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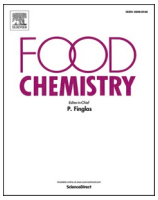

Short term decreases in salinity, combined with the right choice of species, can allow for a more nutritious *sea lettuce* lipid profile

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A B S T R A C T

The sea lettuce *Ulva spp* is becoming an increasingly important macroalgae for aquaculture. Sea lettuce can be grown on- and off-shore, displays high growth rates, and its biomass possesses attractive nutritional benefits. Among those are their fatty acids (FA) and lipid profiles, rich in omega 3 Polyunsaturated Fatty Acids (PUFAs) as well as bioactive lipids. In order to tailor those properties for food applications, we explored the use of a short-term (seven days) low salinity treatment to modulate the lipid profile of two species of *Ulva*. We found large quantitative differences between species, and while a low-salinity treatment negatively affected growth, *Ulva australis'* lipid profile was positively impacted. Total FA particularly ω-3 PUFAs, increased three-fold, as well as most polar lipid species including known bioactive compounds. This study highlights profound differences between species and describes a simple method to increase the nutritional properties of *Ulva* biomass for food applications.

1. Introduction

The sea lettuce *Ulva spp* (Ulvaceae, Ulvophyceae) represents a diverse clade of green macroalgae with increasing importance for aquaculture. *Ulva* species are present throughout the world's oceans (Fort et al., 2022) in a variety of ecosystems, ranging from freshwater to subtidal coastal areas (Mantri et al., 2020), highlighting its cosmopolitan behaviour. *Ulva* is one of the key macroalgal genus currently cultivated in Europe, together with *Alaria esculenta*, *Saccharina latissima*, *Porphyra* sp and *Palmaria palmata*, with the European seaweed market growing at an annual rate of 7–10 % (Mendes et al., 2022). *Ulva* displays high growth rates (Fort et al., 2019), are a good source of nutrients for a balanced diet (Kendel et al., 2015; Ortiz et al., 2006), and can be cultivated off-shore (Steinhagen et al., 2021) or on-shore (Laramore et al., 2022).

Importantly, on-shore cultivation allows for some control of the environmental variables in which the biomass is produced, e.g. nutrient level, salinity, irradiance, dissolved gases, flow rate (Mata et al., 2016; Toth et al., 2020). This offers the possibility to modulate *Ulva*'s growth rate and metabolic content via changes in those environmental factors. For instance, fatty acid, starch and protein content, as well as growth rates, can be modified by varying environmental parameters such as irradiance and nutrient levels (Toth et al., 2020; Traugott et al., 2020). Therefore, using land-based cultivation could allow to tailor *Ulva*'s biomass composition for specific market needs. A relatively simple and inexpensive environmental factor to modify in an on-shore aquaculture system is the salinity level of the raceway/tanks. Salinity has previously been shown to affect the growth rate of *Ulva spp* (Angell et al., 2015; Xiao et al., 2016), concomitant to variations in nutrient uptake, photosynthetic rate, pigments, proteins and amino acid contents (Angell et al., 2015; Bews et al., 2021; Lee & Liu, 1999; Xiao et al., 2016). Importantly, given the negative impact on growth rate, sub-optimal salinity levels are likely to strongly decrease biomass yield over extended growth periods. Therefore, an alternative is to modify salinity over short periods such as a week prior to harvest, to allow for desirable metabolic changes without compromising overall farm yields.

Environmental factors are not the sole factors affecting the quality of *Ulva* biomass. Genetic factors also significantly contribute to variability in growth rate and metabolic content of *Ulva*. Extensive natural variation exists between *Ulva* species as well as between individuals of the same species when grown in similar environmental conditions (Fort et al., 2019; Fort et al., 2020; Lawton et al., 2021). This indicates that both environmental and genetic factors should be considered when cultivating *Ulva* for industrial applications.

