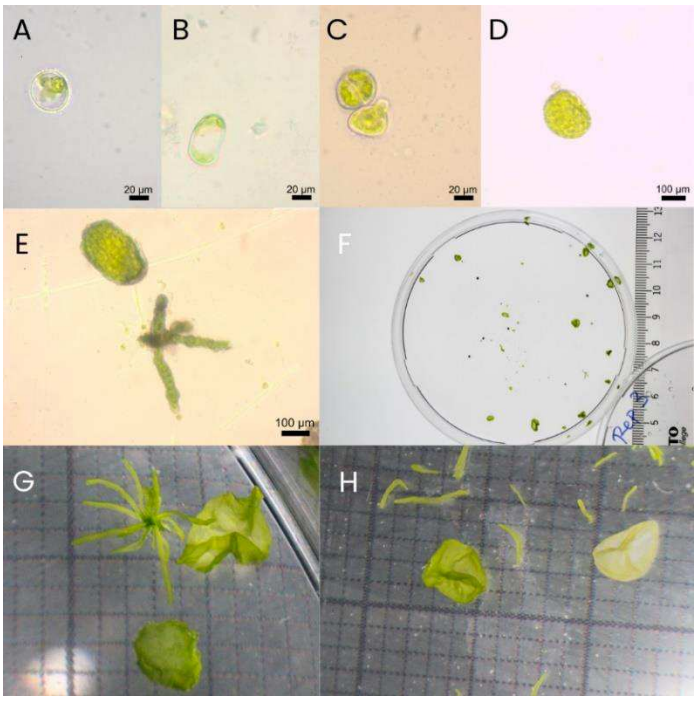

Fig. 9 – Protoplast development from *U. lacinulata* (each picture is from different individuals). A: single protoplast; B: protoplast extending (possible first division); C: two individuals where cell division already occurred – upper cell divided in two and lower cell is extending to the right, suggesting germination; D: cell mass after multiple cell divisions; E: protoplast development, as a cell mass and as a multi-branched germling without visible rhizoid; F: Petri dish from germination experiment with *U. lacinulata* where besides unattached discs and unattached germlings there are also visibly attached single-branched germlings; G: binocular observation of the two developed morphologies originated from protoplasts (unattached discs and unattached germlings). H: unattached discs (green and discoloured) and floating and attached germlings were observed during the germination experiment with protoplasts from *Ulva lacinulata* (1 square is 1 x 1 mm).

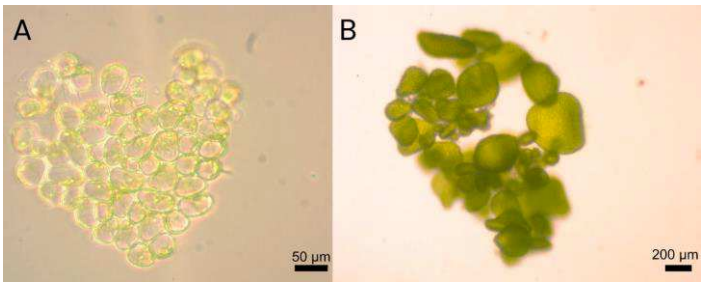

Fig. 10 – Protoplast aggregates' development. A: protoplast aggregates; B: protoplast aggregates development as single discs (from *U. lacinulata*).

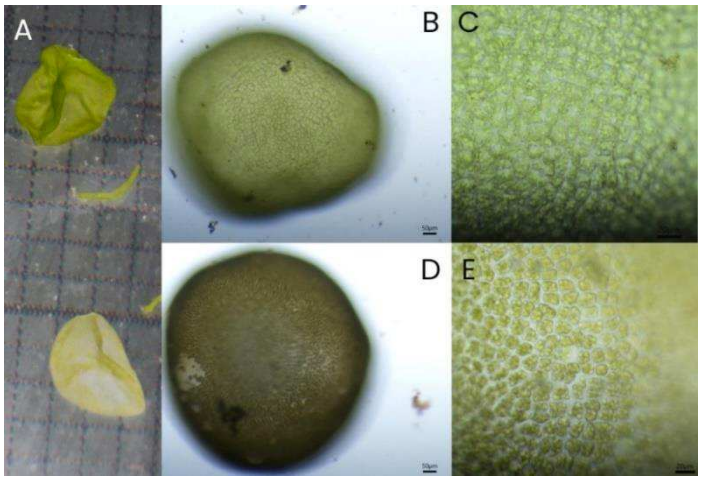

Fig. 11 – Binocular and microscopic observations of the maturity of *U. lacinulata* discs observed at the end of the germination experiment (5 weeks). A: binocular observation and comparison between a green and a brown disc developed from protoplasts (1 square is 1 x 1 mm). B and C: microscopic observations of the green (non-fertile) disc; D and E: microscopic observations of the brown (fertile) discs, gametes were visible and swimming inside of the cells. In the center is visible one single spherical larger cell, similar to the ones that later became protoplasts.

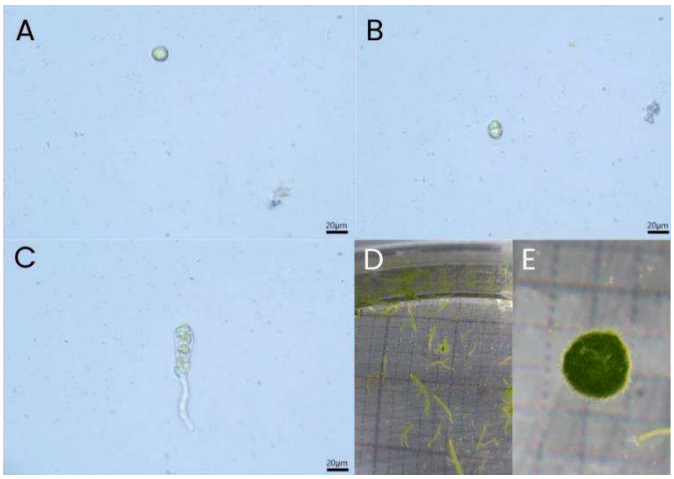

Fig. 12 - Development of *U. lacinulata* gametes released by fertile unattached discs. A: single *Ulva* cell; B: First cell division; C: Germling with a visible rhizoid D: attached germlings with one single filament; E: "germling cluster".

The fertility of the unattached discs was calculated by separating them by colour. The results showed that the majority of discs (81 %) remained vegetative, while 19 % became fertile from the 4th week on (Fig. 13). For each beaker, the ratio of unattached germlings and unattached discs was approximately 1:1 (Fig. 14). Due to contamination issues in the samples from B1, only replicates B2, B3 and B4 were used for the analysis of the germination and follow up experiments.

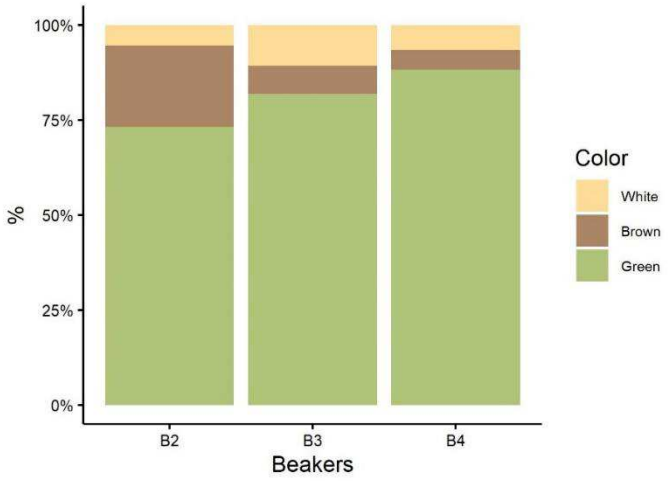

Fig. 13 – Total of *U. lacinulata*'s discs germinated from protoplasts counted at the end of the germination experiment (as 100 %), separated by the relative percentage of the three possible disc colours found.

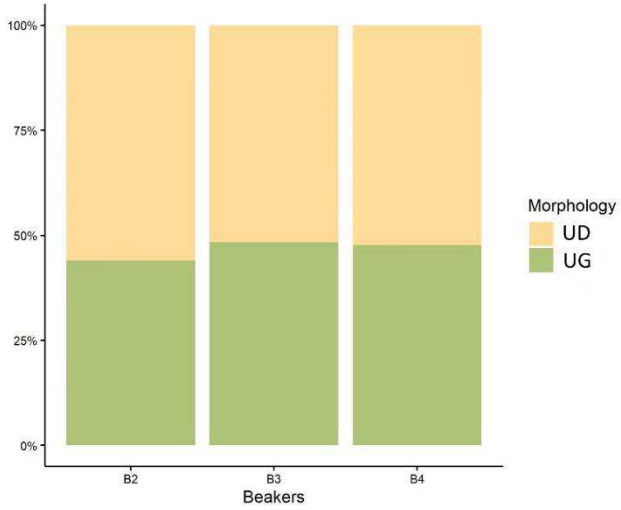

Fig. 14 – Total of *U. lacinulata'*s germinated protoplasts counted at the end of the germination experiment (as 100 %), separated by the relative percentage of the two morphologies found. UD: unattached discs; UG: unattached germlings.

In the same experiment with the *Ulva compressa*, the germination of protoplasts was also observed, for four weeks. On average, germination varied between 1 % and 10 % between replicates, and the average germination was 4 %. No clear ratio was found between replicates. However, in total 54 % of protoplasts germinated into UG and 46 % germinated into UD, suggesting a ratio close to 1:1 (Fig 15).
The presence of *U. compressa* germlings attached to the bottom of the Petri dish was visible by the naked eye in high density after 2 weeks. Discs and unattached germlings were only observed during the 4th week. The attached germlings had a different morphology than the AGs reported in the experiment with the *U. lacinulata*. Instead of one filament, these germlings developed several branches. In this experiment, all discs remained vegetative for the duration of the experiment.

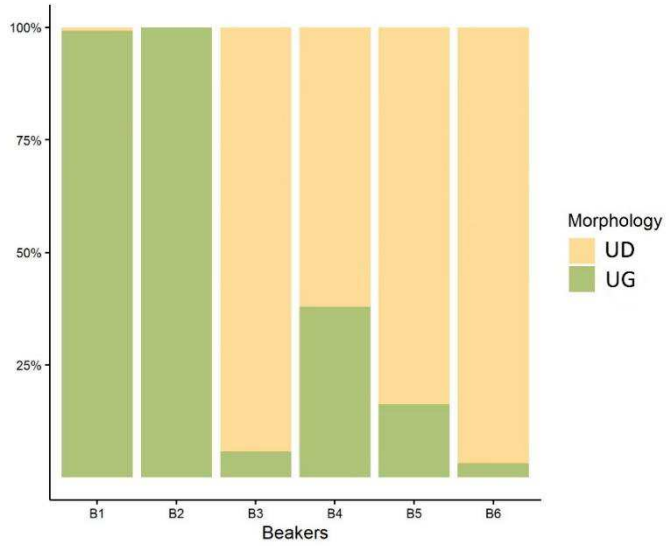

Fig. 15 – Total of *Ulva compressa* germinated protoplasts counted at the end of the germination experiment (as 100 %), separated by the relative percentage of the two morphologies found. UD: unattached discs; UG: unattached germlings.

Discussion

The aims of our work were threefold: 1) to understand and define "degradation events" in non-sporulating *Ulva* species, 2) to demonstrate the existence of naturally occurring protoplasts during degradation and reproduction events in two *Ulva* spp., and 3) to describe and quantify the process of protoplast regeneration, germination and morphological development. Our results provide the first evidence that protoplasts occur naturally in the two *Ulva* spp. tested. In the following discussion, we attempt to convey the significance of our results within the framework of the existing literature and knowledge in the research field.

Demonstration of natural protoplast production in two Ulva spp.

The cells found in the water of the two *Ulva* cultures during degradation and a reproduction event were protoplasts. This was confirmed by the fluorescent staining CFW that showed the total absence of cell walls from large spherical *Ulva* cells. Large spherical cells have been reported before in the work from Bonneau (1978) corresponding to sloughed *Ulva* cells resembling settled swarmers, being spherical instead of polygonal. These cells also resembled protoplasts that have been isolated from *Ulva* spp. (Gupta et al., 2018; Reddy & Seth, 2018). We found the complete absence of cell wall in these cells and the partial absence of cell wall in similar ones found attached to the original thalli. Suggesting that during the degradation observed in the *Ulva* pieces, the cell wall degraded until protoplasts were released into the water. The entire cell wall regeneration from an *Ulva* protoplast usually takes around 3 days (Reddy &

Fujita, 1991; Gupta & Reddy, 2018). We assumed that the observed cells with partial cell walls were either in the process of losing the remaining cell wall or already in the process of regenerating it and that these cells had been released throughout the previous week of the experiment. *Ulva lacinulata* produced protoplasts with an average diameter of 39.36 ± 0.97 µm and *U. compressa* produced protoplasts with an average diameter of 20.53 ± 0.3 µm. While the latter presents sizes in the range of what has been reported by Gupta and Reddy (2018), between 20 and 32 µm, protoplasts from *U. lacinulata* were somewhat larger. However, the sizes reported in this work are just an estimation, as it was not possible to access the regeneration stage of each cell. After regeneration of the cell wall, we demonstrated that these protoplasts germinated. The regeneration and germination of *Ulva* protoplasts have been reported in the literature associated with protoplast isolation methods, and corroborate our observations (Gupta et al., 2018; Reddy & Seth, 2018). Works with protoplasts obtained from higher plants (Grzebelus et al., 2012; Sangra et al., 2019), non-vascular embryophytes (Neubauer et al., 2022) and red and brown seaweeds, reported in the literature, also present similar observations that corroborate our work (Fujita & Saito, 1990; Pak et al., 1991; Kim et al., 2001; Dipakkore et al., 2005; Ye et al., 2005; Avila-Peltroche et al., 2019). Coenocytic and symphonic green macroalgae species such as Bryopsis sp. can spontaneously form protoplast-like structures after simple thallus fragmentation. The occurrence of these structures is associated with the capacity to heal from wounds, a quality that they need in nature (Kim et al., 2001). Provasoli (1958) and Bonneau (1978) presented works with *Ulva* spp., in axenic conditions and wild material, that resemble the findings in our work but do not mention the occurrence of protoplasts. Lin et al. (2008) reported that simple fragmentation of the thalli can cause protoplasts in *Ulva prolifera* but does not describe how or in which circumstances. None of the three works report the number of protoplasts produced or their regeneration rates. Only protoplast isolation methods report yields and regeneration rates (Gupta et al., 2018; Reddy & Seth, 2018).

Protoplast isolation can be achieved through two methods: mechanically or enzymatically. Mechanically, *Ulva* protoplasts can be obtained through fragmentation of the thalli (Lin et al., 2008) or with the assistance of microtools (Fjeld, 1972). The enzymatic method, usually done after thallus fragmentation, is the most common to degrade the seaweed's cell wall (Gupta et al., 2018; Reddy & Seth, 2018). Several enzymes have been tested for the isolation of seaweed protoplasts such as protease P, crude enzymes (e.g., bacterial, abalone), and cellulysin (Fujita & Saito, 1990; Reddy et al., 1990; Björk et al., 1992; Rusig & Cosson, 2001). In most recent years, the isolation of *Ulva* protoplasts has been performed with cellulase Onozuka R-10 (Gupta et al., 2018; Reddy & Seth, 2018; Avila-Peltroche 2021). For tubular morphologies of *Ulva*, the additional use of Macerozyme R-10 has been recommended (Reddy et al., 2006; Gupta et al., 2018; Reddy & Seth, 2018; Wu et al., 2018). After protoplast isolation, the method's success is evaluated using Calcofluor White (CFW), a fluorescent dye that binds with the cellulase in the seaweed's cell wall (Albani, 2011). The absence of fluorescence around the cells indicated the presence of protoplasts (Reddy et al., 1989). By following part of the steps established for protoplast isolation (e.g., use of a mesh with a 30 µm size and confirmation of the absence of the cell wall with CFW) we have found *Ulva* protoplasts in our cultures without requiring enzyme solutions or any alterations to the original cultivation conditions.

The regeneration rates of protoplasts ranged between 1 and 2 % in the experiment with *U. lacinulata* and between 1 and 10 % in the experiment with *U. compressa*. We assume that protoplast handling and culture conditions can have caused the rupture or death of some protoplasts (e.g., during the centrifugation process). In Gupta and Reddy (2018) it is suggested that only glass pipets should be used during protoplast isolation. We used micropipettes with plastic tips to inoculate the protoplasts into the different Petri dishes. Isolation of protoplasts is usually done by incubating the *Ulva* thalli in the dark (or low light) at higher temperatures (e.g.,

25 ˚C) for two to three hours with an enzymatic mixture and later keeping the isolated protoplasts under the same conditions for 24 h (Reddy et al., 2006; Gupta et al., 2018; Gupta & Reddy, 2018). As we aimed to evaluate the natural occurrence and impact of the protoplasts obtained through natural processes, we did not follow the incubation step suggested previously. The entire process was done in the pre-set culture conditions of 15 ˚C and ~ 80 µmol photons m^{-2} s^{-1}. However, low light has been reported to be a requirement for the germination and growth of protoplasts isolated from *Kapaphycus alvarezii* (Salvador & Serrano, 2005), suggesting that this can be a reason for the low number of protoplasts that germinated in our work. Starvation may have been a cause for low germination rates, as the medium in the Petri dishes was not exchanged during the five weeks to not disturb or destroy the cells. This could justify why some of the cell masses and unattached discs underwent gametogenesis. Microscopic cell masses and attached germlings that developed from the cell masses or discs were not counted in the germination rate. Gupta et al. (2018) and Gupta and Reddy (2018) calculated regeneration rates based on the number of protoplasts that developed into germlings, but it is unclear at which stage of development the germlings were counted. Reddy et al. (2006) calculated the regeneration success by counting the number of dividing cells found in 20 random microscopic fields after 7 days. Reddy and Seth (2018) followed the same process but counted the dividing cells after 5 days. In each of these works, regeneration rates were higher than 87 %. The method used by Reddy and Seth (2018) assumes that all microscopic groups of cells found will have the capacity to germinate into new individuals. The method used by Gupta et al. (2018) and Gupta and Reddy (2018) assumes that all germlings found directly originated from protoplasts. This goes against our finding and what has been reported by Bonneau (1978) that cell masses and small discs will become fertile and will not be found developing further into new individuals. Therefore, germlings originating by gametes were also present in the Petri dishes. A similar observation has been reported by Gupta et al. 2018 where some protoplasts, after regenerating their cell walls, became sporangia and released spores before cell division while other protoplasts developed into small distromatic plantlets and sporulated at a very early stage. It is not clear at which moment germlings were counted for the calculation of the regeneration rates and if these sporulation events were considered. Variations in method and unclear definitions between regeneration and germination can cause difficulties when comparing the results of different works. Because of our findings, we defined our calculations as the germination rate instead of regeneration. Our work evaluates the percentage of protoplasts that developed into different morphologies and became fertile. Similar work should be done with the cell masses found, to understand the impact that these masses can have in the calculation of regeneration rates.

Morphological development of protoplasts

The protoplasts collected in our experiment germinated into the same morphologies (discs and germlings) as the ones reported in the protoplast isolation literature (Gupta et al., 2018; Reddy & Seth, 2018) and the ones reported by Provasoli (1958) and Bonneau (1978). However, the presence of rhizoids in these morphologies is not clear. Some articles report the presence of rhizoids in the germlings that originated from protoplasts (Rusig & Cosson, 2001; Reddy & Seth, 2018). In other cases, the germlings and discs do not show any signs of a rhizoid area (Gupta et al., 2012; Gupta et al., 2018; Wu et al., 2018). To our knowledge, most of the published images of regenerated protoplasts isolated from *Ulva* species show germlings and discs without a visible rhizoid area. These images agree with the ones collected during our experiment, where at no point rhizoids were found and all the discs and germlings were found floating in the Petri dishes. Structures similar to the unattached discs (UD) have been reported by Fjeld (1970) and identified as "*bubble*" phenotype and by Provasoli (1958) that described them as pincushions. Germlings with a rhizoid (similar to the AG morphology in our work) were identified as wildtype

phenotype (Oertel et al., 2015). However, it is not clear if the "*bubble*" phenotype exhibited a rhizoidal area. Föyn (1961) reported a recessive mutant in *Ulva mutabilis* Föyn (nowadays named *Ulva compressa* Linnaeus; Steinhagen et al., 2019a) designated "*globose*" with morphology similar to "bubble" (Fjeld, 1970; Oertel et al., 2015) and to the unattached discs in our work. The major difference besides the *Ulva* species, is the strong presence of rhizoid cells in the "*globose*" mutant, which we did not observe (Föyn 1961).

The different morphologies found in our work are usually observed together after protoplast isolation methods are used (Oertel et al., 2015; Gupta et al., 2018; Reddy & Seth, 2018). *Ulva* species have been known to vary their morphology for several reasons such as abiotic factors (Hofmann et al., 2010; Steinhagen et al., 2019b), or the presence of bacteria and molecules (e.g., *thallusin*) that are known to play an important role in the development, growth, and morphology of *Ulva* species (Spoerner et al.,, 2012; Wichard, 2015: Wichard et al., 2015). But we expected that cells that are clones of each other, originating from the same pieces of thallus, growing under the same conditions, in the same Petri dishes, would share the same phenotype. Reddy et al. (1989) suggested that the two different growing patterns (germlings and discs) might be caused by the differentiation of each cell when still attached to the thalli. Previous work from Provasoli (1958) agrees with the assumption from Reddy et al. (1989). The pincushion germlings from *Ulva* sp. found by Provasoli (1958) were formed directly from cells of the thalli, while other cells from the same piece released zoospores. The different potency of these cells was questioned based on the different morphologies, the direct production of germlings without sporogenesis, and because isles of green cells in the bleached thallus of *Ulva* were found (Bonneau, 1978). We believe these islands of green cells to be similar to our observations in Fig. 2A,B, where the same thalli piece shows larger spherical green cells in the middle of pale smaller ones. The findings from Provasoli (1958) and ours, go against the hypothesis that cells from the *Ulva* thallus are irreversibly determined to produce swarmers, assumed by (Fjeld & Løvlie, 1976). Bonneau (1978) considered green islands and sloughed cells to have the capacity to reverse to a state of totipotency and present a swarmer behaviour. Bonneau (1978) described the development of cells from green islands of the thallus as circular distromatic discs released from the thalli with sizes varying between 0.1 and 1.0 mm in diameter. Some of these discs developed rhizoids while others did not. The development of these discs was separated further into two possible outcomes. Some discs separated their distromatic layers and grew into polymorphous sacs (similar to the ones reported in our work), that were grown for two years, occasionally producing swarmers. Other discs did not grow further and released swarmers. The swarmers released by both disc types presented rhizoids. The work from Bonneau (1978) completely corroborates our findings and description of how the isolated protoplasts developed during our work.

