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RESEARCH

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

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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

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(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.

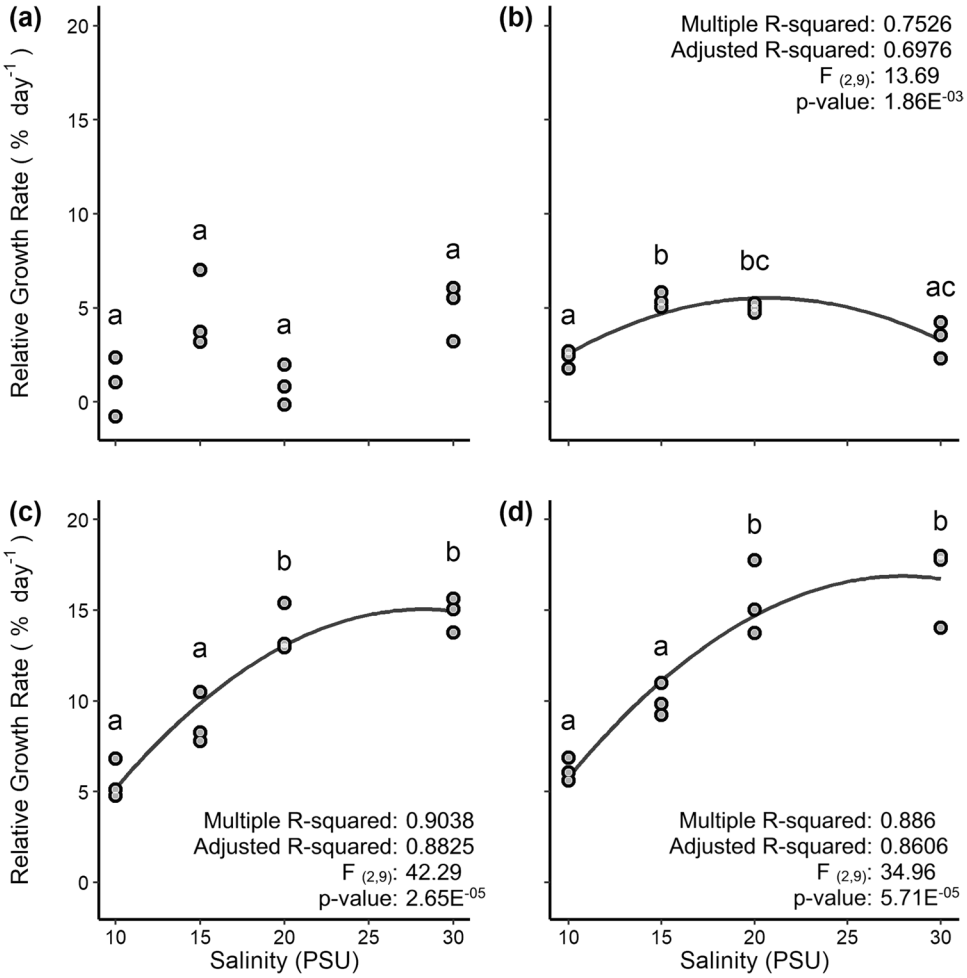

Fig. 1 Relative growth rate (RGR % day $^{-1}$) of adult *Ulva* spp. after 2 weeks of exposure to different salinity conditions (n = 3). Regression analysis performed for three of the four strains. *U. flexuosa* did not present a clear trend. (**a**) *U. flexuosa*, (**b**) *U. linza*, (**c**) *U. lacinulata* (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