One of the important nutritional parameter of *Ulva* biomass for food applications is their fatty acids and lipid content. Generally, *Ulva* species

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contain low amounts of fatty acids and lipids, around 3 % of their dry biomass, (Monteiro et al., 2022; Ortiz et al., 2006), but it was recently shown that both environmental and genetic factors can modulate their accumulation (García-Poza et al., 2022; Monteiro et al., 2022). In addition, important types of fats such as mono- and poly-unsaturated fatty acids as well as bioactive lipids are present in *Ulva* biomass (Monteiro et al., 2022). Polyunsaturated fatty acids (PUFAs) such as omega-3 and omega-6 fatty acids are required for a healthy diet given their health-promoting benefits (Kapoor et al., 2021). Increasing the quantity of nutritionally relevant fats within farmed *Ulva* biomass could therefore represent an important factor for the valorisation of *Ulva* as a food source.

2. Materials & methods

To unravel potential impact(s) of salinity on the lipid profile of *Ulva* biomass, and to detect whether time of harvest could be an important factor to maximise fatty acid and lipid yields, we grew two *Ulva* species, *Ulva australis* and *Ulva lacinulata* at 35 ppt and then transferred the biomass to four different salinities (7.5, 15, 25 and 35 ppt) for one week. We then performed in-depth lipidome analysis on the samples.

2.1. Seaweed biomass and stock culture conditions

Three free-floating *Ulva* individuals were collected in green tide areas of Brittany, France, and sent to the laboratory in Ireland in cool-boxes containing seawater from the site of harvest. Upon arrival in the laboratory, the biomass was thoroughly washed with artificial seawater (Coral Pro Salts, Red Sea) at 35 ppt. ~500 mg biomass (fresh weight (FW)) of each individual was maintained in 500 mL beakers containing an airline for aeration/movement and filled with artificial seawater enriched with Guillard's F2 nutrient media. Light intensity was set at 180 μmol. $m^2.s^{-1}$ photons, photoperiod to 12 h of day, 12 h of night, and temperature at 15 °C. Seawater was changed every week and the individuals were kept in those conditions for >3 months prior to experiments. Excess biomass was discarded to maintain the individuals in constant growth and no sporulation events were detected. Species identification of those three individuals was performed using the Cleaved Amplified Polymorphic Sequences (CAPS) assay described in Fort et al. (2021) from DNA obtained using magnetic beads (Fort et al., 2018). Two individuals belonged to *Ulva australis* and the other one was identified as *Ulva lacinulata*.

2.2. Low-salinity treatment experimental setup

After more than three months acclimation in the growth chamber, the biomass was cut into discs of ~1 cm diameter and grown in the phenotyping platform described in Fort et al. (2019), with 16 discs per salinity and genotype, split into two aquarium tanks. Salinity level was set to 7.5, 15, 25 and 35 ppt by diluting the 35 ppt media with sterile distilled water. Salinity levels were confirmed using a handheld salinity meter. Apart from salinity, temperature, light intensity, nutrients and photoperiod were the same as for the stock cultures.

2.3. Growth rate and water content calculation

Growth rates were obtained from the phenotyping platform, with two measures for growth. First, automated cameras were used to capture images of the aquarium tanks at regular intervals (every 10 min during daylight), and measuring the area of the discs using ImageJ. Discs areas at the beginning of days (8 am) and end of days (8 pm) were used to calculate biomass expansion as Area Specific Growth Rate (Area SGR), representing the area increase per period t (day or night, where t − 1 represents the size of the discs at the previous end of day or end of night period), and calculated as follows:

$$\text{Area SGR}\,[\%\ \text{per period}^{-1}] = \frac{ln(area_t) - ln\left(area_{(t-1)}\right)}{t-(t-1)} \times 100$$