To our knowledge, our work is the first to report a 1:1 ratio between discs and germlings originating from protoplasts. The different morphologies and the ratios should be analysed further as they can shed some light on the mechanisms that determine the morphology of *Ulva* protoplasts. In Provasoli (1958) the green islands in the bleached thalli were avoided when the *Ulva* was grown under inhibitory concentrations of indolacetic acid (IAA; 10 µg %) and gibberellin (100 µg %). The mechanism that determines the fate of each protoplast, which will either become a fertile cell mass, a disc, or a germling-like structure can be a turning point to understanding the *Ulva*'s asexual reproduction strategies.

Between the two germlings types (floating and attached), there were some distinct characteristics. The UGs, which we assume were directly developed from protoplasts, always presented more than three filaments or small blades, with no rhizoid. The AGs, which we understand originated from gametes released by cell masses and unattached discs, presented only one filament growing attached to the Petri dish by a visible rhizoid. As our results show, UD and

UG were observed earlier in the experiment (from the second week on) and the AG appeared later (at five weeks). We hypothesized that the AG were germlings developed through spores or gametes. To test our hypothesis, *U. lacinulata* discs that originated from protoplasts were separated into new Petri dishes and provided with fresh medium and nutrients. The results showed that the green discs changed to brown and eventually white and released gametes (confirmed by the phototaxis technique). All the germlings originated from those gametes grew into the same AG morphology with a visible rhizoid, attached to the Petri dish, thus proving our hypothesis. Our findings are further corroborated by the observations from Bonneau (1978) where all of the germlings originated from discs also presented a rhizoid. The following Fig. 16 summarizes the timeframe of degradation and development of new individuals and details in which moment each of the different morphologies was observed.

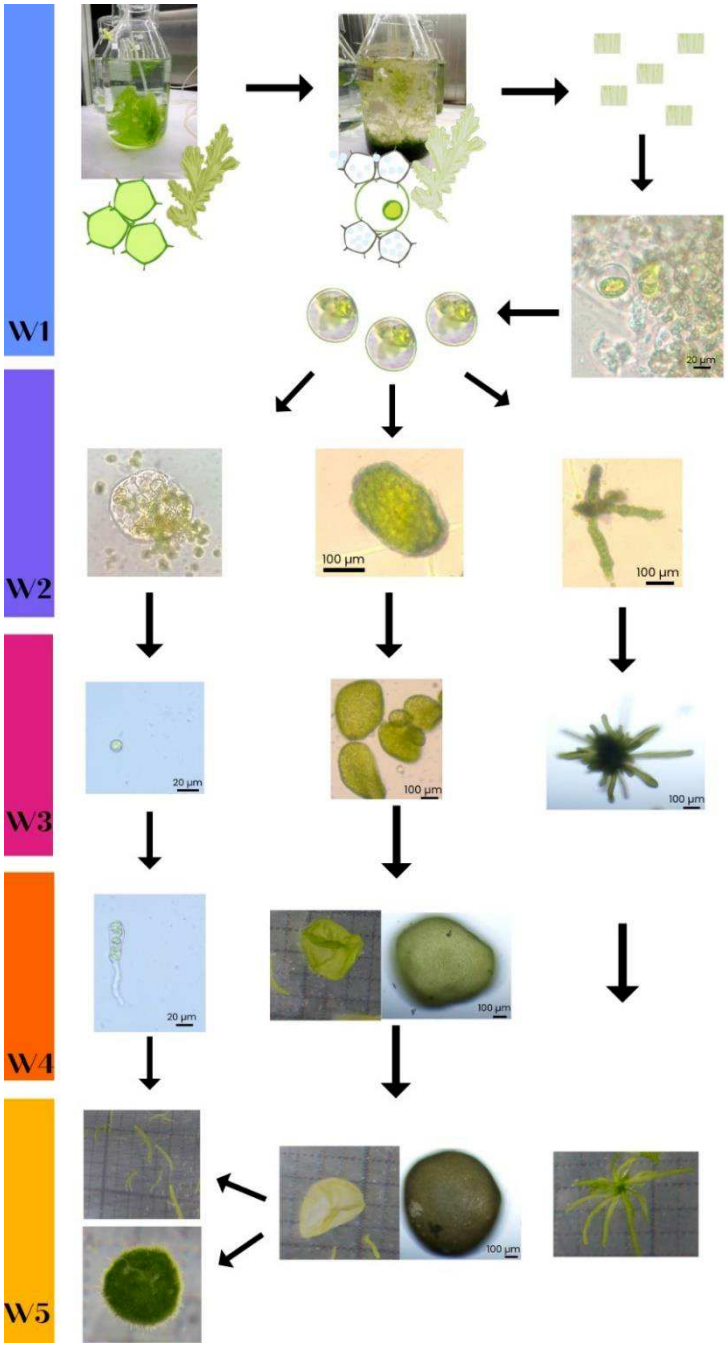

Figure 16 - Visual representation of our observations throughout the two main experiments presented in this work (Degradation experiment and Germination experiment). W1-5 represent the week when the different development stages were observed. W1 indicates the observations made during the degradation experiment. W2-5 indicate the observations made during the germination experiment. [Graphical design created in Canva (Canva, n.d.)].

Distinguishing between reproductive events and degradation events

By describing the degradation process of *U. lacinulata* and *Ulva compressa*, we were able to compare a non-sporulating species that never showed signs of fertility and grew in ASW for two years, with a sporulating species that was collected from the wild and cultivated in NSW. The observation and confirmation of naturally produced protoplasts in both species demonstrates that protoplast production is not just an artifact of cultivation in artificial seawater, poor culture conditions nor it is limited to cultures that have been in the laboratory for years. A similar comparions can be made between the obsevartions from Provasoli (1958) and Bonneau (1978). Provasoli (1958) observed green islands of *Ulva* cells during a laboratory experiment with ASW and plant hormones, in axenic conditions. But Bonneau (1978) observed green islands in bleaching fragments of *Ulva* collected from the wild in Connecticut and New Jersey. In both cases, swarmer release was observed indicating that protoplasts were occurring in sporulating species. Our work distinguishes between a degradation event and a reproduction event and shows that protoplasts are the main outcome of the degradation observed. The degradation event provided new seeding for the next generation of biomass. These events can play an important role in the life cycle of species that are considered non-sporulating (Bonneau 1978). While the trigger for degradation events remains unknown, we were able to rule out our cultivation conditions as the main trigger. *Ulva compressa* released protoplasts only five days after collection from the wild while kept in NSW. Therefore, we hypothesize that the production of protoplasts in the wild may be a common occurrence and an important part of the natural life cycle of some *Ulva* spp. (Bonneau 1978).

Contrary to our expectations that all of the biomass would be lost during a degradation event, *Ulva lacinulata* thalli regenerated and presented high relative growth rates after a period of biomass loss or slow growth. Protoplasts were found to be continually released into the water, confirming that cellular degradation occurred in all the samples while biomass was growing. This suggests that the rates of cell wall degradation and protoplast release were lower than the growth of the biomass and only in some cases protoplast production caused net biomass loss. Because of the high variation in protoplast yields, and the simultaneous growth of biomass, the relationship between protoplast production and biomass was not linear when the biomass continued growing. We hypothesize that the high variability is related to the timing of protoplast production. Each thallus may have begun the degradation process at different times, thus creating variation in protoplast yields. This high variability may be natural and dependent on the size or age.

In the literature, concepts of degradation or similar, are usually associated with the fertility of *Ulva* and the release of gametes and spores (Ryther et al., 1984; Bolton et al., 2008). However, as reported in our work with *U. lacinulata*, and by Bonneau (1978), a species does not have to be fertile to degrade and it does not need to lose weight to generate new individuals. We found that more protoplasts were produced by *Ulva lacinulata* (4.26×10^8 cell g^{-1}) than by *Ulva compressa* (5.54×10^5 cell g^{-1}). *Ulva lacinulata* grew eight times its original weight by the end of the experiment while the original biomass from the sporulating species was lost. We assume the difference between the two species was the reproduction event observed in *U. compressa*, where the majority of biomass was lost during the production of swarmers.

In this work, we separated the concept of degradation event from reproduction and defined a degradation event of a non-sporulating species. We believe that the separation between these two concepts is essential to understanding and working with *Ulva* species. We understand degradation as an entirely separate event that can occur in non-sporulating species, during a period in which the *Ulva* thalli becomes fragile, changes colour from bright green to yellow-green and white and breaks into smaller fragments. These small fragments are visible in the water column creating some turbidity and usually accumulating at the rim of the culture vessel

together with foam. Under the microscope the thallus becomes heterogeneous with two distinct cell morphologies: 1) spherical green cells that will detach and originate protoplasts and 2) polygonal/squared pale cells full of starch. A degradation event stops when biomass starts to regenerate and increase its RGR. Our description of the thallus and the cells matches the description from Bonneau (1978). Waite and Mitchell (1976) found two bacterial isolates with the capacity to penetrate the *Ulva* cell wall and fragment its thallus. Recently 13 *ulvan*-degrading bacteria were isolated from wild decomposed Ulva material, for the first time, indicating that different genera of bacteria can be associated with *Ulva* decomposition in the wild (Tanaka et al., 2022). Waite and Mitchell (1976) and Bonneau (1978) postulated that stressful conditions could lead opportunistic bacteria to degrade and penetrate the cell wall, thus releasing *Ulva* cells into the water that can escape the stressful conditions. More work is necessary to determine the cause of the degradation of *Ulva* and what stops it. It should be studied the *Ulva*-bacteria interactions during stressful circumstances and how they correlate to the protoplast release and cell differentiation in the thalli.

Relevance to the *Ulva* life-cycle

Ulva lacinulata was never found to become fertile during the two years of cultivation in our facilities despite several attempts to induce reproduction. A similar limitation while working with *Ulva lactuca* has been reported before (Bonneau, 1976). Both microscopic cell masses and some of the discs, which originated from protoplasts, became fertile and released gametes (Fig. S1). Under the microscope, gametogenesis was observed for the first time in this species, since it has been in our cultures. Floating and attached germlings did not show signs of reproduction, only the discs and cell masses did. This suggests that discs and cell masses are an intermediate stage during *Ulva*'s development that never develops into adult thalli and its function is to produce swarmers. Our findings are corroborated by the work from Bonneau (1978) and close a knowledge gap in the life cycle of a non-sporulating species, which we now know can sporulate, but not as an adult.

The age of the thalli plays a role in the maturation of *Ulva* species. *U. rigida* becomes more sensitive to stimuli and reproduces more with increasing age (Gao et al., 2017b). Sporulation inhibitors in *U. compressa* play an important role in controlling reproduction (Nilsen & Nordby, 1975; Wichard, 2023). Young *Ulva* pieces have higher levels of sporulation inhibitors (SI-1 and SI-2) than aged *Ulva* pieces, only when the concentration of sporulation inhibitors falls below a certain threshold, sporulation can occur (Stratmann et al., 1996; Wichard, 2015; Obolski et al., 2022). Thus, it has been proposed that, for cultivation purposes, *Ulva* should be grown in a mixed-age culture, keeping high levels of SI's and preventing the loss of biomass caused by sporulation events (Obolski et al., 2022). Stratmann et al. (1996) indicated that when *Ulva* is grown under semi-natural conditions, the spontaneous occurrence of gametogenesis would not occur before the age of 5 weeks. This time constraint, until *Ulva* can become fertile, is commonly known. Huang et al. (2023) reported that *Enteromorpha prolifera* (currently known as *Ulva prolifera*; Hayden et al., 2003), under normal circumstances, takes a long time to grow and become fertile ("dozens of days or even months"). These time frames reported in the literature are significantly longer than what we observed in our work. Visible attached germlings were found after 5 weeks. We estimate that *U. lacinulata* discs and microscopic cell masses, became fertile on their own between the second and the third week of the experiment. By the end of the germination experiment, discs were presenting small sizes of around 4 mm (diameter), and the water in the dishes had not been changed (the presence of SI should have been high). The fact that the discs reproduced in such early stages goes against what is known in *Ulva* species. Gupta et al. (2018) have reported the capacity of isolated protoplasts to sporulate soon after regeneration of the cell wall. More recently, Huang et al. (2023) found that germlings of *U.*

prolifera with only 2.4 mm in length only needed a few days to become fertile, reproduce, and dye. These findings agree with what we have described regarding the size of the discs and cell masses and the timeframe in which they reproduced.

Clusters of germlings were also found in the Petri dishes at the end of the germination experiment with the *U. lacinulata* (Fig. 11d, Fig. 15). Smaller trials were set to try to understand the occurrence of clusters (data not shown). We hypothesized that the clusters were related to the discs. We concluded that these clusters found floating in the Petri dishes were dead *Ulva* discs that had released gametes and were being now used as a surface for the new gametophytic germlings to grow. In the recent work of Huang et al. (2023) a similar explanation for the occurrence of these clusters is given. Reproductive cells of *U. prolifera* would be released into the water if the parental thalli were submerged in it. However, in the circumstance of the parental thalli being exposed to air or only partially submerged, the reproductive cells would germinate on top of the parental thalli (Huang et al., 2023). This information coincides with the circumstances found in our work. The discs were floating in the water, and the water was not changed for the entirety of the experiment which led to evaporation in the Petri dishes. The discs grew into a three-dimensional shape with air in between the cell layers, as described previously by Bonneau (1978), which allowed them to expand and be more exposed. The partial exposure of the discs during gametogenesis could promote the formation of the *Ulva* clusters we observed. Nevertheless, the gametes' development into these clusters should be explored further it can be a potential strategy to obtain nutrients, and protection, and disperse further away and for longer than gametes can swim by themselves (Bonneau, 1978; Miyamura, 2004). For this work, during the germination experiment, each cluster was counted as an individual disc.

Relevance to large-scale cultivation

The use of protoplasts as seeding material for large-scale cultivation has been suggested before by Gupta et al. (2018). While it is a promising technique, its associated costs still confer a limitation to its applicability (Gupta et al., 2018). Finding new ways to decrease its costs and/or increase its effectiveness is needed. In our work, despite not knowing the trigger of this event, we have found that protoplasts occur naturally and germinate without requiring expensive enzyme solutions that increase the costs of the isolation method. But a direct comparison is unfair, because our experiment lasted four weeks, while the isolation method by Gupta & Reddy (2018) and others only took one day. Because of the long duration of our experiment, protoplast yields (from the NE-Atlantic *U. lacinulata*) varied strongly between replicates and weeks. If we consider the original biomass, at the beginning of the experiment, as the sole source of the protoplasts produced during the 4 weeks, the average protoplast production ($2.66 \pm 0.20 \times 10^6$ cell g^{-1}) was only between 3 and 4 % of the protoplast yields reported by Gupta and Reddy (2018). However, if we consider the total protoplast yields obtained at the end of the experiment (4.26×10^8 cell g^{-1}) the results show that we have achieved more than five times the number of protoplasts that Gupta & Reddy (2018) isolated from *U. rigida* and *Ulva lactuca* Linnaeus (wild). Additionally, in the isolation methods reported in the literature, the biomass is completely lost, while in our work the fragmented *Ulva* pieces grew eightfold. Further work is needed to determine what triggers degradation events so that this knowledge can be applied to large-scale *Ulva* cultivation.

Relevance to green tide occurrences

We believe our findings can shed light on what might be happening in some of the reported "green tide" events. Both *U. lacinulata* and *U. compressa* have been associated with such blooms (Kamermans et al., 1998; Largo et al., 2004; Guidone et al., 2012; Potter et al., 2016; Gao et al., 2017a; Wan et al., 2017; Chávez-Sánchez et al., 2018; Bermejo et al., 2023), sometimes, *U.*

lacinulata under the name of *Ulva rigida* (Hughey et al., 2022). The occurrence of "green tide" events is usually associated with seasonality, the life cycle, and the vegetative growth of *Ulva* (Largo et al., 2004; Bermejo et al., 2023). However, the existent explanations for the occurrence of "green tides" do not support all the occurrences. Huang et al. (2023) propose that the capacity for infantile thallus to reproduce in such an early stage can explain the "green tide" forming traits of *U. prolifera*, suggesting that other possible scenarios would not amount to the same degree of blooming. Additionally, Huang et al. (2023) justify the phenomenon of on-year and off-year blooms by suggesting that the large amounts of adult biomass that constitute the blooms are probably the offspring of early infantile thalli. This would mean that the offspring of early-stage reproduction would present high growth rates and would not go into degradation/reproduction until the end of the bloom occurrence. We cannot determine with certainty what happened to the different discs and germlings after the 5-week germination experiment. After the experiment, discs, clusters, floating and attached germlings were placed together in a larger vessel for cultivation purposes. Very soon after, germling-shaped *Ulva* was the dominant morphology (data not shown). This material, all of which directly or indirectly originated from the protoplasts released during the degradation events reported in this work, has been growing with no signs of reproduction or degradation. Similar observations by Bonneau (1978) reported the maintenance of the disc morphology for 2 years. Overall, the behaviour we found in cells originating from protoplasts of *U. lacinulata*, is very similar to what Huang et al., observed. *U. lacinulata* and other *Ulva* spp. may have a similar strategy.

Using the information provided by Zhang et al., (2013) regarding gamete production per cm^2 of *U. prolifera*, we estimated the amount of *Ulva* material that was produced by the original 0.716 g of *Ulva* from our experiment. Fig. 17 presents a comprehensive summary of our observations and conclusions and reports the estimation of total individuals produced directly (e.g. protoplasts) or indirectly (e.g., gametes released by the UD) by the original biomass. The estimation was based on the average protoplast yields obtained after 4 weeks ($2.66 \pm 0.20 \times 10^6$ cell g^{-1}). Based on our results, we assumed the 2 % protoplast germination rate and the 1:1 ratio between the two unattached morphologies. We considered each disc to have an average diameter of 3 mm. Based on our later observations cultivating this material, after the 5 weeks experiment, we found that all of the discs eventually became fertile. Thus, we considered that 100 % of discs became fertile while unattached germlings kept growing. Additionally, fertile discs presented two cell types, and while most of the cells went through gametogenesis, some cells became protoplasts (Fig. 11E). To facilitate the calculations, we did not consider the occurrence of fertile cell masses, or the protoplasts released by unattached discs during gametogenesis. We estimate that $4.64 \pm 0.35 \times 10^{10}$ cells g^{-1}, with the potential to grow as new individuals, were produced by the 0.716 g of the original biomass. If we consider the total of protoplast collected during our work (4.26×10^8 cells g^{-1}), we estimate the total of new individuals created by the original biomass to be 7.42×10^{12}. These results should be considered in addition to the eightfold increase in weight of the original biomass registered at the end of the four weeks. Because the cell masses produced by protoplasts and the small number of protoplasts produced by the discs (while going through gametogenesis) were not counted, we consider the estimation in Fig. 17 an underestimation, and including these two entities will impact positively our estimations.

Following the same estimation, based on the work from Zhang et al. (2013), direct gametogenesis of the 0.716g of original biomass would only produce 1.45×10^9 cells g^{-1} potentially new individuals and the original biomass would have been lost (Fig. 17). Even though these are estimations based on work done with a different *Ulva* species, the extreme difference in results suggests that *Ulva* degradation and protoplast production can be a more efficient strategy than the traditional gametogenesis process.

After the experiments reported in this work, we observed that, even when discs go through gametogenesis, some cells are released into the water as protoplasts. This agrees with our findings regarding the presence of protoplasts in the sporulating *Ulva compressa*. All these observations suggest that the occurrence of protoplasts, while strongly associated with the degradation event reported in this work, can occur in other moments of *Ulva*'s development as well. Bonneau (1978) questioned the importance of motile *Ulva* cells as a strategy of dispersion, based on the occurrence of *Ulva* spp. in eulittoral and high sublittoral areas, where waves and currents could be considered stronger dispersion mechanisms. From Bonneau's (1978) perspective, single cells, such as the ones forming green islands (Provasoli 1958), or protoplasts (in our work) would be as effective in dispersal as swarmers. The totipotency of these cells, the bacterial interactions that can play a role during stressful conditions and the variation in morphologies found when working with these cells, were considered by Bonneau (1978) and in our work as proof of the marvellous *Ulva* spp.'s capacity to survive.