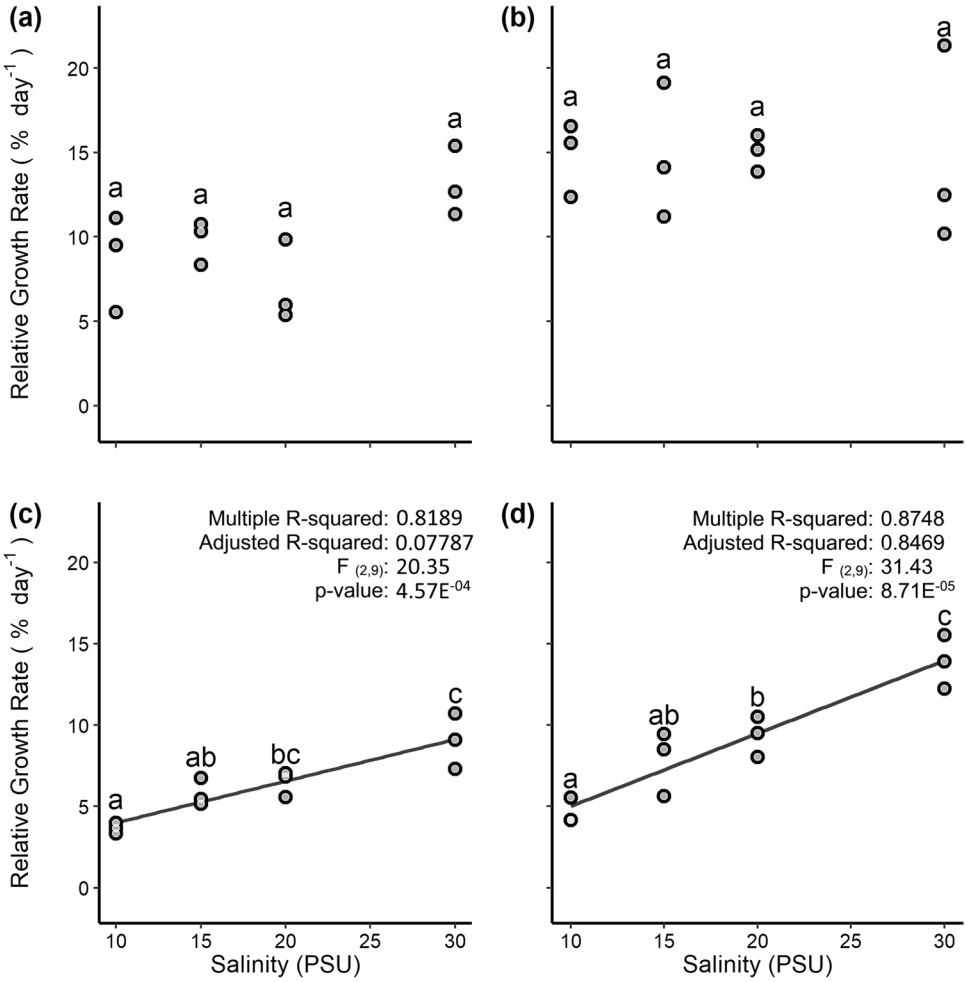

Fig. 2 Relative growth rate (RGR % day $^{-1}$) of germlings of *Ulva* spp. after 3 weeks of exposure to different salinity conditions (n = 3). Regression analysis performed for two of the four species. *U. flexuosa* did not present a clear trend. (**a**) *U. flexuosa*, (**b**) *U. linza*, (**c**) *U. 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.