After seven days of growth in the tanks, discs were harvested at the end of day and at the end of night, quickly rinsed in distilled water, blotted on absorbent paper to remove surface moisture and immediately flash-frozen in liquid nitrogen in pre-weighted screw-caps tubes (Micronics). Then, the tubes + biomass was weighted (taking care to keep the biomass frozen throughout using liquid nitrogen) to obtain the fresh weight (FW) of the discs. The tubes containing the biomass were subsequently freeze-dried and weighted to obtain dry weight (DW) and water content. Relative Growth Rate (RGR), representing the dry biomass increase per unit of dry biomass per day, was calculated by measuring the dry weight (DW) of *Ulva* discs at the beginning (t_0) and at the end of the experiments (t, in days), using the following formula:

$$\text{RGR}\,[\text{mg.mg}^{-1}.\text{day}^{-1}] = \frac{ln(DryWeight_t) - ln\left(DryWeight_{t_0}\right)}{t-t_0}$$

Water content was calculated by dividing the dry weight by the fresh weight of the discs at the end of the experiment.

$$\text{Water content}\,[\%] = \frac{Dry\ Weight\,[mg]}{Fresh\ Weight\,[mg]}\ x\,100$$

2.4. Ash determination and organic matter content normalisation

For ash and organic matter, because more biomass is needed to obtain accurate results, discs from individual 2 of *Ulva australis* and the *Ulva lacinulata* individual were independently grown in 500 mL flasks at the experimental salinities (7.5, 15. 25 and 35 ppt) for one week. Then, discs were harvested as above and freeze-dried, with three biological replicates per salinity and genotype. The dry weight was recorded, the biomass ground to a fine powder using a ball mill (Qiagen TissueLyser II), and 15–20 mg of DW was combusted in a furnace at 550 °C for 24 h. The weight of the combusted samples was recorded and ash content was calculated as follows:

$$\text{Ash}\,[\%] = \frac{Combusted\ Weight\,[mg]}{Dry\ Weight\,[mg]}\ x\,100$$

To normalise the lipidome dataset by organic matter content, we used the following formula:

$$\text{Metabolite per unit of organic matter} = \frac{Metabolite\ per\ unit\ of\ DW}{(100 - Ash\ content\,[\%])/100}$$

2.5. Fatty acids and lipidome analysis

From the discs collected at the end of the experiment, 10 mg of freeze-dried biomass and three replicates (each containing 2–3 discs) were used for lipid and fatty acid analyses. Total lipid extraction, GC–MS and HILIC-LC-MS were performed as described in Monteiro et al. (2022), using the same methodology for the extraction, detection, quantification and identification of fatty acids and polar lipids. Hexane extracts of fatty acid methyl esters (FAME) obtained by alkaline transmethylation were used for fatty acids (FA) characterisation via GC–MS using C19:0 as internal standard (1.125 μg mL^{-1}, CAS number 1731–94-8, Merck, Darmstadt, Germany). GC was performed using an Agilent Technologies 6890 N Network Chromatograph (Santa Clara, CA, USA), with a DB-FFAP column of 30 m, 0.32 mm internal diameter and 0.25 μm film thickness (J&W Scientific, Folsom, CA, USA). In line with the GC, mass spectrometry was conducted using a Mass Spectrometer (Agilent 5973 Network Mass Selective Detector), with an electron impact mode set at 70 eV, a mass range of 50–550 *m/z*, and acquisition using 1 s cycles in full scan mode. Temperatures of 220 °C and 280 °C were used for the injector and detector, respectively. The oven temperature was setup as follows: 80 °C for 3 min followed by three linear increments, first to

160 °C (25 °C per minute), then to 210 °C (2 °C per minute), and finally to 225 °C (25 °C per minute). 225 °C was then maintained for 15 min. Helium (1.3 mL per minute) was used as carrier gas. FA identification was performed by comparing i) the retention times with that of the Supelco 37 Component FAME Mix (ref. 47885-U, Sigma-Aldrich, Darmstadt, Germany), and ii) the mass spectra with lipid databases. Samples were analysed in random order within two days.