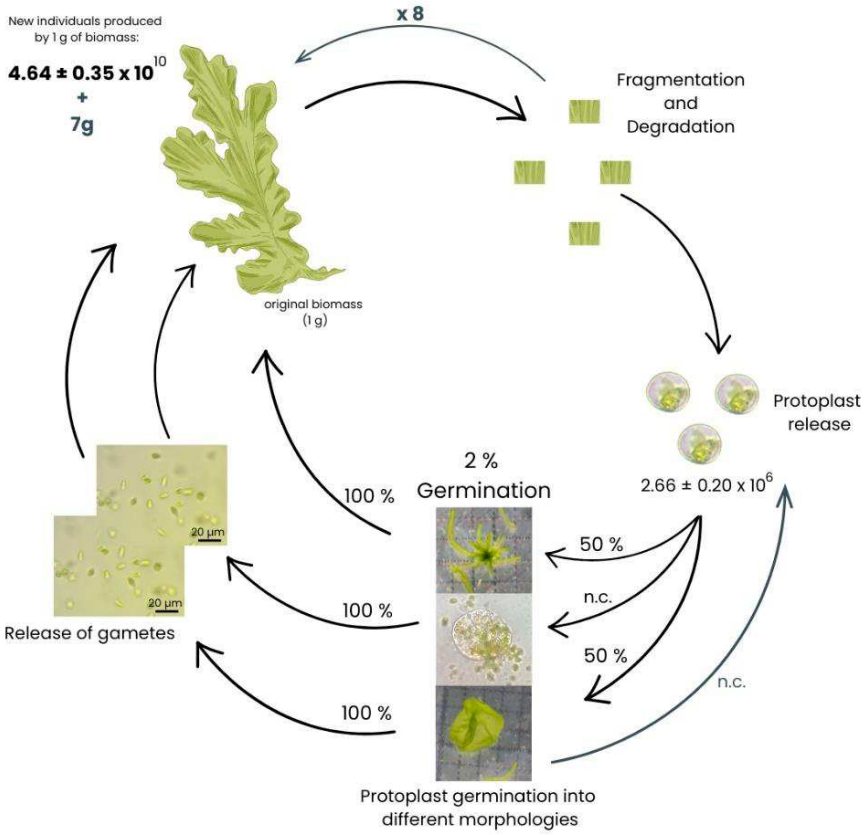

Figure 17 – NE-Atlantic *U. lacinulata* life cycle based on reproduction via protoplasts and estimation of the number of individuals that would be produced, per gram, during a degradation event. This estimation was compared with the number of individuals produced if *Ulva lacinulata* became fertile and directly released gametes (1.45 x10^9 cells g^{-1}). The estimations were calculated based on the average protoplast yields calculated in our work and the average values reported by Zhang et al., (2013). Legend: n.c.: cell masses and protoplasts produced by the fertile discs were not considered for the estimation. [Graphical design created in Canva (Canva, n.d.)].

Conclusion

With our work, we have demonstrated for the first time the production of protoplast in two *Ulva* spp.. Additionally, our work is the first attempt to define degradation events in *Ulva* that occur in controlled cultivation settings. Moreover, we have described for the first time the mode of sexual reproduction in *U. lacinulata*, which was previously considered, by us, as a non-sporulating strain, and the morphological development of naturally occurring protoplasts. The natural protoplast development was extensively corroborated by works from Provasoli (1958) and Boneau (1978) that highlighted the importance of understanding the complexity of *Ulva*'s asexual strategies. In our work we have also shown how protoplasts and degradation events can be used to produce new biomass (an important step for large-scale cultivation) and we have suggested that this mode of reproduction could be related to green-tide formation.

By following the degradation events occurring in the non-sporulating *U. lacinulata* we were able to establish a clear definition that hopefully can be used moving forward to learn how to control such events and use them in our favour. With this definition of degradation, we have distinguished these events from the known reproduction events. Degradation, as it has been reported in this work, can justify how non-fertile strains occur, survive, and even strive by regenerating and producing new individuals through protoplast production, making this method a highly profitable process for the species.

Our work shows that fragments originating from partially degraded pieces of *Ulva* have the capacity to regrow or keep growing even while releasing protoplasts. This information provides new possibilities for the large-scale cultivation of *Ulva* spp.. and understanding the mechanism behind this process can allow us to explore it further and optimize *Ulva* cultivation processes (e.g., increasing RGRs while guaranteeing high amounts of seedling material). Understanding the degradation event can even potentially mean learning how to prevent it from happening at all.

There is still plenty to understand based on our observations regarding what is causing the degradation of the *Ulva* and its cell wall. A common denominator between a species cultivated under laboratory conditions (with ASW) for two years and a species collected from the wild, cultivated briefly (with NSW) is still to be found. Additionally, the real impact of such occurrences in the wild is still to be measured. Nevertheless, we hope our work opens the doors to new possibilities, interesting discussions, and solutions against major bottlenecks in *Ulva* cultivation and the formation of "green tides".

Declarations

Funding:

This research was supported within the framework of the Mak-Pak Scale-Up Project by funds from the Federal Ministry of Food and Agriculture (BMEL) based on a decision of the parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support program (Funding Reference Number 281DL02B20) and the Formas-funded 'A manual for the use of sustainable marine resources' project (Grant no. 2022-00331).

Nagoya Protocol:

We have written confirmation by the Instituto da Conservação da Natureza e das Florestas (ICNF) in its function as ABS National Focal Point as well as Competent National Authority that although Portugal is a party to the Nagoya Protocol no national legislation nor any regulatory requirements drawing from the Nagoya Protocol for access to genetic resources in

mainland Portugal exist presently. As the samples for this project were collected in mainland Portugal, there are no applicable prior informed consent requirements.

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

Data availability:
The raw data used in this work was submitted to PANGAEA and it is currently under revision. The molecular sequences of the species presented in this work are deposited in GenBank with the accession numbers: OP778143 (NE-Atlantic *Ulva lacinulata*).

Code availability:
Not applicable

Author's contributions:
LCH, IC conceptualized and designed the studies, IC drafted the manuscript, LCH supervised the studies, IC carried out the studies, collected and analysed the data. SST conducted the molecular identification of the strains used in this manuscript. LCH, BHB, IB, SST and K-UV provided technical and scientific supervision, BHB & IB provided lab facilities, BHB & LCH provided administrative support, obtained funding for this project and critically revised the manuscript. All authors approved the manuscript for publication.

Acknowledgments:
The authors would like to thank the SeaWheat COST Action and its members for providing such rich opportunities for discussion and their insight into their own experiences cultivating *Ulva*. In particular, we would like to thank Dr. Thomas Wichard for the interesting conversations and helpful insights about *Ulva*'s peculiarities. Furthermore, we thank Dr. Liam Morrison and Dr. Ricardo Bermejo for hosting Isabel Cardoso at the University of Ireland, for the three-month Short-term Scientific Mission (funded by the SeaWheat COST Action) and sharing their expertise in protoplast isolation in *Ulva* species, as it was the motivation for this work. We would also like to thank Claudia Daniel and Andreas Wagner for their technical support during the experiments and Sabine Strieben and Rike Düffert for the help and support provided in keeping the seaweed cultures under optimal conditions. Additionally, we thank Rebecca Aepfler for her support in complying with the regulations of the Nagoya Protocol. Furthermore, we thank the esteemed Reviewers for critically and thoroughly reading this manuscript.

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Supplementary material

Table S1 – Compilation of the statistical information from the regression analysis performed throughout the experiments (non-linear trends). The information is associated with the number of figures presented in the results section.

Figure		**Multiple R-squared**	**Adjusted R-squared**	**F**		**p-value**
5	B1	0.98	0.94	$F_{(2,1)}$	24.12	**0.143**
	B2	1.00	0.99		101.10	0.070
	B3	1.00	1.00		5523.00	0.010
6	B1	0.74	0.74	$F_{(1, 38)}$	109.6	9.44×10^{-13}
	B3	0.53	0.51	$F_{(3, 36)}$	40.81	1.87×10^{-10}

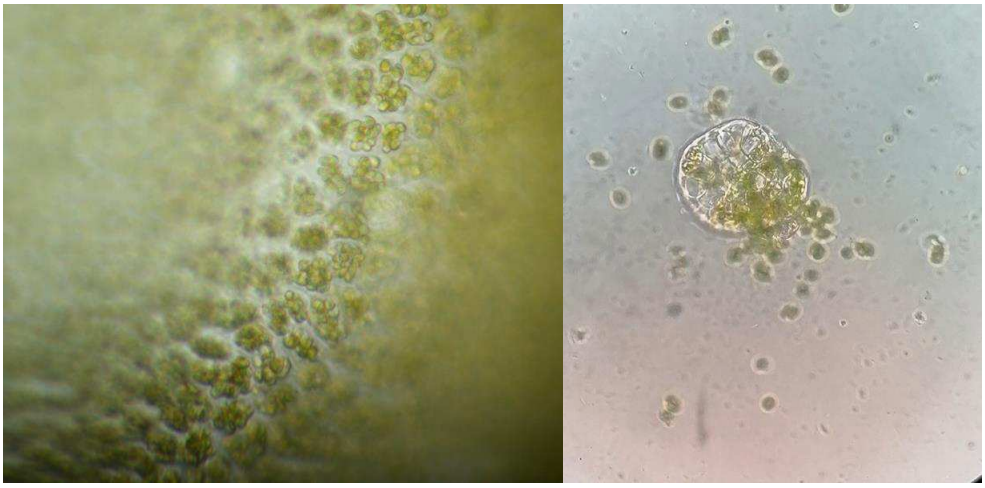

Figure S1 – Two snapshots from the video created to present preliminary results of this work at the SeaWheat COST Action workshop "From Ulva aquaculture to food and feed production: state-of-the-art, bottlenecks, risks and gaps", Lisbon, 2023. On the left: fertile *U. lacinulata* floating disc developed from a protoplast; on the right: Cell mass originated by protoplasts of *U. lacinulata* releasing gametes. The video was It can be accessed at: https://youtu.be/Ulv2LAlUKzw.

6. Discussion

The understanding we have nowadays about *Ulva* and its importance became possible through several eco-physiological studies (Reed & Russell, 1978; Fortes & Lüning, 1980; Lüning, 1984; Sand-Jensen', 1988; Henley & Ramus, 1989; Cohen & Neori, 1991; Orfanidis & Haritonidis, 1996; Lüning et al., 2008), extensive taxonomic analysis (Tanner, 1986; Woolcott & King, 1999; Hayden et al., 2003; Hofmann et al., 2010; J. Wang et al., 2010), genetic and reproductive works (Provasoli, 1958; Föyn, 1960; Föyn, 1961; Fjeld, 1970, 1971, 1972; Hoxmark & Nordby, 1974; Nilsen & Nordby, 1975; Fjeld & Løvlie, 1976; Bonneau, 1978; Løvlie & Bryhni, 1978; Millner et al., 1979; Carl et al., 2014b), the study of *Ulva*-bacteria interactions (Provasoli & Pintner, 1980; Singh et al., 2011; Spoerner, et al., 2012; Wichard, 2015) and the biochemical evaluation (Fleurencel et al., 1995; Lahaye et al., 1996; Flodin & Whitfield, 1999; Kaeffer et al., 1999; Wong & Cheung, 2001; Smit, 2004) of several *Ulva* species. These works established the basis for *Ulva* cultivation (Huguenin, 1976; Fortes & Lüning, 1980; Henley & Ramus, 1989; Cohen & Neori, 1991; Pagand et al., 2000; Neori et al., 2003) and unravelled the complexity of *Ulva*'s life cycle (Hoxmark & Nordby, 1974; Nilsen & Nordby, 1975; Bonneau, 1978; Løvlie & Bryhni, 1978; Lüning et al., 2008). Once established the *Ulva* as a genus with interesting properties to be explored, studies have found that *Ulva*'s biochemical composition and adaptation capacity varies daily, seasonally and within the species and strains (Fort et al., 2019; Steinhagen et al., 2019c; Olsson et al., 2020a, b; Moreira et al., 2021; Jansen et al., 2022). Therefore, some strains can present better properties for large-scale cultivation than others. The variations between species and strains have become clear with the work of Fort et al. (2020a) regarding green tide forming *Ulva* spp. In their work, it was found that green tide-forming strains of *Ulva* present higher growth rates than non-green tide strains. Thus, causing devastating impacts on the environment. However, this nuisance property of green tide forming strains of *Ulva* illustrates the importance of strain selection for *Ulva* cultivation in large-scale. Selecting fast-growing, robust and well adapted *Ulva* strains for each specific cultivation system can increase the profitability of the system. As interest in *Ulva* products keeps increasing, the need for sustainable, reliable, and profitable large-scale cultivation increases as well. Within this framework, the overall objective of this dissertation was to determine the best-suited *Ulva* strains that can be optimized and grown under the required conditions of a large-scale land-based recirculating aquaculture system. It was important that the selected strain would present higher growth rates in higher temperatures and lower salinities, and present high levels of antioxidants. Species or strains that presented RGR below the established 7 % day^{-1} RGR threshold, were considered not ideal for profitable cultivation (Huguenin, 1976). This chapter highlights the major findings of this work and discusses in detail the three steps taken to improve the cultivation of *Ulva* spp.. At the end of the chapter, constraints to this work and perspectives for future *Ulva* research are discussed.

6.1. Major findings

The research presented in this thesis contributes to the production of *Ulva* species and closes some of the knowledge gaps found during *Ulva* cultivation. **Publication I (Cardoso et al., 2023)** and **Publication II (Cardoso et al., 2024)** show that strain selection and strain optimization can increase the overall success of *Ulva* cultivation. **Publication III (Cardoso et al., n.d.)** highlights a previously underestimated proliferation method in *Ulva* spp. that can be further explored to reduce the occurrence of biomass degradation (one of the major bottlenecks in *Ulva* cultivation) and as a low-cost seeding method. **Figure 6.1**. illustrates the three main steps taken to increase the effectiveness of *Ulva* cultivation, derived from the three publications and additional works performed under the scope of this dissertation.

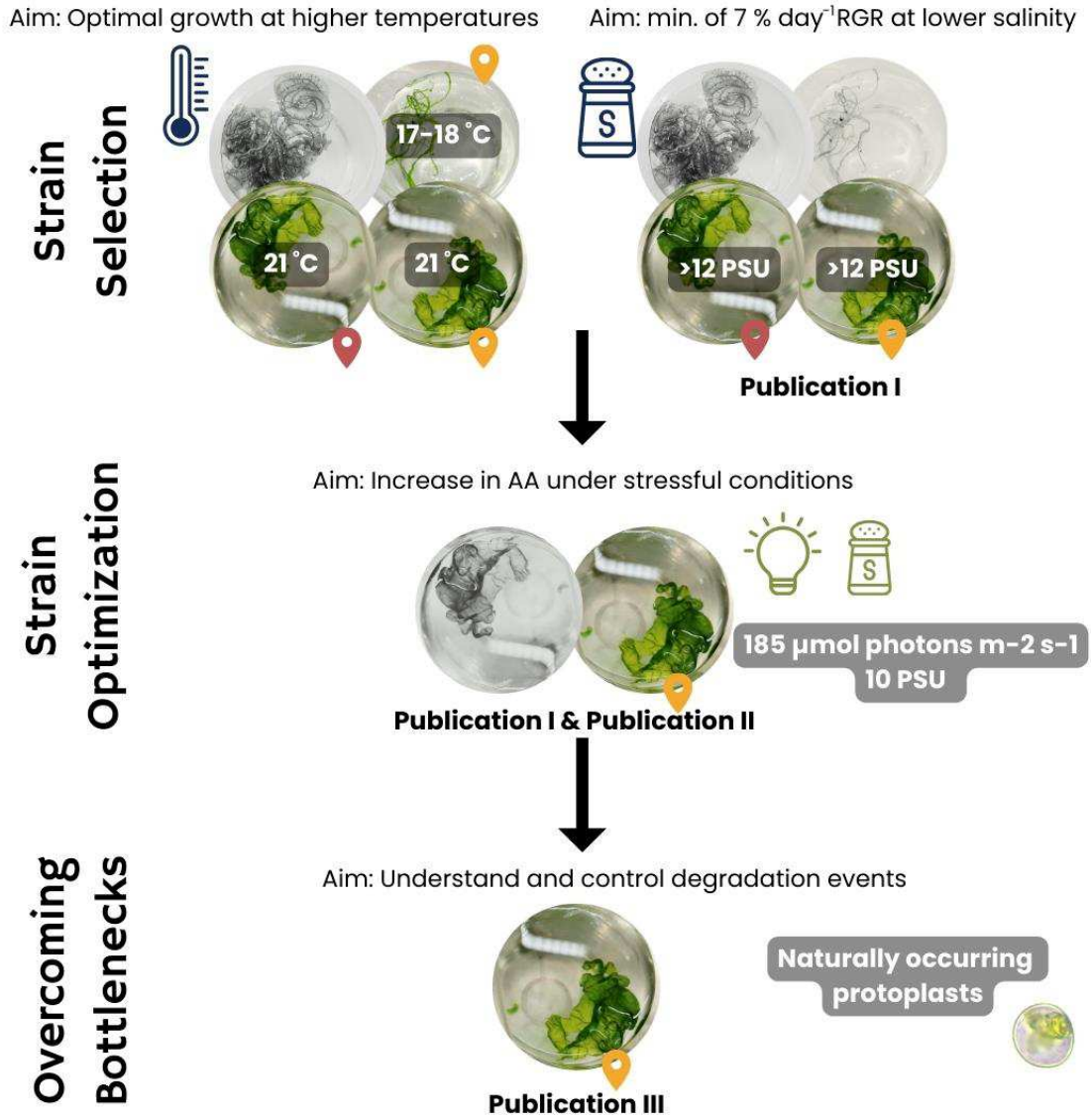

Fig. 6.1 – Major findings reported in this dissertation, divided into the three main steps to increase the success of *Ulva* cultivation. During strain selection, the RGRs from four *Ulva* strains (Red location mark: Mediterranean strains; Yellow location mark: NE-Atlantic strains) were compared after their growth under different temperature and salinity conditions. *Ulva flexuosa* (Mediterranean) and *Ulva linza* (NE-Atlantic) were removed from the strain selection process after presenting RGRs below the required threshold of 7

% day^{-1}. The two *Ulva lacinulata* strains presented higher RGRs under higher temperature conditions and RGRs above the 7 % day^{-1} threshold starting at 12 PSU. During the strain optimization phase, the antioxidant activity (AA) of the two strains was compared after being under different salinity and light treatments for 10 and 5 days, respectively. The optimization of the NE-Atlantic *U. lacinulata* was possible through an increase in AA after exposing this strain to low salinity (10 PSU for 10 days) and high irradiance (185 µmol photons m^{-2} s^{-1} for 5 days). Further cultivation of the NE-Atlantic *U. lacinulata* was limited by the often occurrence of degradation events. The study of such events revealed the natural occurrence of protoplasts of *Ulva lacinulata*, as a novelty reproduction strategy in *Ulva* spp.. [Graphical design created in Canva (Canva, n.d.)].

The salinity experiment reported in **Publication I** was designed to answer the research question regarding **Strain selection**. The difference in RGRs reported by the different strains, while growing under different salinity treatments, allowed the selection of the strains most suited for cultivation in the RAS. Thus, concluding that salinity can be used as a tool for strain selection. From **Publication I**, two strains of *U. lacinulata* were selected for their high RGR and the possibility of cultivating them under lower salinity conditions. These strains can be cultivated at 20 PSU, which corresponds to a 33.3 % reduction in salinity costs, without a significant impact on RGR or AA. Furthermore, they can be cultivated at a minimum of 12 PSU (a 60 % reduction in salinity costs) without the RGR falling below the 7 % day^{-1} threshold necessary to guarantee the profitability of the system. The hypothesis that different *Ulva* species have different salinity preferences was confirmed based on the RGRs obtained and the optimal culture conditions for growth calculated for each species, in **Publication I**. As hypothesized, salinity preference varied between the different life stages of *Ulva*'s development. Germlings of the tubular forms of *Ulva* grow faster during this stage of development than as adults. However, from the three species tested, the *Ulva lacinulata* strains, as adults, present the highest RGRs. The hypothesis that salinity can be used as a stressor and increase antioxidant activity in *Ulva* was corroborated in **Publication I**. Overall, **Publication I** showed how variable growth rates can be between different *Ulva* species and strains, and that different *Ulva* spp. have different salinity preferences. Furthermore, **Publication II** showed different antioxidant responses between the two *U. lacinulata* strains under the saturating light treatment. Both publications corroborate the importance of strain selection before cultivation.

Publication II was designed to compare the impact of irradiance and light dose on the antioxidant activity of the two *U. lacinulata* strains and answer the research questions regarding **Strain optimization**. It was found that the NE-Atlantic *U. lacinulata* can be optimized if irradiance is increased to 185 µmol photon m^{-2} s^{-1} for five days before harvesting. During this time, the antioxidant activity (AA) increased by 89 %. Only the NE-Atlantic *U. lacinulata* strain could be optimized through this method, thus contradicting the original hypothesis that the optimization method would have a similar impact on both strains. The hypothesis that the saturating irradiance treatment used in the experiment would impact the photosynthetic efficiency of both strains was confirmed, but the treatment was not saturating for any of the strains at the end of the experiment. Based on the Imaging PAM measurements, both strains

adjusted to the treatment, presenting similar and healthy photosynthetic efficiency, corroborating the hypothesis that photosynthetic efficiency would be similar between strains. Nevertheless, the strains presented different adaptations to the irradiance treatment, proving that different strains have different adaptations to the same abiotic factor, thus showing the importance of strain selection and optimization.

The research question regarding **Overcoming cultivation bottlenecks** was answered in **Publication III.** In this work, a degradation event occurring in the NE-Atlantic *U. lacinulata* and a reproduction event occurring in the wild *U. compressa* were followed. **Publication III** defines degradation event, separating it from the concept of reproduction, confirms the natural occurrence of protoplasts in two *Ulva* spp., and quantifies the process of natural protoplast production and germination. Naturally occurring protoplasts were found in both *Ulva* species despite their different origins, capacity to become fertile, time spent in culture conditions or the water quality each species was growing in. Therefore, **Publication III** confirms the hypothesis that protoplasts occur naturally in *Ulva* spp.. Based on the original biomass at the start of the experiment, *U. lacinulata* produced a total of 4.26 x 10^8 protoplasts g^{-1} and *U. compressa* produced 5.54 x 10^5 protoplasts g^{-1}. While this work confirms the hypothesis that *Ulva* degradation caused protoplasts production, the fact that protoplasts were found in *U. compressa* suggests that this asexual reproduction strategy is not specific to non-sporulating species. Despite not finding a clear trigger for degradation, **Publication III** proves that degradation can produce higher biomass yields than during reproduction. The observations reported in **Publication III** prove that protoplasts can regenerate and reproduce at a fast pace, making them ideal as seeding material. This information can be used to guarantee a higher number of seeding material without requiring the control of the *Ulva*'s reproduction cycle or high amounts of biomass to start cultivation.