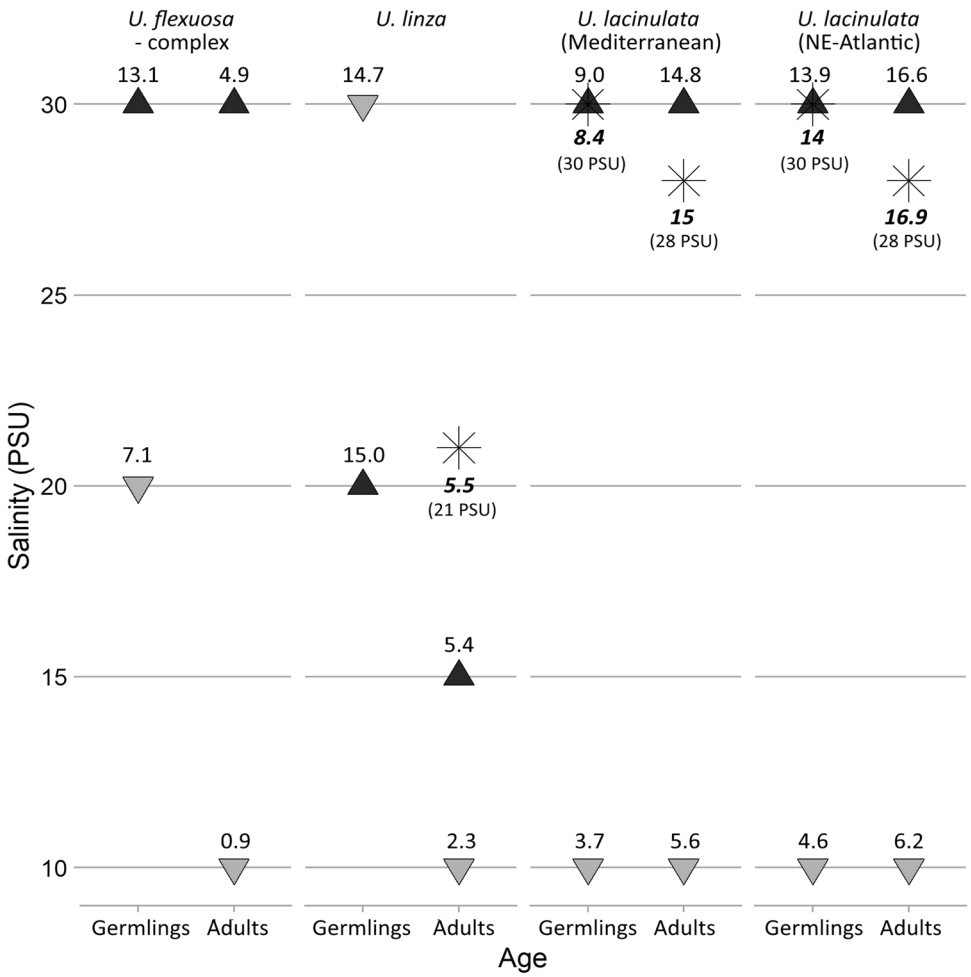

Fig. 3 Salinity treatments in which the lowest and highest RGR were measured in adults and germlings (n = 3). Results are shown as the mean of the replicates. Dark grey triangle: maximum RGR observed during the experiments; light grey upside down triangle: minimum RGR observed during the experiments; 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.

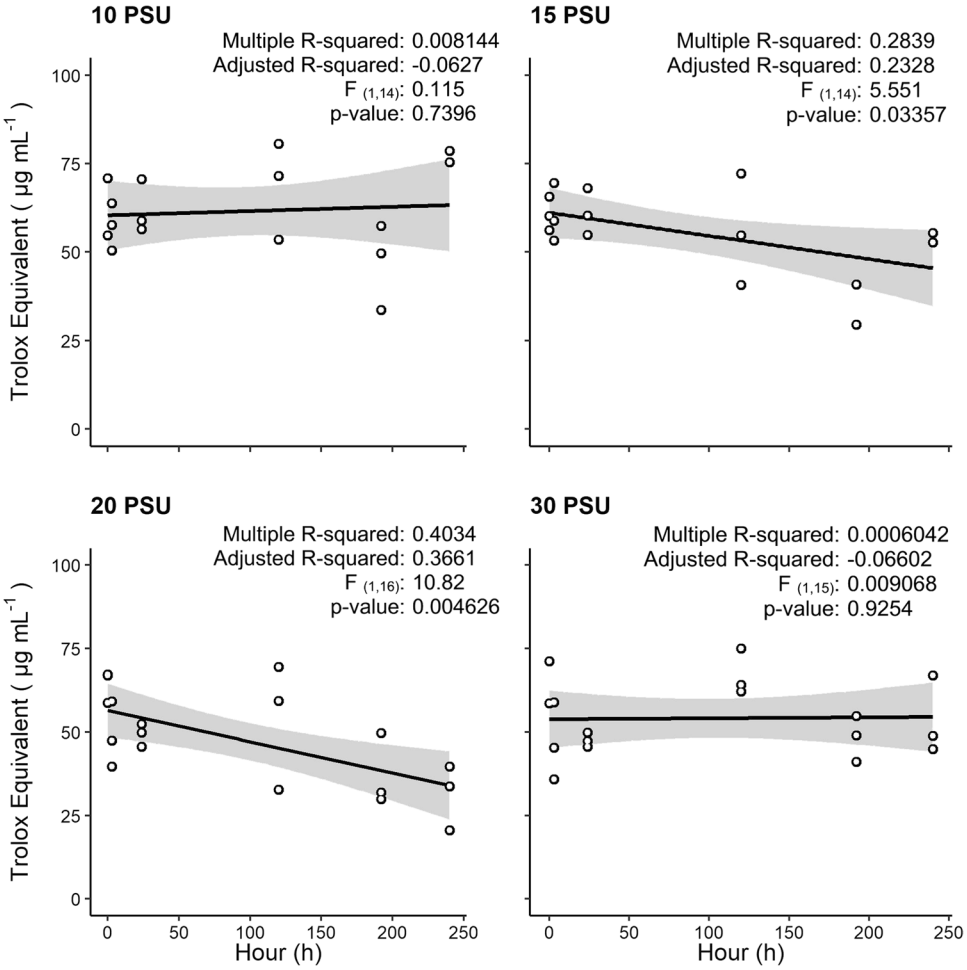

Fig. 4 Antioxidant activity based on the antioxidant concentration (Trolox Equivalent in µg mL^{-1}) of adult *U. lacinulata* (NE-Atlantic) under different salinity conditions over 10 days (samples taken at 0 h, 3 h, 24 h, 120, 192 h, and 240 h) (n = 3). Linear regressions were performed for each salinity 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.

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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.

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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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