Dichloromethane extracts were used for polar lipid analysis via HILIC-LC-MS & MS/MS using an internal standard for each classes of polar lipids, described in detail in Monteiro et al. (2022). The polar standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA), and contained: dimyristoyl phosphatidylcholine (dMPC), dimyristoyl phosphatidylethanolamine (dMPE), lysophosphatidylcholine (19:0 LPC), dipalmitoyl phosphatidylinositol (dPPI), dimyristoyl phosphatidylglycerol (dMPG), dimyristoyl phosphatidylserine (dMPS), tetramyristoyl cardiolipin (tMCL), sphingomyelin (17:0 SM (d18:1/17:0)), dimyristoyl phosphatidic acid (dMPA), and *N*-heptadecanoyl-D-erythro-sphingosine (Cer (d18:1/17:0)). The liquid chromatography was performed using an Ultimate 3000 Dionex HPLC (Thermo Fisher Scientific, Bremen, Germany), coupled to Mass Spectrometry with a Q-Exactive hybrid quadrupole mass spectrometer (Thermo Fisher, Scientific, Bremen, Germany). MS data acquisition was performed in positive and negative modes (3 kV and −2.7 kV, respectively), 70,000 resolution, AGC target of $1e^6$, capillary temperature of 350 °C, sheath gas flow of 20U. MS/MS analysis used a different resolution of 17,500 and AGC target of $1e^5$. Peak integration was performed using MZmine and assigned using an in-house database. Ions within <5 ppm of the lipid species exact mass were assigned a putative match.

The Fatty Acids dataset contained data for both *Ulva australis* and the single *Ulva lacinulata* individuals, while the polar lipid data only contained the data for one of the two *Ulva australis* and the *Ulva lacinulata* individuals. We only used one of the two *Ulva australis* samples given their identical fatty acid profiles. Importantly, while the FA analysis is quantitative and expressed in mg.g DW^{-1}, the polar lipid is semi-quantitative and is expressed in Arbitrary Units.mg DW^{-1}. Internal standards for each lipid class were used for quantification (dMPC − 0.04 μg, dMPE − 0.04 μg, LPC − 0.04 μg, dPPI − 0.16 μg, dMPG − 0.024 μg, dMPS − 0.08 μg, tMCL − 0.04 μg, NPSM(17:0/d18:1) – 0.04 μg, dMPA – 0.16 μg, Cer(d35:1) – 0.04 μg, all added to 15 μL of lipid extracts), which only allows to compare the quantities within the same class of lipids, as each lipid class is compared with its representative standard. Therefore, the methodology allows to compare the quantities within lipid classes, for example PI(40:7) versus PG(36:5), two phospholipids, but not between a phospholipid and a sulfolipid.

2.6. Statistical analysis

All statistical analyses were performed using R. To determine the impact of species, salinity and time of harvest on FA/lipid profiles, we used a Permutational Multivariate Analysis of Variance (PERMANOVA) on a scaled matrix containing all FA or lipid data using the "vegan" package (adonis2 function), with 9999 permutations and pairwise distances calculated via Euclidean dissimilarity. ANOVAs and Tukey's tests for post-hoc pairwise comparisons were preformed using the base R packages. Correction for multiple testing via False-Discovery Rate (FDR) calculation was performed using the p.adjust function of R base. Alpha = 0.05 throughout. Clustered heatmaps were generated from the scaled datasets using the "pheatmap" package, and all other graphs using ggplot2.

3. Results

3.1. Short-term low salinity treatments impact Ulva growth

To investigate the effect of a short-term low-salinity treatment on *Ulva* growth, we grew three foliose *Ulva* individuals (two *Ulva australis* and one *Ulva lacinulata*), at four different salinities for seven days in the phenotyping platform described in Fort et al. (2019) and Fort et al. (2020). All three individuals showed a biomass accumulation (Relative Growth Rate) of ~0.28 mg.mg DW^{-1}.day^{-1} at 35 ppt (Fig. 1**A**). At 25 ppt, RGR was similar to 35 ppt except for one of the two *Ulva australis* samples which showed a small decrease in RGR to ~0.25 mg.mg DW^{-1}.day^{-1} (One-way ANOVA for each genotype with Tukey's test, $p < 0.05$). At 15 ppt, all genotypes had slower RGR than at 35 ppt, which further declined at 7.5 ppt with RGRs of ~0.17 mg.mg DW^{-1}.day^{-1}.