6.2. Strain Selection

Publication I and **II** demonstrate the occurrence of inter and intra-specific variations in *Ulva* that should be considered before their cultivation. In both works it became clear that differences in RGRs and AA were present between species and between the two strains of *U. lacinulata*. Similar variations between *Ulva* spp. have been reported in the literature as an argument for pursuing strain selection to increase biomass yields, biomass quality, and the productivity of *Ulva* cultivating systems (Fort et al., 2019, 2020a, 2020b).

Publication I concluded that the RGRs varied considerably between different *Ulva* spp.. However, no significant differences were found between the two *U. lacinulata* strains. Thus, indicating inter-specific variations in response to salinity conditions. In **Publication II**, the antioxidant responses from the two *U. lacinulata* strains to an elevated irradiance varied significantly. Thus, indicating that intra-specific variations also occur and that, within the same

species, strains can have different adaptions and biochemical compositions. The findings in **Publication I** and **II** can be justified by the inter and intra-specific genetic variations reported by Fort et al. (2020a, b) and Liu et al. (2020). Fort et al. (2020b) reported high levels of inter-specific genetic diversity but low levels of intra-specific genetic variability between foliose *Ulva* species from Europe, but Fort et al. (2020a) reported that European green tide strains of foliose *Ulva* species presented higher protein and pigment content, lower starch concentrations and grew faster than non-forming green tide strains with the same geographical origin. Additionally, Liu et al. (2020) reported a strong intra-specific variability in different samples of *U. compressa* with different morphologies, collected in the United States of America and several locations in China. Therefore, testing *Ulva* species and strains before scaling-up the cultivation can help determine those that would be better suited to the specific conditions of each cultivation system (Jansen et al., 2022). Furthermore, **Publication I** established the optimal growth conditions for the cultivation of each strain, showing that with strain selection it is possible to further customize each particular system to achieve optimal levels of growth.

Because of the settings of the large-scale land-based RAS, high temperatures were expected during summer, which would require the placement of a cooling system, and increase the costs of production. For this reason, as a first strategy to select potential *Ulva* candidates, species from temperate climates were selected (Mediterranean and NE-Atlantic). Before the experiments reported in **Publication I**, the same *Ulva* strains were tested in an eco-physiological experiment with temperature treatments ranging between 10 and 25 ˚C. This experiment compared the RGRs of the four strains, following the same 7 % threshold established in **Publication I**. Three of the four strains were considered suited for cultivation in the RAS, based on the results of the temperature experiment (**Fig. 9.1**, in **Appendix**). The Mediterranean *U. lacinulata* reported the highest RGR at 20 ˚C (14.1 % day^{-1}) and its optimal temperature at 21 ˚C. This strain can be cultivated at a minimum 12 ˚C and a maximum 30 ˚C, for three weeks, without crossing the 7% day^{-1} threshold. *Ulva linza* reported the second highest growth rate at 15 ˚C (considered the control; 13.7 % day^{-1}) and its optimal temperature was between 17 and 18 ˚C. This species can be cultivated between 9 and 26 ˚C, for three weeks, without reducing their RGR below the necessary threshold. The RGRs of the NE-Atlantic *U. lacinulata* did not vary significantly between treatments (RGR between 9 and 10.8 % day^{-1}) and were above the necessary threshold (**Fig. 9.1** and **Table 9.3**, in **Appendix**).

This preliminary experiment was designed with natural seawater supplemented with half-strength provasoli's medium (PES). These two factors (seawater type and nutrient source) would be changed in the land-based RAS where there is no access to seawater, and PES is too expensive to be used on large-scale. To approximate our laboratory experimental conditions to those in the RAS, the salinity experiment reported in **Publication I** was set up with artificial seawater supplemented with a commercial fertilizer (Blaukorn, COMPO SANA®, Germany). Because

the use of artificial salt would increase the costs of production, **Publication I** aimed to select *Ulva* strains that present high RGR under low salinities. This way, finding a strain that shows high growth rates under low salinity would amount to a reduction in the costs of salt and eventual desalinization treatments (Sharrer et al., 2007; Liu, 2013; Chang et al., 2022). The two *U. lacinulata* strains presented similar results (14.8 and 16.6 % day^{-1}) and the highest RGRs at the highest salinity tested (30 PSU, considered the control) with the optimal salinity for growth at 28 PSU. In both strains, significant differences between treatments were only found between the two lower salinities and the two highest ones. Despite the higher RGRs being found in the strains with optimal growth at high salinity levels (28 PSU), the *U. lacinulata* strains can be cultivated at a minimum of 12 PSU at which the 7 % day^{-1} RGR would still be achieved (60% reduction in salt costs). Furthermore, based on the antioxidant analysis in **Publication I**, the 15 and 20 PSU treatments did not cause antioxidative stress to the biomass, suggesting that reducing the salinity in the system would not negatively impact the *Ulva*.

Based on the results from the preliminary temperature experiment and **Publication I**, *Ulva flexuosa* and *U. linza* were removed from further experiments. However, the elimination of these two species from further works does not indicate that these species are not relevant for cultivation. Rather these species would be suited for cultivation in systems with different conditions. The interest in cultivating and utilizing tubular *Ulva* spp. exists as they have been used around the globe for food, feed, fertilization of crops and medicine (Prud'homme van Reine & Trono, 2001). In 2019, *Ulva intestinalis* Linnaeus represented 63.6 % of the world's wild cultivation (Cai & Galli, 2021). In **Publication I**, *U. flexuosa* and *U. linza* presented higher growth rates as germlings than as adults, suggesting that a cultivation with more harvesting time points would generate a rapid overturn and fast production of the biomass. Moreover, *U. linza* can be cultivated, with no associated costs, under lower temperature (**Fig. 9.1**, in **Appendix**) and lower salinity conditions (**Fig. 3**, in **Publication I**). Thus, being suited for cultivation in cold climate regions and in mesohaline (5-18 PSU), instead of polyhaline conditions (18-30 PSU; Rybak, 2018). However, for the particular case study presented in this dissertation, the two *U. lacinulata* strains were considered the best suited candidates for cultivation.

The Mediterranean strains were collected in Thessaloniki Bay, Greece where temperature varies annually between 7 and 27 ˚C (Kambezidis, 2021) and salinity varies between 36 and 39 % (Haritonidis, 1978; **Table 9.1**, in **Appendix**). In both Mediterranean species, the highest RGRs were found within the reported range of temperatures (**Table 9.1**, in **Appendix**). However, *U. flexuosa* preferred medium temperatures, closer to the average of 17 ˚C, while the Mediterranean *U. lacinulata* preferred higher temperatures (21 ˚C), close to the maximum temperature registered in that area (**Fig. 9.1**, in **Appendix**). The salinity treatments tested in **Publication I** were all below the salinity in Thessaloniki Bay (**Fig. 1** and **2**, in **Publication I**). However, samples of both *Ulva* species have been kept under laboratory conditions for several years at 30

PSU, which is why 30 PSU was established as the control group during the salinity experiment. The assumption that the species should be adapted to 30 PSU conditions was confirmed by the results from the Mediterranean *U. lacinulata* which reported an optimal salinity at 28 PSU. However, the same was not observed for *U. flexuosa* which showed an unclear pattern of growth between the treatments tested. Rybak (2018) reported that *U. flexuosa* can occur in a wide range of salinities, ranging from freshwater (< 0.5 PSU) to euhaline (30-40 PSU) environments. It is possible that under higher salinity treatments *U. flexuosa* could increase its RGRs. Nevertheless, this work aimed to select a strain that could produce high biomass yields under the lowest salinity possible, causing this species to not be a suitable candidate.

The NE-Atlantic strains, collected in Óbidos Lagoon, Portugal, were growing in an area where temperatures varied annually between 6 and 24 ˚C and salinity varied between 25 and 35 PSU (Mendes et al., 2021; **Table 9.1**, in **Appendix)**. Both NE-Atlantic species reported the highest or optimal growth at temperatures within that range. *U. linza* had an optimal growth close to the average temperature in Óbidos and the temperature set in the culture room (15 ˚C). The NE-Atlantic *U. lacinulata* reported its highest RGR at a higher temperature (20 ˚C), close to the maximum temperature registered at the Óbidos Lagoon. This strain reported its optimal growth at 28 PSU, within the salinity range in Óbidos Lagoon and close to the culture conditions (30 PSU). *U. linza* reported an optimal growth at 21 PSU, below the salinity range in Óbidos and below the salinity conditions in the culture room. This can be justified by the work from Rybak (2018) where *U. linza* can occur in a range of salinities between oligohaline (0.5-5 PSU) to euhaline (30-40 PSU) conditions, with a preference for polyhaline (18-30 PSU) conditions. Both the cultivation conditions at 30 PSU and the calculated optimal condition (21 PSU) fall within the polyhaline conditions, corroborating the results in **Publication I**. Additionally, the distance from the Óbidos lagoon to the sea causes salinity variations within the lagoon (Pedro et al., 2015). Inside the lagoon, where the two species were collected, lower salinities (14 PSU) have been reported (Pedro et al., 2015).

The different preferences found in the *Ulva* species collected at the same location (NE-Atlantic and Mediterranean) can be justified by what has been reported in multispecific green tides (Bermejo et al., 2023). Bermejo et al. (2023) found that a mix of green tide-forming *Ulva* species present temporal successions, based on variations of different abiotic factors (e.g., temperature and photoperiod). As an example, Yabe et al. (2009) reported a mixed *Ulva* community (*Ulva pertusa* and *Ulva ohnoi*) where the different species cause green tides at different moments of the year, depending on the environmental conditions. Besides corroborating the importance of strain selection this shows that species collected in the same geographical location have different preferences, even after being acclimated for a long period under different laboratory conditions.

In **Publication I** and the preliminary temperature experiment, salinity and temperature preferences varied between the two morphologies (foliose and tubular). The *U. lacinulata* strains

(foliose morphology) reported higher RGRs in higher temperatures (20-21 ˚C) and higher salinities (28 PSU). The tubular strains, as adults, preferred medium temperatures (15-18 ˚C) and reported low RGRs in all the salinity treatments tested. The impact of salinity in *Ulva* species with different morphologies has been reported previously. Rybak (2018) reported that *Ulva* species, known to present a tubular morphotype, occurred in a wider range of salinity, with a preference for lower salinity levels, compared with species often associated with their foliose morphotype. Moreover, foliose species tend to present optimal growth and photosynthetic activity under salinities ranging between 20 and 35 PSU, while tubular species present optimal results in salinities ranging between 10 and 32 PSU (Choi et al., 2010; Chen & Zou, 2015; Xiao et al., 2016; Li et al., 2017; Bastos et al., 2019; Bews et al., 2021; Kim et al., 2021). These observations corroborate the results presented in **Publication I**. However, the occurrence of each morphotype is species-specific and depends on several factors besides salinity (e.g., nutrient concentration, microbiome, attachment to a substrate; Blomster et al., 2002; Hofmann et al., 2010; Spoerner et al., 2012; Wichard, 2015; Steinhagen, 2018; Steinhagen et al., 2019c; Ulrich et al., 2022).

Stressful conditions can induce *Ulva*'s reproduction (McArthur & Moss, 1979; Dan et al., 2002; Corradi et al., 2006; Carl et al., 2014). Establishing the optimal cultivation conditions can reduce stress and, therefore, the number of reproduction events. Nevertheless, it was expected a certain reproduction rhythmicity caused by the provided photoperiod (16:8 h light:dark; Lüning et al., 2008) or the age of the *Ulva* biomass (Stratmann et al., 1996; Wichard & Oertel, 2010; Obolski et al., 2022). Because reproduction is associated with biomass loss (Gao et al., 2010; Bruhn et al., 2011; Ryther et al., 1984; Bolton et al., 2008; Obolski et al., 2022), the fast development of a new generation is important as it can accelerate the recovery of the lost material. Therefore, RGRs of germlings were evaluated in **Publication I**. Having germlings with high growth rates at similar optimal conditions as the adult material would allow for direct seeding of the cultivation system. Thus, removing the need for a nursery and guaranteeing constant cultivation (Praeger & de Nys, 2017, 2018). The only strain that showed similar and high growth rates as germlings and adults, at conditions close to optimal (28 PSU) was the NE-Atlantic *U. lacinulata*. In the case of reproduction, the germlings from this strain would be able to grow fast without requiring any changes to the cultivation conditions.

In **Publication II** the two *U. lacinulata* strains were tested for their antioxidant activity (AA) under a saturating light irradiance. While the aim was to use such treatment to increase AA and optimize biomass quality, the results showed that the two *U. lacinulata* strains responded differently to the irradiance treatment. The correlation between alpha values and AA found in the Mediterranean *U. lacinulata* suggests that this strain was adapted to lower irradiance conditions (**Fig. S4** and **Table S3** in **Publication II**) while the NE-Atlantic strain was better adapted to higher irradiances. The AA response to the same treatment also varied between the

strains, with the Mediterranean strain not presenting any visible change to AA while the NE-Atlantic strain increased its AA significantly. This difference could be justified by the species and strain-dependent carotenoid yields reported by Eismann et al. (2020). Despite the method used in **Publication II** not being suited to evaluate carotenoid concentrations (see **Considerations and constraints** section), this shows that differences between strains can impact the quality of the biomass during cultivation, as previously stated by Fort et al. (2019). Thus, corroborating the importance of strain selection before cultivation.

6.3. Strain Optimization

Nowadays, on-shore IMTA systems are recognized as one of the most profitable methods for achieving high macroalgae biomass yields (Bolton et al., 2008; Zertuche-González et al., 2021). Monoculture land-based systems for macroalgae are still considered expensive and require a reduction in costs and an increase in biomass quality with consistent and reliable production (Hafting et al., 2012; Suthar et al., 2019). The process of strain optimization can guarantee biomass quality (Henley & Ramus, 1989; Magnusson et al., 2014; Chye et al., 2017; Fort et al., 2019; Suthar et al., 2019; Fort et al., 2020a; Olsson et al., 2020; Sugumaran et al., 2022). Additionally, if the increase in quality can be achieved by a decrease in costs (e.g. low salinity) then the profits can increase further (Marques et al., 2020).

Publications I and **II** demonstrate that optimization of *Ulva* strains is possible by exposing the biomass to potentially stressful or non-optimal conditions. In both works, AA increased after the NE-Atlantic strain was exposed to low salinity (10 PSU; **Publication I**) for ten days and high irradiance (185 µmol photons m^{-2} s^{-1}; **Publication II**) for five days. **Publications II** focused on the impact of light irradiance and light dose on the AA of both strains of *U. lacinulata*. From the start of the experiment, the antioxidant concentration of the Mediterranean strain was similar between treatments. The AA in this strain was significantly higher than the AA in the NE-Atlantic strain from the beginning, despite the Fv/fm values indicating the good health of both strains throughout the experiment. This significant difference in AA, previously considered to be caused by a variation of strain-dependent carotenoid content (Eismann et al., 2020) had not been found previously during other experiments with the same strains. Meiβner (2022) evaluated the antioxidant concentration of the Mediterranean strain during the salinity experiment reported in **Publication I**. At the time, both strains of *U. lacinulata* reported similar antioxidant concentrations at the beginning of the experiment (Meiβner, 2022). Such a significant difference between the two strains in **Publication II** suggests that other factors besides irradiance and light dose can influence AA. Between **Publication I** and **II**, only the light quality was changed. Both treatments were composed of white, blue, and red light. This was necessary to achieve the irradiance needed for the saturating treatment in **Publication II**. Exposure to blue light has been reported to increase AA in *Ulva* sp. after 44 days (Schwoerbel, 2019), indicating that light quality

also plays a role in AA. In **Publication II**, both strains were acclimated to the new light quality for a week before the experiment. This could justify the difference in AA between the Mediterranean strain reported by Meiβner (2022) during the salinity experiment and the AA from the same strain reported in **Publication II**.

The higher AA found in the Mediterranean strain indicates a clear and direct advantage for its cultivation. This strain could be cultivated at lower irradiance with an extended photoperiod (16 h light:8 h dark), ideal for vegetative growth of *Ulva* spp. (Lüning et al., 2008). However, **Publication II** aimed to evaluate if light irradiance could improve AA when light dose is kept the same. The NE-Atlantic was the only strain that showed an increase of 89 % in AA under the saturating light treatment at the end of the experiment with a tendency to keep increasing (**Fig. 1**, in **Publication II**). Thus, proving that optimization of this strain was possible and that an increase in irradiance for five days before harvesting can increase the quality of the biomass.

In the literature, several studies can be found regarding the effect of irradiance on the biochemical composition and growth rates of *Ulva* species (Fortes & Lüning, 1980; Xiao et al., 2016; Olsson et al., 2020b; Toth et al., 2020). However, these works usually change the irradiance levels but keep the photoperiod. This causes light dose to vary between treatments, together with irradiance. In **Publication II**, the light dose was kept the same. Therefore, a direct comparison between **Publication II** and other works cannot be done. However, this reveals the importance of **Publication II** in separating two factors that have been previously tested together. Works with other macroalgae species have shown the importance of looking at light dose and day length separately. For example, higher light dose increases the growth rates and the concentration of pigments of *Codium tomentosum* Stackhouse (Marques et al., 2020) and of *Gracilaria vermiculophylla* (Ohmi) Papenfus if the light dose is kept constant despite the day length (Weinberger et al., 2008). In *Ulva*, the day length has been proven essential to guarantee high growth rates, larger reproductive areas, and higher concentrations of chlorophyll (W. Li et al., 2018; Schwoerbel, 2019; Yue et al., 2019). The maximum day length to grow *Ulva* has been established at 16 h (Fortes & Lünning, 1980). **Publication II** shows that light irradiance and light dose can act alone and that different irradiance settings can be considered to achieve similar results. In the NE-Atlantic strain, short days (day length of 6 h) at a high irradiance (185 µmol photons m^{-2} s^{-1}) had a similar impact to the one observed in the Mediterranean strain during long days (day length of 16 h) at a lower irradiance (70 µmol photons m^{-2} s^{-1}). The choice between the given options must be made based on the *Ulva* strain being used and the specificity of each system. Based on the purpose of **Publication II** and the scope of this dissertation, the strain selected for further analysis was the strain that responded significantly to the different irradiance treatments tested during the strain selection and optimization steps, and that presented higher RGRs in **Publication I**, the NE-Atlantic *U. lacinulata*.

6.4. Overcoming cultivation bottlenecks

Despite working with NE-Atlantic *U. lacinulata* germlings and reporting their growth rates in **Publication I**, this strain was considered a non-sporulating strain because signs of reproduction (e.g., change in colour or swarmer release) were never observed. Germlings could be found rarely in the culture vessels but motile *Ulva* cells, which would indicate sporulation, were never found. To overcome the lack of reproduction, induction attempts (e.g., fragmentation of the thalli, temperature shock, dehydration) were tested but unsuccessful. This limitation prevented further optimization attempts (e.g., hybridization experiments for strain optimization). Therefore, this strain was cultivated vegetatively in the small-scale laboratory setting, which was considered the optimal way to cultivate it later in the large-scale land-based RAS. The vegetative growth of *Ulva* is a recommended practice for large-scale cultivations and the use of non-sporulating *Ulva* species has been reported in the literature as a strategy to avoid reproduction events and associated biomass loss (Lüning et al., 2008; Gao et al., 2017b; Praeger et al., 2019; Obolski et al., 2022). However, the NE-Atlantic *U. lacinulata* often and spontaneously degraded (without the clear release of offspring), limiting cultivation through vegetative propagation, and causing strong biomass loss.

During a short-term scientific mission (STSM) in Ireland, funded by the SeaWheat COST Action (CA20106), protoplast isolation methods were tested to overcome the limitations faced when working with a non-sporulating and often degrading strain such as NE-Atlantic *U. lacinulata*. A successful protocol for protoplast isolation was developed by testing and adjusting the different variations of the method reported in the literature (Reddy et al., 2006; Gupta & Reddy, 2018; Reddy & Seth, 2018; Gupta et al., 2018). By the end of the three months, protoplasts from the Irish *Ulva* species named in this work "*Ulva*-2" were successfully obtained. The corrections to the original protoplast presented in **Chapter 2** can be found in **Table 9.5**, in the **Appendix**. Because protoplast isolation is a method most suited for small laboratory scales and is considered expensive for large-scale settings (Reddy et al., 2006; Gupta et al., 2018), an optimization of the technique was attempted. Protoplast isolation requires the removal of the *Ulva*'s cell wall, usually done by expensive enzyme solutions (e.g., Cellulase Onozuka R-10 and Macerozyme R-10; Gupta et al., 2018; Gupta & Reddy, 2018). Previous work by Holzinger et al. (2015) reported structural cell wall changes when *Ulva* was placed under stressful desiccation conditions. As an intertidal species, *Ulva* must tolerate the strong variations in environmental conditions caused by the tides (e.g., desiccation, salinity variation, and light exposure; Smith & Berry, 1986; Kirst, 1990). Therefore, salinity and light treatments were tested in this work to evaluate their capacity to reduce *Ulva*'s cell wall thickness and reduce the dependence on expensive enzymes during protoplast isolation. The aim was to use the salinity or light treatments that successfully reduced the cell wall thickness as pre-treatments to be implemented for a short time before protoplast isolation. Two *Ulva* species (*Ulva*-1 and *Ulva*-2) were cultivated for 5 days under three salinity

treatments (10, 34 as the control, or 40 PSU) and two light treatments (10 µmol photons m^{-2} s^{-1}, LL, or 100 µmol photons m^{-2} s^{-1}, HL; **Fig. 9.2** and **9.3**, in **Appendix**). The cell wall thickness increased or stayed the same in all pre-treatments tested, except one (40 PSU, in *Ulva*-1; **Fig. 9.3**, in **Appendix**). Because of time constraints, it was not possible to test the 40 PSU pre-treatment together with the isolation protocol. Only the LL treatment, where a slight reduction of the cell wall width was observed (in *Ulva*-2), was tested as a pre-treatment before protoplast isolation (**Fig. 9.4**, in **Appendix**). A new set of *Ulva* samples was placed under LL for 8 days to evaluate the cell wall width/thickness after a longer period. The cell wall width from *Ulva*-2, at the end of the experiment, was similar to the one reported on day 5 of the first experiment, indicating that low light did not impact the cell wall thickness. Protoplasts from *Ulva*-1 and *Ulva*-2 were isolated after the 8-day LL pre-treatment. The protoplast yields obtained with the pre-treatment were similar between the two species (**Fig. 9.4**, in **Appendix**), but lower than the protoplast yield from *Ulva*-2 without a pre-treatment (**Fig. 9.4**, in **Appendix**). Since the light did not impact the cell wall, it was expected that protoplast yields would be similar with and without pre-treatment. Additionally, while protoplasts from *Ulva*-2 were observed through microscopy, confirming the successful isolation of protoplasts, no protoplasts from *Ulva*-1 were observed. Thus, suggesting that the method used for cell counting required further adjustments. A detailed discussion about the limitations faced during this work can be found under the **Considerations and constraints** section of this chapter, where is discussed the method selected for cell counting as a potential limitation for the results obtained.

For successful protoplast isolation, it is important to use healthy, non-fertile *Ulva* material in an exponential growth phase (Gupta et al., 2018; Gupta & Reddy, 2018). However, when attempting to use the previously reported protoplast isolation protocol to isolate protoplasts from the NE-Atlantic *U. lacinulata*, back at the Alfred Wegener Institute, the biomass was visibly degrading, and protoplast isolation was not possible.

Instead of protoplast isolation, the process of degradation of the NE-Atlantic *U. lacinulata* was followed. **Publication III** reports the observations during what was called a "degradation event" and establishes a clear description that separates a degradation event from a reproduction event. At the beginning of the experiment, the *Ulva* thalli became pale and fragmented. A strong accumulation of debris and foam became visible on the seawater surface (**Fig. 1**, in **Publication III**). Under the microscope, *Ulva* thalli presented two different cell types: 1) polygonal, small, grey cells, full of starch; and 2) spherical, large, and green cells with a parietal plastid (**Fig. 2B-D**, in **Publication III**). The larger cells, later determined to be protoplasts, were observed detaching from the original thalli during degradation (**Fig. 2D-E**, in **Publication III**). Degradation was considered to be over if protoplast yields were low, bleached *Ulva* fragments were not visible and the RGRs of the *Ulva* pieces in the original beakers were high.

The debris and foam found at the beginning of degradation varied in colour (green, yellow, and orange) and presented a strong smell, characteristic of decomposing *Ulva* (Dong et al., 2023). This was indicative of an accumulation of organic compounds and cell particles in the cultivation vessels (**Fig. 1**, in **Publication III**). The release of organic compounds into the water can justify the colours found in the decomposed material as this could lead to an explosion of *Ulva*-associated microbes (Qu et al., 2021; Tanaka et al., 2022; Zhao et al., 2022). Moreover, *Ulva* spp. are known to release dimethyllsulfonio-propionate (DMSP; Dickson et al., 1982; Stefels, 2000; van Alstyne et al., 2023). DMSP is a volatile organic compound associated with the osmotic protection of the macroalgae cells (Dickson et al., 1982; Stefels, 2000) and known to attract bacteria, such as *Roseovarius* sp. (Kessler et al., 2017, 2018), a genus of bacteria associated with morphogenesis determination in *U. compressa* (Spoener et al., 2012; Wichard, 2015, 2023). The smell present at the time of degradation can be associated with the production of dimethylsulphide (DMS) a volatile compound found in seaweeds that derives from DMSP and has been associated with a sulphurous, fishy aroma (Sugisawa et al., 1990; van Durme et al., 2013; Villar et al., 2020; Urlass et al., 2023). Han et al. (2020) reported average concentration levels of DMSP and DMS to be two and four times higher, respectively, during an *Ulva prolifera* bloom in Qingdao, China, and concluded that DMSP was being produced by *U. prolifera*. The release of DMSP was, therefore, associated with the *Ulva* decomposition (Han et al., 2020). The production of DMS, DMSP, and acrylic acid by *U. prolifera* has also been associated with moments of environmental stress and loss of membrane integrity (van Alstyne et al., 2016). Thus, corroborating the description of the degradation event in **Publication III**.

Publication III reports the occurrence of protoplasts during a degradation event in the NE-Atlantic *U. lacinulata* and a reproduction event in *U. compressa*. The work with *U. compressa* aimed to evaluate if the observations from NE-Atlantic *U. lacinulata* were a product of the cultivation conditions in which it was growing. Thus, *U. compressa* was collected from the wild and cultivated in natural seawater for about a week when reproductive blades were detected, and the release of swarmers and protoplasts was observed. This proves that protoplasts occur naturally in different *Ulva* species, and it is not specific to non-sporulating species or moments of degradation/decomposition. Similar observations detected during cultivation of the non-sporulating Mediterranean *U. lacinulata* and the sporulating *Ulva*-3 (from Helgoland) corroborate that protoplasts occur in different *Ulva* species with different reproduction patterns (**Fig. 9.5**, in **Appendix**).

The released protoplasts from NE-Atlantic *U. lacinulata* and *U. compressa* were collected based on the knowledge acquired during the STSM. Following a methodology similar to the successful protoplast isolation method, the absence of cell wall was confirmed by using CFW, and protoplast yields were estimated, this time with a hemacytometer (**Fig. 3**, **5** and **8**, in **Publication III**). Total protoplast yields were higher in the NE-Atlantic strain (4.26×10^8 cells g^{-1}) than in

U. compressa (5.54 x 10^5 cells g^{-1}). This represents that the NE-Atlantic *U. lacinulata* uses a different method of reproduction and dispersion which does not involve the release of swarmers, while *U. compressa* allocates most of its cells to swarmer formation. The method of reproduction through protoplasts found in the NE-Atlantic *U. lacinulata* can justify what had been previously reported in **Publication I**. **Publication I** reports an experiment with germlings of different *Ulva* strains but states that the *U. lacinulata* did not respond successfully to induction of reproduction. However, because germlings were found in the cultivation vessels, at the time, it was assumed that the biomass had become fertile. But no signs of fertility were ever observed. It is possible that at that time both strains of *U. lacinulata* reproduced via protoplast formation instead of sporo/gametogenesis.

Degradation of *Ulva* tissue and reports of sloughed somatic *Ulva* cells obtained from non-sporulating *Ulva*, have been reported by Provasoli (1958) and Bonneau (1978). Provasoli (1958) used the term "green islands" to describe cells from the *Ulva* thalli with the capacity to generate new germlings without going through the process of swarmer formation. The cells forming the "green islands" could be released into the water by the death of the cells surrounding them (Provasoli, 1958; Bonneau, 1978). Provasoli (1958) stated that "there are two types of cells: one which bleaches and dies easily, and a very resistant one", similar to what is reported in **Publication III** regarding the heterogeneity of the thallus during degradation (**Fig. 2**, in **Publication III**). These "green islands" were observed by Provasoli (1958) in germlings obtained under laboratory conditions, bleaching blades, and the original foliose pieces of thalli. Provasoli (1958) assumed that the original pieces of thalli should be only composed of one cell type that can produce swarmers. However, Provasoli (1958) found that besides swarmers, a second cell type was released into the water and developed into a pincushion form, resembling the disc morphology reported in **Publication III (Fig. 9G-H, 10** and **11**, in **Publication III**). Morphologies similar to the pincushion described by Provasoli (1958) were later reported by Föyn (1961) and Fjeld (1970) as "globose" and "bubble", respectively, and considered spontaneous mutants found in *Ulva mutabilis* (nowadays known as *U. compressa*; Oertel et al., 2015; Steinhagen et al., 2019a). Additionally, Bonneau (1978) observed "green islands" in bleaching fragments of wild-collected *Ulva* material, corroborating the observations in **Publication III** that this cell differentiation occurs in the wild.

The morphologies found in the germinated protoplasts from **Publication III** are similar to the ones reported by Provasoli (1958) and Bonneau (1978). Furthermore, these morphologies confirm that the two *Ulva* spp. released protoplasts. The obtention of discs and germling morphologies (**Fig. 9**, in **Publication III**) is typical of protoplast development after protoplast isolation methods reported in the literature (Reddy et al., 1989; Reddy et al., 2006; Gupta et al., 2012; Gupta et al., 2018; Gupta & Reddy, 2018; Reddy & Seth, 2018). Moreover, the capacity of some of the protoplasts to become fertile after protoplast isolation, like the cell masses and

discs reported in **Publication III** (**Fig. 11** and **S1**, in **Publication III**), has also been described in the literature (Reddy et al., 1989; Gupta et al., 2012, Gupta & Reddy, 2018; Reddy & Seth, 2018).

During the work reported in **Publication III**, preliminary experiments were performed to evaluate the occurrence of three morphologies found during the protoplast germination experiment (fertile discs, attached germlings with one single branch and germling clusters). Green (non-fertile) and brown (fertile) discs developed from protoplasts were separated into different Petri dishes. In all the Petri dishes the discs became fertile (green discs became brown and later white) and released gametes into the water, confirmed by phototaxis. All the individuals obtained from the release of gametes presented the same morphology of attached single-branch germlings (**Fig. 9.6**, in **Appendix**). These observations confirmed that the attached germlings found during the germination experiment in **Publication III** were not directly developed from protoplasts. Gupta et al. (2012), evaluated similar discs obtained after protoplast isolation and reported the discs becoming fertile and the formation of single-branch germlings developed from the released swarmers, thus corroborating the observations in the preliminary experiment. The occurrence of germling clusters reported in **Publication III** at the end of the germination experiment was analysed further during a preliminary experiment where mature discs were separated into Petri dishes. To each Petri dish was added a dead piece of NE-Atlantic *U. lacinulata* (after being submerged in a battery of ethanol solutions from 40 to 100 % for two hours). All the gametes released from the fertile disc were later found attached to the original discs (after it bleached and died) or attached to the bottom and sides of the Petri dish, but never attached to the added piece of *Ulva* (**Fig. 9.6** and **9.7**, in **Appendix**). These results confirmed that germling clusters had previously been a single disc. Therefore, germination rates were calculated assuming that each cluster represented one disc. The results of this preliminary work will be further discussed in the **Prospects for future research** section of this chapter.

Despite Provasoli (1958) and Bonneau (1978) describing similar events to the ones reported in **Publication III** and corroborating the conclusions of this work, **Publication III** is the first work where these "green island" forming cells and "sloughed" cells were identified as protoplasts and its yields have been reported. Furthermore, germination rates were calculated after the germination of the protoplasts was followed for 5 weeks. The NE-Atlantic *U. lacinulata* reported germination rates between 1 and 2 %, while *U. compressa* reported germination rates between 1 and 10 %. The low germination rates will be discussed further in the **Considerations and constraints** section of this chapter. Despite the low number of germinated protoplasts, **Publication III** brings to light this method of reproduction that has been overseen. Protoplasts germinated into different morphologies (unattached germlings, unattached discs, and cell masses), and the cell masses and discs from the NE-Atlantic *U. lacinulata* were found to become fertile from the second week of the experiment on. A comprehensive summary of the

observations made during the germination experiment is shown in **Fig. 16**, in **Publication III**. Based on the average protoplast yields from the NE-Atlantic *U. lacinulata*, the germination rates, and an estimation of the gametes released by the cell masses and discs (Zhang et al., 2013), the degradation event observed could yield more offspring ($4.64 \pm 0.35 \times 10^{10}$ cells g^{-1}) than if the same amount of *U. lacinulata* had become fertile and directly released swarmers (1.45×10^{9} cells g^{-1}; **Fig. 17**, in **Publication III**). Additionally, the degraded thalli regrew and increased eightfold, while direct sporogenesis would cause most of the biomass to be lost. Thus, a degradation event can be positive for land-based recirculating cultivation, as protoplasts will be kept in the culture and germinate or reproduce quickly. Understanding such events could help establish further seeding methods that do not require as much biomass or are dependent on the complexity of *Ulva*'s life cycle. Gupta et al. (2018) attempted to overcome such limitations by optimizing the protoplast isolation method and reutilizing the expensive enzyme solutions. This would amount to a reduction in the costs of the method so it can be applied on a large scale (Gupta et al., 2018). However, **Publication III** shows that *Ulva* protoplasts can be obtained without enzymes being used. The biomass that produced protoplasts grew eightfold and the total number of protoplasts obtained in the NE-Atlantic *U. lacinulata* during the four-week experiment was five times higher (4.26×10^{8} cell g^{-1}) than the yields reported in the literature after protoplast isolation (Gupta & Reddy, 2018). Additionally, the results from **Publication III** indicate that degradation events and reproduction via protoplasts can produce higher biomass yields (eight times more) and *Ulva* offspring ($4.64 \pm 0.35 \times 10^{10}$ cell g^{-1}) than reproduction through gametogenesis (1.45×10^{9} cells g^{-1}), which can have implications for the formation of green tides. During disc maturity, it was found that some cells did not go through gametogenesis and presented a similar shape to the ones that became protoplasts (**Fig. 11E** and **S1** in **Publication III**). A similar observation was made by Bonneau (1978) who reported cells dividing at an angle to the plane of the blade that would develop to form new discs and continue the process of propagation. These observations suggest that this method of dispersion through protoplasts is not a mistake during *Ulva*'s development but rather a constant strategy of successful reproduction. The impact that this strategy can have in the formation of green tides has been considered previously by Bonneau (1978) who questioned the importance of motile cells in an intertidal species such as *Ulva* and concluded that, for dispersion purposes sloughed cells, such as protoplasts, would have a similar impact to motile cells.

To provide a broader perspective on *Ulva*'s bottlenecks and limitations to cultivation, an online survey was developed and sent to the members of the SeaWheat COST Action. From the original 36 survey participants, 32 sets of answers were considered reliable and used. *Ulva lactuca* (16 reports) and *Ulva rigida* (14 reports) were the species most worked with, followed by *Ulva intestinalis* and *Ulva compressa*, with 11 and 10 reports respectively (**Fig. 9.8**, in **Appendix**). Molecular identification of the different *Ulva* species is reported in **Fig. 9.9**, in **Appendix**.

Because not all the species reported in the survey were molecularly identified, it should be considered that there are still misapplications of the species' names. Despite identifying *U. rigida* as being restricted to European waters, Hughey et al. (2022) considered the species *U. rigida*, to be part of the *U. lacinulata* taxon, based on the lack of morphological and genetic distinctiveness. Hughey et al. (2019) showed that *U. fasciata* and *U. lobata* are heterotypic synonyms of *U. lactuca*, which occurs in warmer regions while *Ulva fenestrata* occurs in the northern hemisphere in cold temperate areas. However, the survey did not inquire about the geographical origin of each species being cultivated.

The survey's main aim was to understand how re-current degradation is during *Ulva* cultivation and if protoplasts have been found previously occurring naturally during cultivation. A correlation was found between the species in which degradation occurs often (**Fig. 9.10**, in **Appendix**) and the species where "spontaneous" new generations of *Ulva* were found (i.e., without the occurrence of reproduction; **Fig. 9.11** and **9.12** in **Appendix**). *Ulva lacinulata* was the species in which degradation and "spontaneous" germlings occurred the most. *U. lactuca* and *U. intestinalis* showed a strong association between these two events, as well. The results suggest that these events occur more frequently in species presenting a foliose morphotype than species commonly presenting a tubular morphotype. These results agree with **Publication III** where the two species analysed presented a foliose morphology.

The "spontaneous" occurrence of new *Ulva* generations was understood in this work as an indicator of the potential occurrence of natural protoplasts. Thus, following the observations reported in **Publication III**, where naturally occurring protoplasts were found in the NE-Atlantic *U. lacinulata*, after "spontaneous" observations of germlings had been reported in **Publication I**.

At the end of the survey, it was clear that *Ulva'*s spontaneous degradation is a bottleneck for its cultivation since 60 % of the participants failed to deal with those events (**Fig. 9.13**, in **Appendix**). Moreover, there is no clear understanding between the participants on how to deal with degradation. The success cases were divided into 22.86 and 25.71 % between the options of changing cultivation conditions or just waiting for degradation to stop, respectively (**Fig. 9.13**, in **Appendix**). Nevertheless, 11 % of the participants found the option to change the cultivation conditions unsuccessful, showing that culture conditions might not be the only influencing factor for degradation.

6.5. Considerations and constraints

The results reported in **Publication I** show the RGRs of each species after 2 weeks since the start of the experiment. In the third, and last week of the experiment, a slight decline in RGRs was observed. It is possible that, with the growth observed during the first two weeks, the biomass density in each beaker became a limitation for its growth. Additionally, during the

experiment, the nutrient concentration provided weekly did not change, which could have caused a nutrient limitation and reduced growth. The settings for the experiment were established based on the cultivation conditions available in the culture room that had been effective in cultivating different species for several months. Changing the biomass to new and larger vessels during cultivation could have caused the need for the biomass to acclimate to the new conditions (as discussed in **Publication II**), which could have impacted the RGRs. Increasing the nutrients based on the amount of biomass in each beaker could have increased the RGRs, thus shadowing the effect of salinity on *Ulva*'s growth. These two limitations, density, and nutrient concentration can be corrected in the large-scale system where the biomass will have more space to grow and the nutrient concentration can be calculated based on the biomass present in the tank and the amount of nitrogen required for that biomass to grow (Gevaert et al., 2007; Msuya, 2007; Alencar et al., 2010; Luo et al., 2012; Obolski et al., 2022; Zollmann et al., 2023). The experiments presented in **Publication I** were performed on a laboratory scale to select strains for large-scale cultivation. Several factors, such as the use of commercial fertilizer and artificial seawater, aimed to approximate the conditions of those in a large-scale system. Nevertheless, as exemplified by the previously reported limitations, the conclusions taken from this work are limited by the laboratory scale, and further works with the selected strains should be developed in the large-scale RAS.

Publication II used the ABTS assay to evaluate the concentration of antioxidants in the *Ulva* strains. However, the results reported were restricted by this method, as it lacks specificity, does not report variations within the antioxidant compounds, and does not indicate which antioxidants were present. Additionally, antioxidant affinity varies between extraction methods (Chakraborty & Paulraj, 2010; Heo et al., 2005; Mezghani et al., 2013; Srikong et al., 2017). For this work, it was sufficient to report the total antioxidant concentration, as it was expected the irradiance treatment tested to be saturating, thus causing a stress response to the *Ulva*. However, as the results showed, at the end of the experiment, the two strains adapted to the treatment. A strong response was still visible in the NE-Atlantic *U. lacinulata*, but not in the Mediterranean strain, suggesting that the irradiance tested was below the necessary threshold to cause an antioxidant response. Eismann et al. (2020) reported species and strain-dependent carotenoid yields in *Ulva* sp., which could justify the differences observed in AA between the two strains. Moreover, the Mediterranean strain presented similar AA between the control group and the treatment. This result might have been caused by the variation of the different pigments while the total pigment ratio was kept the same, as reported by Ramus et al. (1976). However, because of the method used to measure AA, it was not possible to evaluate the variations between pigments (e.g., carotenoids). It would be interesting to investigate if variations between pigments occurred as a subtle response to the treatment (Chakraborty & Paulraj, 2010; Magnusson et al., 2015). Additionally, in both **Publication I** and **II**, a strong decrease in antioxidant activity was observed

after three hours since the start of the experiment, in all the tested treatments, indicating that daily variations of AA occurred in both strains. Daily variations have been reported in the literature about brown macroalgae which suggests that more sampling points should have been included in this work (Abdala-Díaz et al., 2006; Connan et al., 2007). This way, it would be possible to establish the highest and lowest AA achieved in the treatments and remove the impact of the daily variations from the results. An increase in the electron transport rate (rETR) in the Mediterranean strain after 48 h was found in both treatments. This result suggests that the one-week acclimation period to the experimental conditions was not sufficient. Nevertheless, if only the last two days of the experiment were to be considered, a 30 % increase in AA would still be observed in the NE-Atlantic *U. lacinulata* under the saturating irradiance treatment.

In **Publication III**, the method of protoplast isolation learned during the STSM was partially used for the collection of the cells being released into the water during the degradation and reproduction events. However, as this was the first time developing a methodology specific for isolating naturally occurring protoplasts throughout several weeks, some of the methods used might have limited the calculation of protoplast yields and germination rates. The mesh used to filter the water in each beaker (30 μm) was the same as the one used in the standard protoplast isolation method (Gupta, 2018; Gupta & Reddy, 2018). However, in **Publication III** the degradation of the thalli and cell wall removal were continuous processes. Based on the literature, the regeneration of a protoplast cell wall takes two to three days (Reddy & Fujita, 1991; Gupta & Reddy, 2018). Because the filtration of the water was done once a week, it is to be expected that cells with partial or complete cell walls were filtered. Based on the estimated size of the NE-Atlantic protoplasts (39.36 ± 0.97 μm) it is possible that larger cells or cells in which the cell wall was present (i.e., in the process of cell wall removal or regeneration) were retained in the filter. This could limit the calculation of the protoplast yields obtained. Despite these limitations, the protoplast yields were still comparable to the ones reported in the literature (Gupta & Reddy, 2018; Reddy & Seth, 2018).

The germination rates were extremely low compared to the literature (1-3 % in the NE-Atlantic *U. lacinulata* and 1-10 % in *U. compressa*). This difference was potentially caused by the methodology and equipment used in **Publication III**. During a typical protoplast isolation, the biomass is incubated in a small volume of enzyme solution and the resultant protoplasts are collected after the protoplast solution is centrifuged twice (Gupta & Reddy, 2018; Reddy & Seth, 2018). In **Publication III**, approx. 1 L of water per beaker, was centrifuged each week to collect the totality of protoplasts. This required the protoplast solutions to be centrifuged and resuspended several times, which could have caused damage to the protoplasts (e.g., rupture of the cell membrane). In the germination experiment, plastic falcon tubes and micropipettes with plastic tips were used to inoculate known concentrations of protoplasts in each Petri dish. The use of plastic material could have negatively impacted the germination rates reported in

Publication III. It has been suggested by Gupta & Reddy (2018) that only glass material should be used to handle protoplasts. Moreover, protoplasts were observed clustering together (**Fig. 10**, in **Publication III**) after their isolation, which could cause a variation in the protoplast concentrations placed in each Petri dish. In the literature, after isolation, protoplasts are incubated under low light and high temperature (25 ˚C) for at least 24 h (Reddy et al., 2006; Gupta et al., 2018; Gupta & Reddy 2018). However, because the degradation and reproduction of the two *Ulva* species occurred under the cultivation conditions set in the cultivation room, the cultivation conditions for growing the protoplasts were kept the same. Gupta et al. (2012) determined the optimal regeneration rate at 20 ± 1 ˚C, for protoplasts obtained from *Ulva reticulata* Forsskål collected in India, where the seawater temperature was 21 ˚C. Because the temperature for cultivation (15 ˚C) in **Publication III** was below the temperatures tested by Gupta et al. (2012) it is not possible to determine how the temperature impacted the germination rates reported.