Tissue expansion (Area Specific Growth Rate) in the two *Ulva australis* individuals showed a similar pattern as that of biomass accumulation, with a decrease in Area SGR as salinity decreases (Fig. 1**B**). Area SGR was found to be ~30 % per day at 35 ppt in both samples, and was halved (~15 % per day) at 7.5 ppt (One-way ANOVA for each genotype with Tukey's test, $p < 0.05$). However, the *Ulva lacinulata* individual did not show any significant difference in tissue expansion ($p = 0.133$) across all four salinities, with an area SGR of ~18 % per 24-hour period regardless of the experimental conditions. This data indicates that the *Ulva lacinulata* individual used in this study favours biomass accumulation over tissue expansion and that in this genotype tissue expansion remains unaffected by salinity. As found previously (Fort et al., 2019), tissue accumulation was higher during the night than during the day in all samples and conditions and day/night expansion ratio was similar in all samples and salinities, apart from 15 ppt in the first *Ulva australis* individual (Fig. S1).

The water content of the *Ulva* discs at the end of the seven-day period (Fig. 1**C**) was lower at 35 ppt than in the other salinities in all three genotypes (One-way ANOVA for each genotype with Tukey's test, $p < 0.05$). Water content was similar between 7.5, 15 and 25 ppt in the first *Ulva australis* individual and *Ulva lacinulata* ($p > 0.05$), but highest at 7.5 ppt in the second *Ulva australis* sample. This data indicates that lowering the salinity below 35 ppt increases the water content of *Ulva* biomass.

Altogether, the growth experiments using short-term (seven days) low salinity treatments show that biomass accumulation is strongly negatively affected by salinity, particularly when the salinity drops below 15 ppt.

3.2. Fatty acid profile of Ulva biomass exposed to decreasing salinity levels

At the end of the seven-day growth period, the biomass collected at the end of day and end of night was extracted to study whether short-term low-salinity treatments, harvest time and species could impact fatty acids (FAs) profiles of *Ulva* biomass.

Twenty-five fatty acids were extracted and quantified from the samples (Table S1). The most abundant FA across the dataset was palmitic acid (16:0, average of 2.92 μg.mg DW^{-1}), followed by stearic acid (18:0, average of 1.81 μg.mg DW^{-1}). The lowest abundance FA was pentadecylic acid (15:0), with 0.0091 μg.mg DW^{-1} on average. All FAs were present in all samples at all salinities.

In *Ulva lacinulata*, total FA quantity remained unchanged by either salinity or the time of harvest (end of day or end of night), Fig. 2**A**. Indeed, the sum of FAs remained stable between 6.3 and 7.7 μg.mg DW^{-1} (Two-Way ANOVA, $p > 0.05$). However, the two *Ulva australis* individuals showed significant differences for both salinity and timepoint. For timepoint, end of day samples grown at 15 ppt showed higher total FAs than end of night ones (13.2 versus 9.2 μg.mg DW^{-1} and 14 versus 7.5 μg.mg DW^{-1} for *Ulva australis* individuals 1 and 2, respectively, both $p < 0.05$ with Two-Way ANOVA per genotype and Tukey's test). This indicates that at 15 ppt, diurnal differences exist in terms of FA metabolism. Both *Ulva australis* individuals responded similarly to the treatments, with no significant difference (Three-Way ANOVA, $p = 0.7$). For this reason, we merged the FA data of both *Ulva australis* individuals for the rest of this analysis. To highlight species and salinity impact on total FA accumulation, we calculated the sum of all FAs for both species at the four different salinities (Fig. 2**B**). In *Ulva australis*, low