The results from the germination experiment were further restricted by the count of only visible individuals after five weeks of the experiment. During protoplast regeneration and development, microscopic cell masses, with a few cell divisions, were found to become fertile very soon after regeneration. As were only counted the germinated individuals visible to the naked eye, these microscopic cell masses were not counted as germinated protoplasts. The inclusion of this data would have increased the germination rates reported in **Publication III**.

Because contamination was found in all the Petri dishes from one of the original replicates (B1) soon after the start of the germination experiment, in **Publication III**, the contaminated beakers were removed from the experiment. To avoid the loss of more replicates, the Petri dishes were kept untouched until the end of the experiment, which could have led to starvation. Starvation has been reported as a potential stressor that can reduce growth rates in *Ulva* (Zollmann et al., 2023). While the impact of this decision should be considered as a limitation to the work reported, the impact of starvation was ruled out as a cause for the reproduction observed in the cell masses and discs originating from protoplasts. Cell masses were observed becoming fertile two weeks after the beginning of the experiment when nutrients were expected to still be available, suggesting that their behaviour followed what has been reported in the literature about sporulating protoplast cells (Gupta et al., 2012; Gupta et al., 2018, Reddy & Seth, 2018) rather than being a response to the cultivation conditions. A short experiment performed after the germination experiment showed that even after the replacement of the medium (with fresh artificial seawater and nutrients), discs originating from protoplasts still became fertile (**Fig. 9.6**, in **Appendix**). Finally, throughout the germination experiment, only cell masses and discs became fertile, not individuals with the typical germling morphology, suggesting that the cultivation conditions were not stressful. If the culture conditions had been the main factor for reproduction, it would be expected that all the individuals would have shown signs of stress or

reproduction. Nevertheless, abiotic conditions might have played a role in the maturity of cell masses and discs and their germination success. Gupta et al. (2012) found that regeneration is dependent on temperature and that different morphologies (i.e., discs and filamentous germlings, in **Publication III** reported as unattached germlings) present different regeneration rates at different temperatures. Filamentous germlings (i.e. unattached germlings) had a higher regeneration rate at 20 ˚C, while the discs had a higher regeneration rate at 30 ˚C. Moreover, the discs were kept for over a year without becoming fertile under the three temperatures tested (20, 25, and 30 ˚C) by Gupta et al. (2012), even when the culture medium was not changed for a long period. Only at 30 ˚C, Gupta et al. (2012) reported a sporeling phase (i.e. cell masses). Aeration also played a role in the filamentous germling development and increased the daily growth rate of the filamentous germlings. In **Publication III** aeration was not provided and cultivation was done at 15 ˚C.

Despite the previously listed limitations to the methodology used in **Publication III**, one major limitation was the direct comparison between this work and the works reported in the literature (Reddy et al., 2006; Gupta et al., 2012; Gupta et al., 2018; Gupta & Reddy, 2018; Reddy & Seth, 2018). The methodology used to determine the protoplast yields was similar to the methodology in the literature and presented higher results (if total protoplast yields are considered). However, strong variations in protoplast yields were observed between replicates, which could indicate that degradation and protoplast isolation were not happening at the same rate in all of the replicates, reducing the average protoplasts yields to $2.66 \pm 0.20 \times 10^6$ cell g^{-1}, only representing 3 to 4 % of the protoplast yields reported by Gupta & Reddy (2018). The comparisons to the literature stop at the germination experiment. The discussed literature is not clear about when regeneration or germination rates were calculated, how germination was defined, or what was considered to be the direct result of protoplast germination. It is known in the literature that some protoplasts sporulate soon after the regeneration of their cell wall, and once empty from reproductive cells, die (Gupta et al., 2012; Gupta & Reddy, 2018, Reddy & Seth, 2018). However, a separation between *Ulva* individuals directly originated by protoplasts and the ones originated by gametes or spores released during protoplasts' sporulation is usually not clear. The final estimations in **Publication III** considered the total of new *Ulva* individuals originating directly or indirectly by protoplasts produced by 1 g of biomass ($4.64 \pm 0.35 \times 10^{10}$ cell g^{-1}, **Fig. 17**, in **Publication III**). However, if 100 % of the protoplasts would germinate during the germination experiment, the direct protoplast germination would amount only to 4.26×10^8 cell g^{-1}. A clear separation between individuals obtained directly or indirectly through protoplasts is important as this information will influence the percentage of regenerated/germinated protoplasts. If indirectly produced *Ulva* (i.e. developed by gametes released by discs or cell masses) is counted to determine protoplast regeneration/germination, the results will be misleading, as those germlings were produced by dead protoplasts.

6.6. Prospects for future research

Based on the restrictions reported in **Publication I**, future work should focus on developing longer experimental designs where the development of the *Ulva* can be followed from germling to adult, having in consideration to maintain density throughout the experiment so the nutrient concentration can be kept constant without impacting the biomass growth. Additionally, the germling growth of the species reported in **Publication I** should be tested under different temperature conditions. Germlings of *U. intestinalis* were found to grow better at 20 and 15 ˚C than at 25˚C (Kim et al., 2021).

During the preliminary temperature experiment, only RGRs were evaluated for strain selection. However, this abiotic factor should be considered as a strain and biomass optimization method. High temperatures were found to increase the amino acid content in *U. rigida* (nowadays identified as *U. lacinulata*; Gao et al., 2018; Hughey et al., 2022), suggesting that the manipulation of temperature conditions could increase biomass quality that would therefore result in a more competitive product.

The saturating irradiance treatment was found to be a possible way to optimize the NE-Atlantic *U. lacinulata*. Based on the trend reported in **Publication II**, the samples under higher irradiance tended to keep increasing their antioxidant activity. A longer experiment and an experiment with higher levels of irradiance should be used to test the maximum AA that can be achieved in this strain before harvesting. Cost-effective methods should also be considered as a way to increase biomass quality and simultaneously reduce production costs (e.g., optimization under low irradiance).

The lack of variation in AA observed in the Mediterranean strain in **Publication II** suggests that in both treatments the Mediterranean strain responded to light dose and not to light irradiance. The light dose should be considered separately from irradiance and be tested as an influencing factor when it comes to *Ulva*'s biochemical composition and growth. Further works focusing on light dose would be beneficial for the clear determination of the influencing factors behind the growth and biochemical composition of *Ulva*. These works should be associated with photosynthetic analysis and the use of assays (e.g., DPPH or FRAP) that can provide a wider overview of variations in pigment concentration (Chakraborty & Paulraj, 2010; Magnusson et al., 2015). Aqueous and ethanolic extracts should be tested together as well (Chakraborty & Paulraj, 2010; Heo et al., 2005; Mezghani et al., 2013; Srikong et al., 2017). This will provide a deeper understanding of the role of each antioxidant on the photosynthetic capacity of *Ulva*.

Light quality (i.e., colour) has been reported to impact *Ulva*'s growth, cell integrity, and photosynthetic efficiency (Muthuvelan et al., 2002; Schwoerbel, 2019; Wan et al., 2022; Lee et al., 2024). It would be interesting to further investigate the impact that different light colours have on the AA of *Ulva*. Blue light has also been reported to increase growth and influence the reproduction in *Laminaria*, *Saccharina*, and *Undaria* spp. (Lüning & Dring, 1972; Klenell et al.,

2002; Dring & Lüning, 1975; Mizuta et al., 2007; W. Wang et al., 2010). The exploration of how different light colours can control the reproduction of *Ulva* spp. could lead to promising results. While **Publication III** does not provide a clear answer to what caused the reported degradation event, the *Ulva* holobiont and the mutualistic and symbiotic relations between *Ulva* and bacteria should be considered as a possible cause. The *Ulva*-bacteria interactions have been studied for decades and are known to vary during green-tide events or stressful environmental conditions (Qu et al., 2021; Zhao et al., 2022; Dong et al., 2023). Some bacteria have the capacity to degrade *ulvan*, a compound found in the *Ulva*'s cell wall (Waite & Mitchell, 1976; Tanaka et al., 2022). It has been considered by Waite & Mitchell (1976) and Bonneau (1978) that stressful conditions can lead opportunistic bacteria to penetrate the *Ulva* cells and cause thallus degradation, thus releasing *Ulva* cells into the water, as reported in **Publication III**. This interaction could help *Ulva* cells and their associated bacteria to be dispersed and escape stressful conditions (Waite & Mitchell, 1976; Bonneau, 1978). Further work is necessary to test this hypothesis and determine what causes *Ulva* degradation during cultivation, how it is regulated, and what stops it from happening.

Despite considering a degradation event to be over when protoplast yields were low and the RGRs of the *Ulva* pieces were high, this was not observed at the end of the four-week experiment. At the end of the experiment, protoplast yields were still high while the RGRs of the original fragments of *Ulva* were also high. To confirm the hypothesis that protoplast yields will drop to zero once degradation stops, a longer experiment is required. The fact that the protoplasts were released by the same fragments that kept mostly positive RGRs during the four-week experiment suggests that degradation is a slower process than regrowth. The occurrence of protoplast yields outside the delimited degradation event should be evaluated further.

Because cell masses were not considered when calculating the protoplast germination rates in **Publication III**, further work should count the occurrence of cell masses and evaluate their importance in the process of protoplast germination and reproduction. Sporulation in protoplasts (as cell masses or small discs) occurred sooner than it is expected in *Ulva* (Stratmann et al., 1996; Huang et al., 2023). The mechanism behind this sporulation capacity should be studied for the impact it can have in the wild (e.g., formation of green tides; Huang et al., 2023) and for the possibility of developing faster seeding methods for *Ulva* cultivation. Huang et al. (2023) reported the reproduction of infantile thallus from *U. prolifera* as a strategy to form green tides. As strains of both *U. lacinulata* and *U. compressa* have been associated with green tides, and because the sporulation of young discs as cell masses matches the infantile reproduction described by Huang et al. (2023), further work should be done, to evaluate the importance of natural protoplast production in the formation of green tides.

The protoplast germination rates reported in **Publication III** were low compared to the ones reported in the literature after protoplast isolation. The possible reasons for these results are

listed in the **Considerations and constraints** of this chapter. Because of the novelty of **Publication III**, the overall methodology should be optimized. In particular, the importance of low light should be tested as a way to achieve higher protoplast germination rates. Low light was considered a requirement for the development of protoplasts from *Kappaphycus alvarezii* (Doty) L. M. Liao (Salvador & Serrano, 2005) and it is a common practice to incubate *Ulva* protoplasts for 24 h under low light after their isolation (Reddy et al., 2006; Gupta et al., 2018; Gupta & Reddy, 2018).

Publication III reported a 1:1 ratio between the two morphologies originated directly from protoplasts (unattached discs and unattached germlings). Gupta et al. (2012) reported variations in the occurrence of these two morphologies when protoplasts grew under different temperature conditions, suggesting that abiotic factors could play a role in determining the morphology of germinated protoplasts. Based on the observations in **Publication III** and the work from Gupta et al. (2012), the two morphologies developed differently since the first cell division (**Fig. 9C**, in **Publication III**). Future work should focus on determining the influencing factor causing the formation of cell masses and including them in the ratios reported in **Publication III**. Additionally, Gupta et al. (2012), tested the development of the two morphologies at 20, 25, and 30 ˚C and only found a ratio close to 1:1 at 25 ˚C, while **Publication III** reported the 1:1 ratio at 15 ˚C. The effect of temperature and other abiotic factors should be explored further to find the mechanisms that determine the protoplast development into the different morphologies. Reddy et al. (1989) hypothesized that the different morphologies could be caused by cell differentiation before cells detach from the original thalli. A similar hypothesis has been used to justify the degradation pattern in the cell thallus where the cells either die or become protoplasts. Provasoli (1958) called the areas of the thalli that did not bleach “green islands”, which were, in **Publication III**, understood as the areas composed of the same cells that became protoplasts. Following the findings from Provasoli (1958), Bonneau (1978) reported sloughed cells and their development into the same morphologies reported in **Publication III**. Both Provasoli (1958) and Bonneau (1978) hypothesized that the totipotency of those cells was behind the development of the two morphologies. Based on the hypotheses suggested by Provasoli (1958) and Bonneau (1978), the totipotency of protoplasts should be evaluated further. However, Gupta et al. (2012) found a difference in methylation modifications between filamentous germlings (i.e., unattached germlings) and discs, with discs presenting hypermethylation conditions, suggesting that epigenetic causes are behind the occurrence of these two morphologies. Because seaweed-associated bacteria are known to influence the morphology of *Ulva* spp. (Marshall et al., 2006; Wichard & Oertel, 2010; Singh et al., 2011), abiotic and biotic factors should be explored further as possible influencers to the occurrence of these two morphologies. The control over the morphologies obtained from protoplasts can increase the potential of using this material for seeding large-scale cultivation systems.

In **Publication III** the presence of naturally occurring protoplasts was detected in a blade morphotype of *U. compressa*, a species known to vary its morphology between tubular and foliose (Hofmann et al., 2010; Steinhagen et al., 2019c). The NE-Atlantic *U. lacinulata*, in **Publication III,** and the *Ulva lactuca* samples studied in Provasoli (1958) and Bonneau (1978), presented a foliose morphology as well. Protoplast isolation methods for species presenting a tubular morphology usually require the use of an additional enzyme (Macerozyme) to guarantee the success of the method (Gupta & Reddy, 2018). This suggests that the cell wall structure of tubular morphotypes and protoplast formation can be different. It would be interesting to evaluate the occurrence of degradation and protoplast formation in tubular morphotypes of *Ulva* spp..

As of now, it is not clear what triggers either the degradation of the biomass or the release of protoplasts observed in **Publication III**, and which of these events is the catalyst for the other to occur. Therefore, a long-term analysis to determine the timing in which degradation occurs when non-fertile *Ulva* is grown under constant and optimal conditions would be interesting. The timing in which protoplasts and protoplast-originated germlings occur after degradation should also be evaluated under the same conditions. This will allow the possible corroboration of the correlation found in the survey (**Fig. 9.12**, in **Appendix**) and test the hypothesis of this event to be a successful alternative asexual reproductive method in *Ulva* spp. Furthermore, future attempts to optimize and reduce the costs of protoplast isolation methods should consider the results from **Publication III** and the results from the pre-treatments tested, during the STSM in Ireland, that caused the reduction of the cell wall thickness (e.g., 40 PSU; **Fig. 9.3** in **Appendix**). In most treatments tested as pre-treatments to protoplast isolation, a reduction in cell wall width was observed after 3 days. This suggests that a change in cultivation conditions for a short time can reduce the cell wall thickness and facilitate protoplast isolation.

7. Conclusion

The objective of this dissertation was to investigate successful methods of strain selection and optimization of *Ulva* species and overcome some of the major bottlenecks faced in *Ulva* cultivation nowadays. In this dissertation, *Ulva* strains were selected and optimized to be suited for cultivation in a large-scale land-based recirculating aquaculture system. These methods can increase the profitability and reduce the costs of a system never used before for *Ulva* cultivation.

Strain selection enables the selection of robust strains that fit the particular characteristics of each system. For *Ulva* cultivation in a RAS, under high temperature and low salinity, the two strains of *Ulva lacinulata* tested were the ones with the highest growth rates. These strains can be cultivated at optimal conditions of 21 ˚C and 28 PSU, but salinity can be reduced to 12 PSU (**Publication I**), a 60 % reduction in salinity, without their relative growth rates reducing below the minimum daily growth required for profitable cultivation.

Optimization of strains during cultivation can increase biomass quality. The NE-Atlantic strain increased its antioxidant activity when exposed to low salinity (10 PSU, **Publication I**) for ten days or a high irradiance (185 µmol photons m^{-2} s^{-1}; **Publication II**) for five days. These treatments can be used for a short time before harvesting the *Ulva* so as to not cause an undesirable impact on its growth. Antioxidant activity, used in this dissertation as a method to evaluate biomass quality and the success of *Ulva* optimization, proved the importance of strain selection by showing differences in the antioxidative response of the two *U. lacinulata* strains (**Publication II**).

For the reasons presented above, the NE-Atlantic *U. lacinulata* was the chosen strain for further cultivation in the large-scale land-based RAS. Despite presenting desirable characteristics, the lack of fertility and constant degradation of the biomass limited its cultivation. During the degradation, an overlooked reproduction strategy was found. *Ulva* can naturally produce protoplasts as a method of dispersion and asexual reproduction. Depending on the morphology that the protoplasts take, they can become fertile, even if originated by a considered non-sporulating strain such as the NE-Atlantic *U. lacinulata*. Protoplasts were also found in pieces of fertile *U. compressa* collected from the wild. Thus, proving that protoplasts occur naturally in different *Ulva* species.

The interest in *Ulva* spp. keeps rising with the belief that this genus can become an important feature as food or as a plastic substitute in the future to answer the needs of the global population. However, several steps are still a limitation for *Ulva*'s cultivation. Discussion has been initiated on how to overcome such bottlenecks. Our understanding

of this genus, our capacity to provide the necessary conditions for its optimal growth in large-scale systems, and the associated high costs of such systems are examples of limitations that the scientific community has been working with. The concepts, experiments, and findings discussed in this dissertation contribute to closing a knowledge gap regarding *Ulva* reproductive strategies and to the growing knowledge of *Ulva* successful cultivation. With this work, I hope to inspire further discussion and research to allow the *Ulva* community to achieve our collective goal of successful and sustainable *Ulva* cultivation.

When descends on the Atlantic
The gigantic
Storm-wind of the equinox,
Landward in his wrath he scourges
The toiling surges,
Laden with seaweed from the rocks:

From Bermuda's reefs; from edges
Of sunken ledges,
In some far-off, bright Azore;
From Bahama, and the dashing,
Silver-flashing
Surges of San Salvador;

From the tumbling surf, that buries
The Orkneyan skerries,
Answering the hoarse Hebrides;
And from wrecks of ships, and drifting
Spars, uplifting
On the desolate, rainy seas; —

Ever drifting, drifting, drifting
On the shifting
Currents of the restless main;
Till in sheltered coves, and reaches
Of sandy beaches,
All have found repose again.

So when storms of wild emotion
Strike the ocean
Of the poet's soul, erelong
From each cave and rocky fastness,
In its vastness,
Floats some fragment of a song:

From the far-off isles enchanted,
Heaven has planted
With the golden fruit of Truth;
From the flashing surf, whose vision
Gleams Elysian
In the tropic clime of Youth;

From the strong Will, and the Endeavor
That forever
Wrestle with the tides of Fate;
From the wreck of Hopes far-scattered,
Tempest-shattered,
Floating waste and desolate; —

Ever drifting, drifting, drifting
On the shifting
Currents of the restless heart;
Till at length in books recorded,
They, like hoarded
Household words, no more depart.

Seaweed, by Henry Wadsworth Longfellow

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9. Appendix

Supporting information to Chapter 6 (Discussion)

Table 9.1 - List of *Ulva* species and strains used during this dissertation. The list includes morphological data from each species, geographical location, environmental conditions reported in each sampling site and optimal conditions calculated during the temperature and salinity experiments (**Publication I**)

Species name	**Morphology of the thallus**	**Origin**		**Abiotic Conditions**				**Optimal Conditions of growth based on strain selection experiments**		**References**
		Location	**GPS coordinates**	**Temperature (°C)**	**Salinity (PSU)**	**Light (daylength)**	**Irradiance**	**Temperature (°C)**	**Salinity (PSU)**	
U. flexuosa	tubular	Mediterranean Thessaloniki Bay, Greece	40°33'57.4"N 22°57'28.0"E	7-27	36-39	09h16 - 15h05	623.5 to 645 µmol photons m^{-2} s^{-1}; 290 to 300 W m^{-2}	15 *	30 *	(Haritonidis, 1978; Kambezidis, 2021) based on Global Horizontal Irradiation data
U. linza	tubular	NE-Atlantic Óbidos Lagoon, Portugal	39°23'41.5"N 9°12'48.9"W	6-24	25-35	09h24 - 14h57	392.69 µmol photons m^{-2} s^{-1}; 1600 kWh m^{-2} (annual)	17-18	21	(Cavaco et al., 2016; Mendes et al., 2021)
Ulva-1	tubular	Clonakilty Bay, Ireland	51°36'45.2"N 8°52'33.5"W	8-21	31-35	08h - 17h	0.6 kWh (min) – 6.2 kWh (max)	--------	--------	(Irish Water, 2021; Sunrise Sunset, n.d.; Sea Temperature, n.d.a;)
U. lacinulata	foliose	Mediterranean Thessaloniki Bay, Greece	40°34'11.2"N 22°57'12.5"E	7-27	36-39	09h16 - 15h05	623.5 to 645 µmol photons m^{-2} s^{-1}; 290 to 300 W m^{-2}	21	28	(Haritonidis, 1978; Kambezidis, 2021) based on Global Horizontal Irradiation data

Table 9.1 ***(cont.)***

Species name	Morphology of the thallus	Origin		Abiotic Conditions				Optimal Conditions of growth based on strain selection experiments		References
		Location	GPS coordinates	Temperature (°C)	Salinity (PSU)	Light (daylength)	Irradiance	Temperature (°C)	Salinity (PSU)	
U. lacinulata	foliose	NE-Atlantic Óbidos Lagoon, Portugal	39°23'41.5"N 9°12'48.9"W	6-24	25-35	09h24 - 14h57	392.69 µmol photons m^{-2} s^{-1}; 1600 kWh m^{-2} (annual)	20 *	28	(Cavaco et al., 2016; Mendes et al., 2021)
Ulva-2	Foliose	Clonakilty Bay, Ireland	51°36'45.2"N 8°52'33.5"W	8-21	31-35	08h - 17h	0.6 kWh (min) – 6.2 kWh (max)	--------	--------	(Irish Water, 2021; Sunrise Sunset, n.d.; Sea Temperature, n.d.a; WeatherSpark, 2024)
Ulva-3	Foliose	Helgoland, Germany	54°11'04.0"N 7°53'28.0"E	2-18	29-35	07h20 - 17h11	0.5 kWh (min) – 1.6 kWh (max)	--------	--------	(Munda, 1977; Raabe & Wiltshire, 2009; WeatherSpark, 2024)
U. compressa	Foliose/tubular	Dorum (Wurster North Sea coast, Lower Saxony), Germany	53°44'30.8"N 8°30'52.4"E	5-18	29-34	07h33 - 16h57	0.5 kWh (min) – 6.1 kWh (max)	--------	--------	(Scheurle et al., 2005; Sea Temperature, n.d.b; WeatherSpark, 2024)

* Treatment where higher RGR was found, between the four treatments tested during the temperature and salinity experiments for strain selection. No optimal conditions could be found based on regression analysis, for these species.

9.1. Temperature experiment

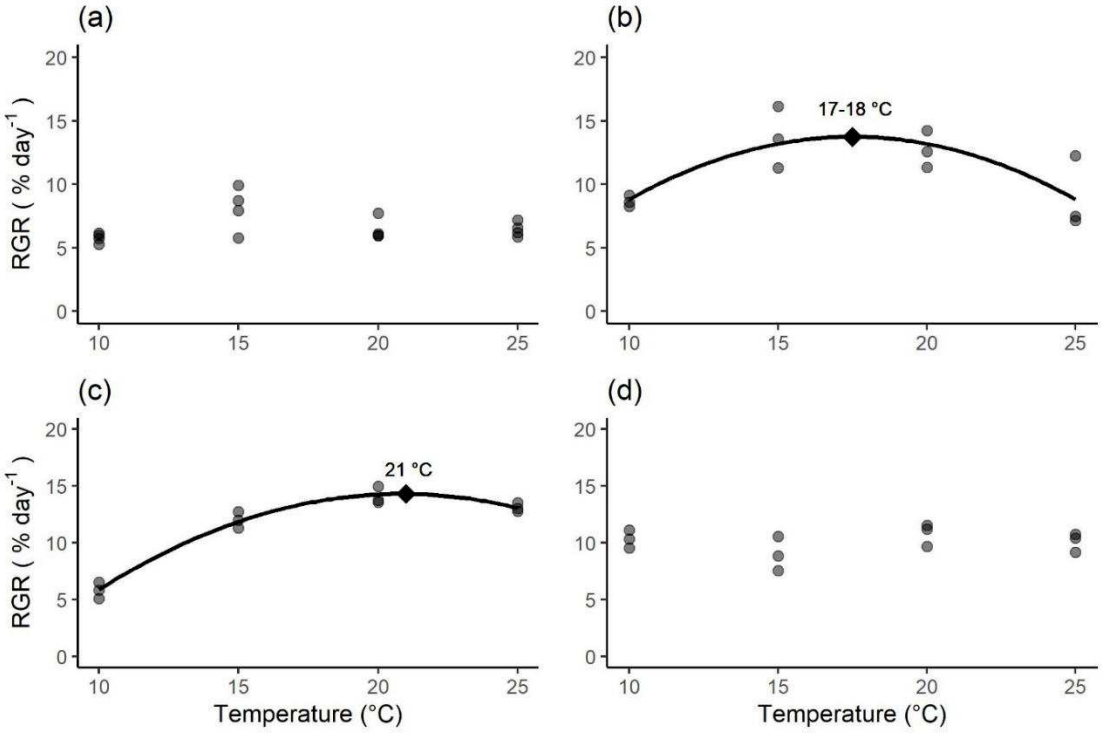

Fig. 9.1 – Relative growth rate of *Ulva* species and strains growing for three weeks under different temperature conditions. a. *U. flexuosa*/*californica* complex; b. *U. linza*; c. Mediterranean *U. lacinulata*; d. NE-Atlantic *U. lacinulata*. Black lines represent the second-degree polynomial functions as non-linear correlations found through regression analysis. Results from the regression analysis can be found in **Table 8.2**.

Table 9.2 – Results from the regression analysis performed between the temperature treatments and RGRs (% day $^{-1}$) for *U. linza* and the Mediterranean *U. lacinulata*. Non-linear regressions were determined by second-degree polynomial functions as represented in Fig. 8.1.

Species	**Multiple R-squared**	**Adjusted R-squared**	**$F_{(2,9)}$**	**p-value**
U. linza	0.6267	0.5438	7.556	0.0119
U. lacinulata (Mediterranean)	0.9714	0.9651	153.1	< 0.0001

Table 9.3 – Results of the One-Way PERMANOVA analysis of the effect of temperature on relative growth rates of adult *Ulva* spp. and pairwise comparisons between temperature treatments. RGRs of each species, in the different treatments, were compared between weeks (Week 1, 2 and 3). "Total RGR": RGR calculated based on the weight of the biomass at the beginning of the experiment and its weight at the end of the experiment (after 3 weeks of exposure to the treatments; n = 3) "Adj. p": comparison between temperature treatments represented by the adjusted p-value after Bonferroni correction. Statistically significant differences between treatments are presented in bold.

Species	**Differences between weeks**	**F-exp**	**Adj. P**					
			10-15	**10-20**	**10-25**	**15-20**	**15-25**	**20-25**
U. flexuosa		**$F_{(3,12)}$**						
	Week 1	2,48	0,14	0,51	1,00	1,00	1,00	1,00
	Week 2	4,14	**0,02**	1,00	0,42	0,15	1,00	1,00
	Week 3	1,89	1,00	1,00	1,00	0,58	0,33	1,00
	Total RGR	3,61	**0,04**	1,00	1,00	0,21	0,27	1,00
U. linza		**$F_{(3,8)}$**						
	Week 1	9,38	**0,04**	**0,01**	0,26	1,00	1,00	0,35
	Week 2	7,20	**0,03**	0,72	1,00	0,55	**0,04**	0,47
	Week 3	3,68	1,00	0,56	0,10	1,00	0,32	1,00
	Total RGR	4,80	0,10	0,36	1,00	1,00	0,18	0,32
U. lacinulata (Mediterranean)		**$F_{(3,8)}$**						
	Week 1	41,44	**0,01**	**0,01**	**0,01**	**0,05**	1,00	0,58
	Week 2	3,13	0,41	0,32	0,24	1,00	1,00	1,00
	Week 3	0,63	1,00	1,00	1,00	1,00	1,00	1,00
	Total RGR	95,16	**0,01**	**0,01**	**0,01**	**0,03**	0,43	0,67
U. lacinulata (NE-Atlantic)		**$F_{(3,8)}$**						
	Week 1	5,87	1,00	0,10	0,12	0,14	0,14	1,00
	Week 2	1,39	0,73	1,00	0,67	1,00	1,00	1,00
	Week 3	4,34	1,00	0,18	0,16	0,43	0,32	1,00
	Total RGR	1,52	0,93	1,00	1,00	0,37	1,00	1,00

9.2. Protoplast isolation and pre-treatments

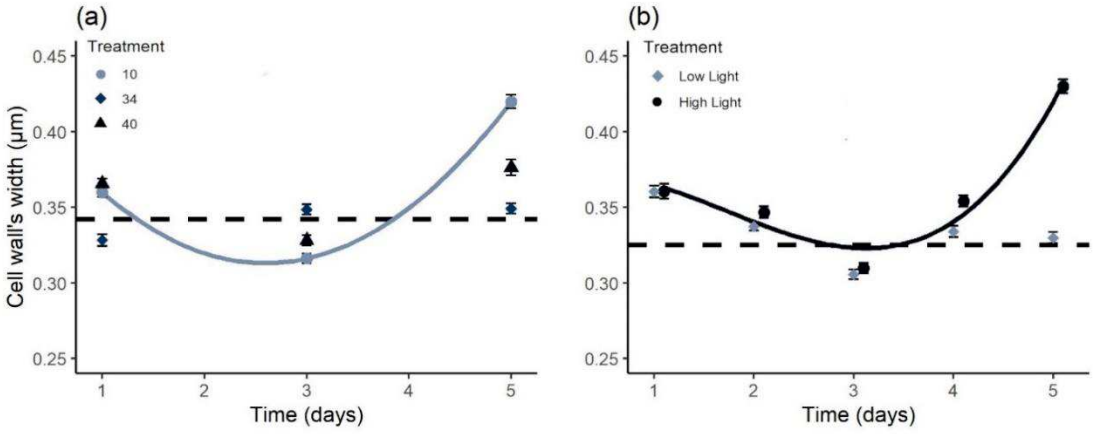

Fig. 9.2 – Cell wall thickness (in µm) of *Ulva*-2 under different salinity and light treatments during a five-day experiment. A) Salinity experiment. The green line represents the 2nd degree polynomial as a correlation between time and width at 10 PSU. The dashed line represents the mean width at 34 PSU throughout the experiment; B) Light experiment. The yellow line

represents the 3rd degree polynomial as a correlation between time and width at High Light (HL) treatment. Results from the regression analysis are presented in Table 9.3. The dashed line represents the mean width at 34 PSU throughout the experiment. In both figures, points represent the mean and bars the se.

Table 9.4 – Results from the regression analysis performed between the different pre-treatments and cell wall width (μm) shown in Fig. 9.2.

Treatment	Multiple R-squared	Adjusted R-squared	F		p-value
10 PSU	0.6467	0.6435	(2, 222)	203,2	< 0.0001
HL	0,5213	0,5174	(3, 371)	134,7	< 0.0001

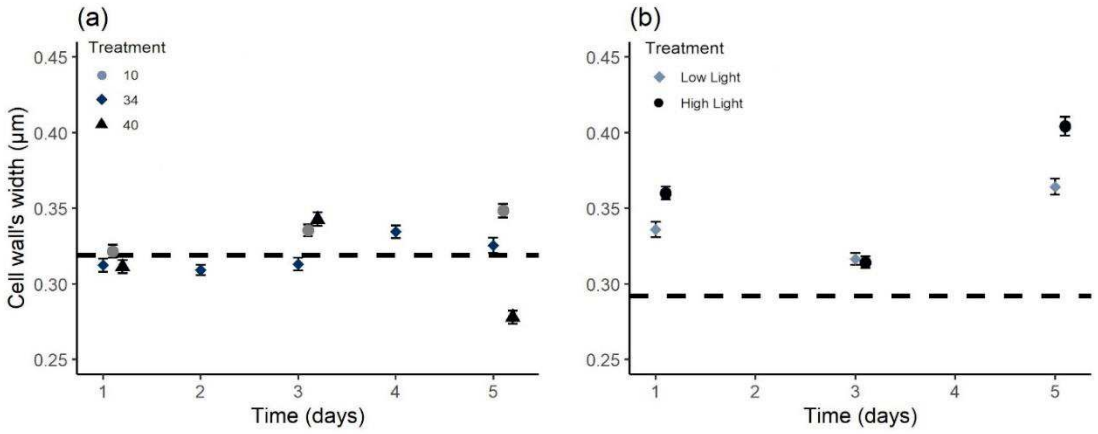

Fig. 9.3 – Cell wall thickness (in um) of *Ulva*-1 under different salinity and light treatments during a five-day experiment. A) Salinity experiment; B) Light experiment.

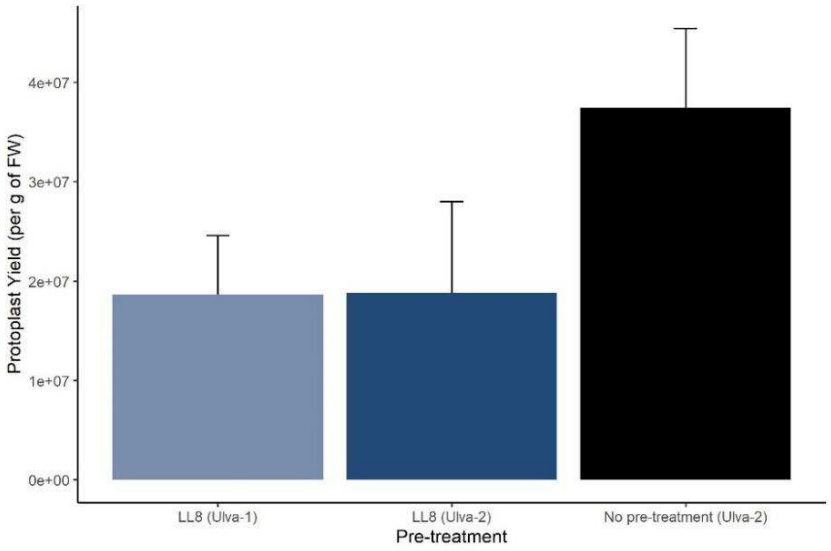

Fig. 9.4 – Protoplast yields obtained using the successful protoplast isolation methods developed before the experiment. Comparison between the protoplast yields obtained from the two *Ulva* species (*Ulva*-1 and *Ulva*-2) after an 8-day pre-treatment of low light. Protoplast yields obtained from *Ulva*-2 without a pre-treatment are used to compare the efficiency of the pre-treatment. No significant differences were found.

Table 9.5 – Corrections made to the original protoplast isolation protocol. Protoplast isolation was successful after implementing these corrections. The "Ref." column reports the list of references upon which the original protocol was based.

Steps for protoplast isolation	Original Protocol	Ref.	Corrections
Preparations of enzyme mixture (distilled water:ASW ratio)	1:1	Personal communication with Dr. Ricardo Bermejo	Only distilled water
Biomass used [mg]	100	Gupta and Reddy 2018; Gupta et al.2018	30 mg
Volume of enzyme solution [mL per mg of biomass; mg g^{-1}]	0,05	Gupta et al. 2018	0,08
Incubation (Temperature; [°C])	25	Gupta et al. 2018	20
Incubation (Time; [h])	3	Gupta et al. 2018	2
Incubation (shaker; [rpm])	80	Gupta et al. 2018	60
Centrifugation (protoplasts collection; [xg])	120 (swinging-bucket)	Gupta and Reddy 2018; Gupta et al. 2018	500 (fixed angle)

9.3. Degradation event and naturally occurring protoplasts in *Ulva* sp.

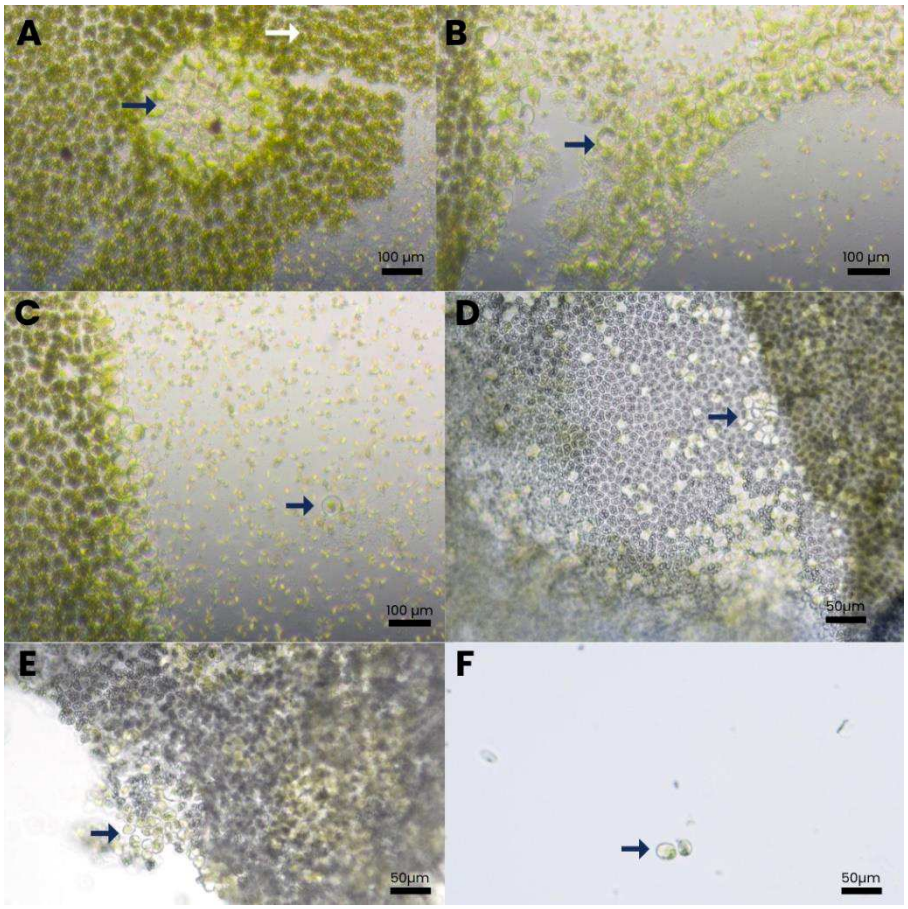

Fig. 9.5 – Heterogeneity of the thallus and protoplast-like cells found in the *Ulva*-3 and Mediterranean *U. lacinulata* during a reproduction and degradation event, respectively. A-C: reports from *Ulva*-3, the only cell types observed in the thallus are reproductive cells and spherically, mostly empty green cells with a parietal plastid, similar to what was reported in *U. compressa*; D-F: reports from Mediterranean *U. lacinulata*, the thallus is divided into two different cell types, grey cells full with starch and larger green spherical cells with a parietal plastid, similar to what was reported in NE-Atlantic *U. lacinulata*. The white arrow indicates the cell that went through swarmer formation and release; the blue arrows indicate cells that were released to the water, similar to the protoplasts observed in **Publication III**.

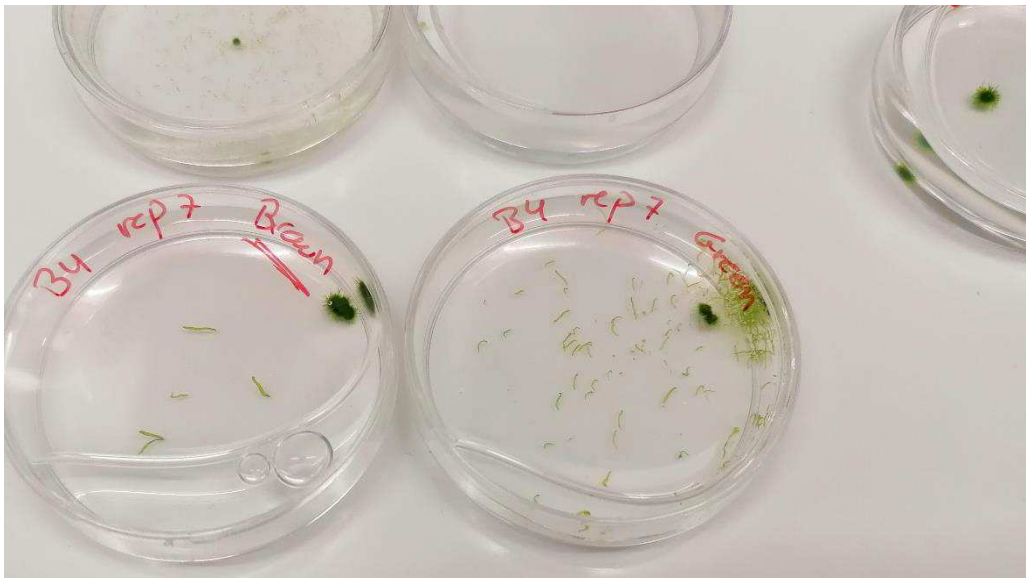

Fig. 9.6 – Preliminary experiment with green and brown discs originated from NE-Atlantic *U. lacinulata* protoplasts. The experiment aimed was twofold: 1) to confirm the capacity of green discs to become fertile (brown) and release swarmers; 2) identify the swamers as gametes or spores through phototaxis and observe their morphology after development. The presence of singular branched germlings and germling clusters permitted the conclusion that these morphologies were not a direct product of protoplast development. Additionally, germling clusters were found to be formed on top of the original bleached discs placed in the petri dishes.

Fig. 9.7 – Preliminary experiment to evaluate the formation of germling clusters. In this experiment, a known number of discs between 1 and 3 were placed in petri dishes together with a previously bleached piece of NE-Atlantic *Ulva lacinulata* thalli (from adult *U. lacinulata* being cultivated at the time in the culture room). The piece of *U. lacinulata* was bleached by being placed in a gradient of ethanol solutions ranging from 40 % to 100 %. This experiment aimed to test if *Ulva* swarmers released from the protoplast-originated discs would chose to attach to the bleached piece of *Ulva*. The observations showed that germlings settled either on top of the original disc (yellow arrow) or in the petri dish, but never in the bleached *Ulva* piece (yellow circle).

9.4. Survey

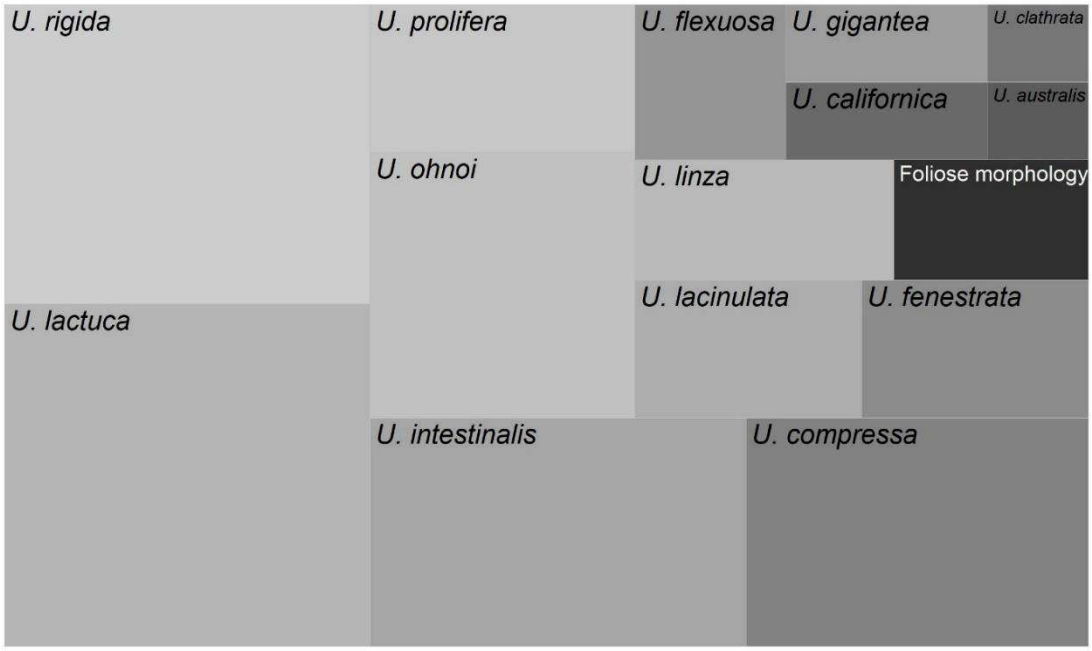

Fig. 9.8 – Treemap chart of species used by the participants of the survey (based on the number of times a species was selected).