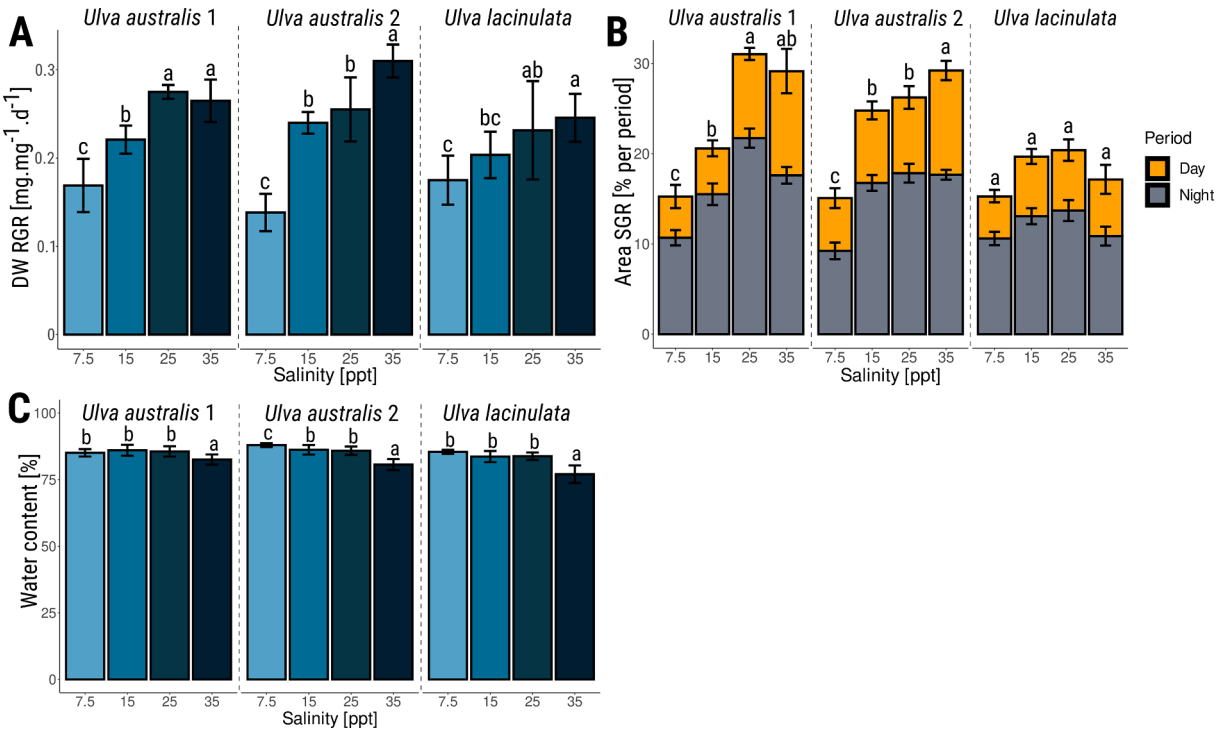

Fig. 1. *Ulva* growth is affected by salinity. A) Relative growth rate per unit of dry weight per day at different salinities. Data represents the mean ± s.d, letters indicate significance groups within each genotype, with a One-Way ANOVA and Tukey's test. B) Area Specific Growth Rate per period. Data represents the mean ± s.e.m, letters indicate significance groups within each genotype based on day + night growth (24 h period Area SGR), with a One-Way ANOVA and Tukey's test. C) Water content of *Ulva* biomass. Data represents the mean ± s.d, letters indicate significance groups within each genotype, with a One-Way ANOVA and Tukey's test.