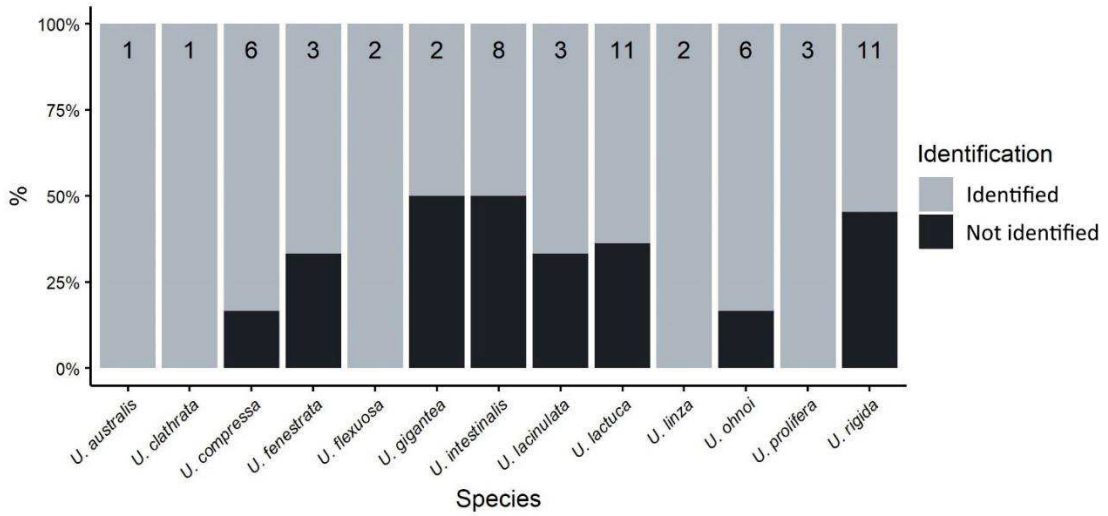

Fig. 9.9 – 100 % stacked columns of the species cultivated by the participants of the survey, separated by molecularly identified and not molecularly identified. The numbers on top of each column represent the total times each species was reported to be cultivated.

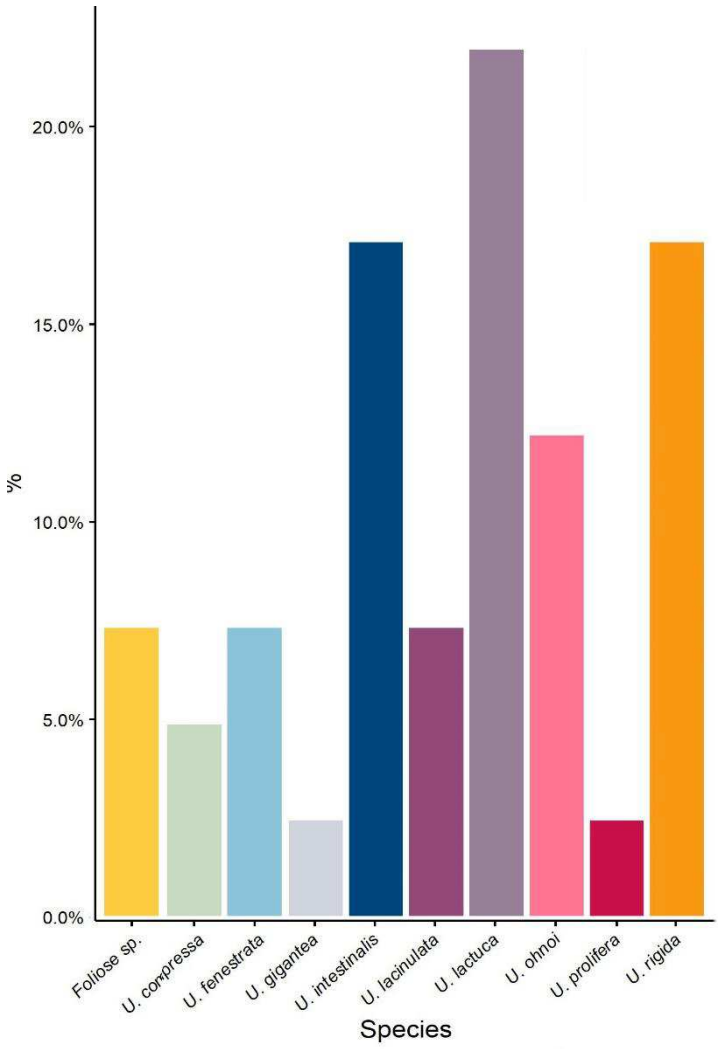

Fig. 9.10 – Percentage of *Ulva* species in which degradation was observed, based on the total of each cultivated species.

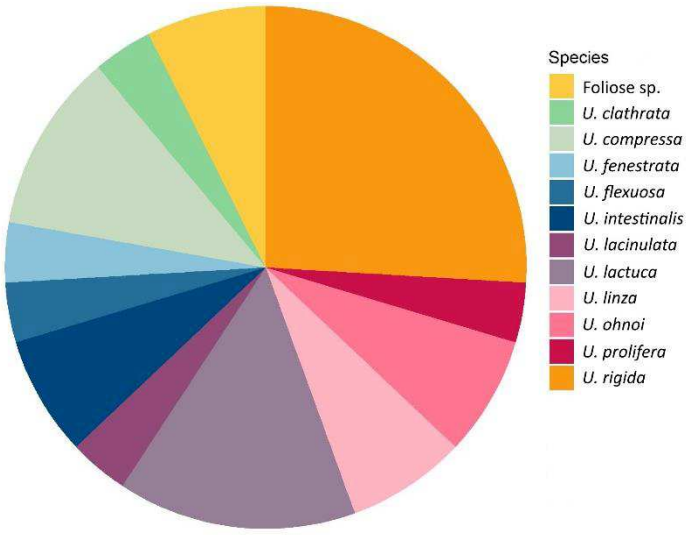

Fig. 9.11 – Percentage of *Ulva* species cultivated in which it was observed the occurrence of germlings not associated with sexual reproduction.

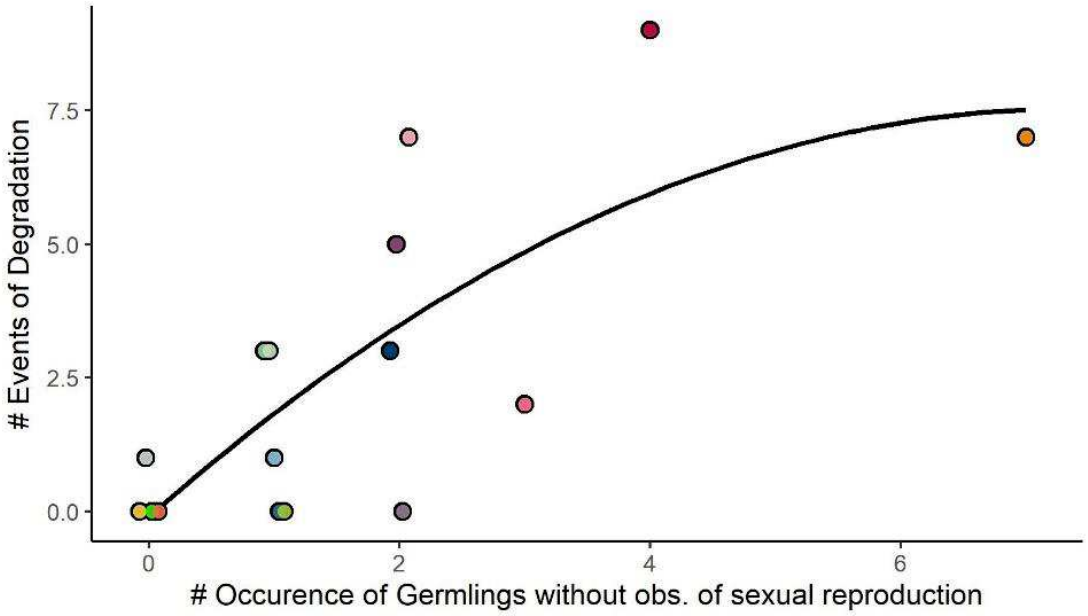

Fig. 9.12 – Correlation between *Ulva* species that were reported to degrade (Fig. 9.10) and *Ulva* species that were reported to generate germlings without signs of sexual reproduction (Fig. 9.11).

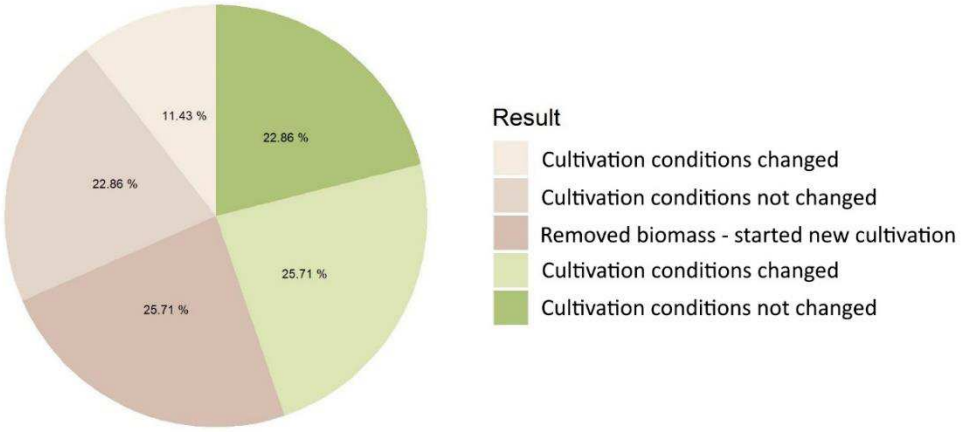

Fig. 9.13 – Percentage of reports of biomass loss (brown colours) and cultivation success (green colours) after degradation was observed. The chart is divided into the different ways the survey participants tried to control (or not) the degradation. "Cultivation conditions changed" represents the participants who changed the cultivation conditions as an attempt to stop degradation. "Cultivation conditions not changed" represents the participants who observed degradation but did not attempt to stop it by changing the cultivation conditions. "Removed biomass – started new cultivation" represents the participants who observed degradation and immediate removed the biomass, therefore eliminating the possibility for the *Ulva* to recover.

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10. Acknowledgments

I would like to express my immense gratitude to my Doktorvater, Prof. Dr. Bela H. Buck for giving me the opportunity and support to develop my PhD thesis in his working group. His supervision and valuable advice were essential for the success of my work. Likewise, I express my deep appreciation to Prof. Dr. Laurie Hofmann who always gave me the freedom to explore different (sometimes crazy) ideas and participated in some of them. I will never forget our conversations, especially those where neither of us knew what was happening with "our" *Ulva*. I am thankful for her support and incentive which led me to take part in some of the best experiences so far in my career, such as the STSM in Ireland or the possibility to meet other amazing scientists and fellow *Ulva* enthusiasts. Both Bela and Laurie always found time to listen to my ideas and my worries, and for that, I am most thankful. Thank you both, very much.

A PhD is not a PhD without the TAC members. I extend my acknowledgment to my Thesis Advisory Committee and co-authors, Dr. Inka Bartsch, Dr. Klaus-Ulrich Valentin, and Dr. Sophie Steinhagen who helped me navigate through my PhD during the last three years. To each of you, I am very thankful. I will never forget the trip to Britanny with Klaus, which reminded me of where my love for seaweed started, at the beach surrounded by them. I will never forget the meeting with Sophie, where via Webex she took me around her lab to show me where and how to start this journey. Thank you, Sophie, I wish there was more time to keep learning with you. Most of all, I will never forget the hours and days spent borrowing lab space, cultivation rooms, and equipment from Inka, and all the stops she made to check on me. Thank you for all of your support and for making me feel part of your team.

I would like to extend my gratitude to Dr. Ricardo Bermejo Lacida for agreeing to review this thesis, Prof. Dr. Kai Bischof for his support and agreeing to be the Commission chair for my thesis defense, to Dr. Nora Diehl for accepting my invitation to be part of the examination commission, and to the Bremen University student Janka Jokay for her interest in being part of this moment.

Many thanks to my AWI section head Marteen Boersma for always signing my travel applications and supporting my work.

I also thank POLMAR for the amazing work supporting PhD students and giving us so many opportunities to succeed. I have lost count of the number of workshops I took and how much I learned because of you.

I would like to thank my two co-authors and bachelor students whom I had the privilege to help supervise, Anneke Meißner and Anja Sawicki. I am very proud of what you accomplished and to have the opportunity to share part of your work in my dissertation. Thank you for understanding every time I responded with an "I don't know" and thank you for going with me in the search for answers. A special thank you to Anneke who took such good care of "my" *Ulva* while I was away. To all of the other students I had the pleasure to help, Mascha, Jesse, Clarissa, thank you for letting me share with you what I have learned so far and my interest in seaweed.

I thank Dr. Sotiris Orfanidis for allowing me to use his *Ulva* cultures in my work and for providing me with the information needed. I thank Rebecca Aepfler, from AWI, for her amazing work in helping me follow the Nagoya Protocol. Half of the work presented in this dissertation depended on it.

For a great three-month experience in Galway, Ireland, I must thank Dr. Liam Morris and Dr. Ricardo Bermejo Lacida. You gave me the space and freedom I needed to try new methods and, meanwhile, you allowed me to meet such amazing people. Nessa, Ana, Camille, Manuel, Alice, and Sara, I had such a lovely time with you. Thank you for one of the best birthdays ever!

I thank the SeaWheat COST ACTION (CA20106) for funding my STSM to Ireland and for the opportunity to join the group and share my work. The SeaWheat COST ACTION inspired me throughout my Ph.D. studies, and I learned so much every time I had the chance to meet the SeaWheat COST Action members. Thank you, Karina and Viviana, for letting me be part of the Communications Team, I cannot wait to keep working and learning with you.
To Andreas Wagner, Claudia Daniel, and Sabine Strieben, I thank you for all your support and patience. Without your help, this work would have not been possible. I also thank all my working group that I feel I only had the opportunity to really meet in the last year, during the section meeting on Helgoland. I keep close in my heart the day we all came together to clean Bremerhaven and have some fun along the way.
I am grateful for the support I had from Prof. Dr. Teresa Mouga, who was supposed to be my PhD supervisor if I had stayed in Portugal. Not only did she believe in me but she also helped me collect the *Ulva* that I ended up selecting as the best candidate for cultivation and that gave me so many headaches. My appreciation is extended to her excited and interested group of students who helped me clean so much *Ulva*, no questions asked. That *Ulva* that you helped me clean, was essential to start my adventure. My Portuguese mind and heart will always be in debt with you.
I thank all my friends, in Germany and Portugal for their support, either by being a constant presence in my day-to-day life or by just accepting the fact that it will take me a while to reply to your messages. Thank you, Cocas, for always thinking the best of me and for the long conversations. I miss Coimbra and I miss you. Thank you Tânia, who is an “expat” just like me, for sharing the enthusiasm of culture shock and the typical PhD frustrations. Thank you, Claudia, for achieving the title of my oldest friend and always being you, despite everything that life throws at us. Thank you, Kiri, for just showing up in my life now, three years of a Ph.D. with you would have been way too much fun! I wish you all the best in your academic adventure. Thank you Rike for the long conversations and reminders to just chill. Keep taking good care of “our” *Ulva*! More importantly, thank you, Freitag, for always letting me enter my own office and receiving me with such excitement. To both Rike and Kiri, never forget, «When life gives you lemonade, make lemons. Life will be like “What?!”». A huge thank you and an enormous hug to Luisa, for such an easy and growing friendship. I still have the first bottle of Flensburger we drank together, and I am looking forward to having more great moments with you. Prost!
I would like to thank my family for teaching me how to leave and inspiring me to become a better person. Whatever success I had in life was because of you. I especially would like to thank my mom for always having a fun story to tell and for learning English so she can speak with my German partner. Thank you for always doing your best and supporting me, no matter what.
Finally, I must thank the most patient partner a person could ask for, Kai. You met me in one of the most challenging years of my life. I was living in a new country, during the pandemic, where I did not know the language or culture and was struggling to make friends. More than anything, I thank you for being my friend and allowing me to navigate through insecurities, a chronic illness, and a PhD with you. I cannot wait to share new adventures with you. I would also thank you for giving me a new little family, you, Rosa, and Karla. And I thank them for always waking me up way too early.
To everyone, thank you!

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12. Erklärungen zur elektronischen Version und zur Überprüfung einer Dissertation

Hiermit betätige ich gemäß §7, Abs. 7, Punkt 4, dass die zu Prüfungszwecken beigelegte elektronische Version meiner Dissertation identisch ist mit der abgegebenen gedruckten Version.
Ich bin mit der Überprüfung meiner Dissertation gemäß §6 Abs. 2, Punkt 5 mit qualifizierter Software im Rahmen der Untersuchung von Plagiatsvorwürfen einverstanden.

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Changes to the thesis's submitted version:

Page – Line/Figure	In the submitted version	Corrected to
Cover page	Missing information	"Thesis supervised by Prof. Dr. Bela H. Buck and Prof. Dr. Laurie C. Hofmann"
Cover page (front and back) – University Logo	The old logo was used	The new logo was added
Thesis Reviewers – Line 28-30	"Examiner: Dr. Nora Diehl Marine Botany, University of Bremen, Bremen, Germany"	"Faculty member: Dr. Nora Diehl Marine Botany, University of Bremen, Bremen, Germany"
Thesis Reviewers – Line 31-33	"Student member: Bjarner Fobel, Bachelor's student, University of Bremen, Bremen, Germany"	Deleted
Table of contents – 3rd page	"Table of contents" shown in an empty page as heading	Deleted
Page iii and iv - Zuzammenfasung	Entire text presented unclear and non-scientific sentences (wrong translation from the English summary)	Entire text was reviewed and corrected to present clears sentences and scientific wording
Page 4 – Line 1	"Lormatire and Gonçalves, 2022a"	"Lormatire & Gonçalves, 2022a"
Page 6 – Line 13	"macroalagae"	"macroalgae"
Page 7 – Line 16	Spacing error	Spacing corrected
Page 9 – Line 15	"Wichard et al. 2015; Balar and Mantri, 2020"	"Wichard et al., 2015; Balar & Mantri, 2020"
Page 9 – Line 21	"Balar & Mantri 2020"	"Balar & Mantri, 2020"
Page 9 – Line 23	"Balar & Mantri 2020"	"Balar & Mantri, 2020"
Page 9 – Line 25	"Balar & Mantri 2020"	"Balar & Mantri, 2020"
Page 10 – Figure 1.2	"re-presented", "diploidi-zation", "partheno-genesis"	"represented", "diploidization", "parthenogenesis"
Page 20 – Lines 25 and 29	Consecutive sentences start with "While". First sentence: "While and overview […]"; Second sentence: "While the potential use […] was previously mentioned, its antioxidant extracts can also be […]".	Correction for clarity, Second sentence corrected to: "The potential use of Ulva as a whole in different industry sectors (e.g., food) was previously mentioned, but its antioxidant extracts can also be incorporated in different industries"
Page 28 – Line 4	"[…] limitations of the two methods […]"	Correction for clarity: "[…] limitations of the methods […]"
Page 37 – Line 15	"Steinhagen et al. 2019b"	"Steinhagen et al., 2019b"
Page 37 – Line 15 - 16	"The identification of this species will be discussed further in the Discussion chapter of this dissertation I."	"The identification of this species is discussed further in Publication I."

Page 37 – Line 26	"The Greek species were collected from partners from […]"	"The Greek species were collected by partners from […]"
Page 37 – Line 28	"[…] unialgal cultures.."	"[…] unialgal cultures."
Page 39 – Line 8	"The variation in the initial fresh weight used for each species will be discussed further in the Discussion."	Sentence deleted
Page 49 to 73	Low document quality	Increased document quality
Page 77 to 92	Low document quality	Increased document quality
Page 137 – Line 17 (excluding legend of Fig. 6.1)	"Publication I and II."	"Publication I."
Page 137 – Line 17 (excluding legend of Fig. 6.1)	"But this was only visible for the NE-Atlantic *U. lacinulata*. In Publication II, the Mediterranean *U. lacinulata* presented high levels of antioxidants throughout the experiment, but it did not show a clear response to the stressor. On the other hand, the NE-Atlantic *U. lacinulata* increased its AA under the saturating light treatment (Publication II). Thus, corroborating the importance of strain selection before cultivation."	Correction for clarity: "Overall, Publication I showed how variable growth rates can be between different *Ulva* species and strains, and that different *Ulva* spp. have different salinity preferences. Furthermore, Publication II showed different antioxidant responses between the two *U. lacinulata* strains under the saturating light treatment. Both publications corroborate the importance of strain selection before cultivation."
Acknowledgments (starting on Page 253)	Species names not italicized	Species names italicized
Overall document changes	Horizontal pages with the wrong headings and missing the page number	Headings and page numbers of horizontal pages were corrected throughout the thesis.
Corrections list		A list of the corrections made to the original submitted thesis was added on page 257 of the thesis.
Declaration of consent		A Declaration of consent for electronic publishing and for the transfer of the distribution rights of doctoral thesis and Habilitations was added on page 259 of the thesis.

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