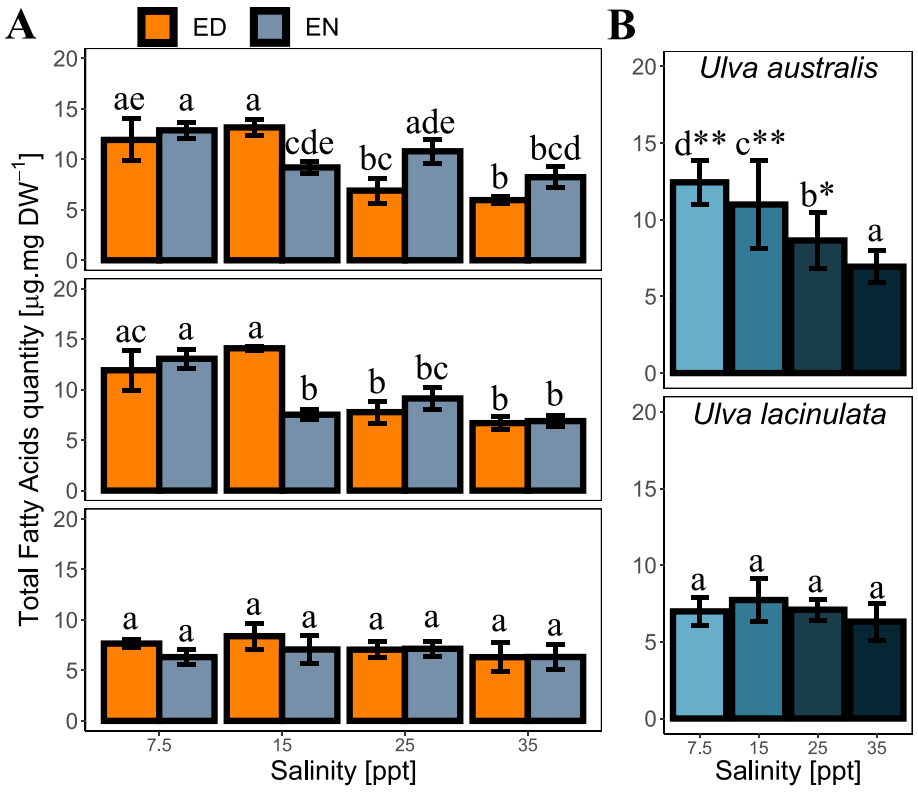

Fig. 2. Total Fatty Acid amount in *Ulva* biomass grown at different salinities. A) Sum of all FAs found in *Ulva* biomass, depending on time of harvest (ED = End of Day, EN = End of Night). Data represents mean ± s.d, $n = 3$. Letters represent significance groups within each genotype with Two-Way ANOVA and Tukey's test. Top: *Ulva australis* 1, Middle: *Ulva australis* 2, Bottom: *Ulva lacinulata*. B) Same data but with both timepoints and species merged. Letters represent significance groups within each species with Two-Way ANOVA and Tukey's test, asterisks indicate whether the difference between species is significant at each salinity (Two-Way ANOVA and Tukey's test, **: $p < 0.001$, *: $p < 0.05$).

salinity treatment significantly impacted FA accumulation, with higher total FA as salinity decreases (Two-Way ANOVA per species and Tukey's test, $p < 0.05$). No significant differences were observed in *Ulva lacinulata*. Finally, total FA was similar for both species at 35 ppt, but the higher FA accumulation at lower salinities in *Ulva australis* led to significant species differences at 7.5, 15 and 25 ppt (Two-Way ANOVA, $p < 0.05$, Fig. 2**B**).

To further characterize the impact of low salinity treatment on FA accumulation, we used a multifactorial Permutational Multivariate Analysis of Variance (PERMANOVA) to compare the impact of species, salinity and timepoint on the fatty acids profile of *Ulva*. Table 1 describes the PERMANOVA results. The species, salinity and timepoint variables were all found to significantly impact the fatty acid profile of the dataset (with R^2 values of 0.22, 0.27 and 0.03, respectively, all $p < 0.05$). The interaction between species and salinity was also significant ($p < 0.05$), indicating a differential response to salinity from the two species. There was no interaction between species and timepoint ($p = 0.17$), but the interaction between salinity and timepoint was significant ($p < 0.05$), which showed that under certain salinities, the time of harvest of the biomass impacts its FA profile. Finally, the three-way interaction (species, salinity and timepoint) was also significant.

Clustered heatmap (Fig. 3**A**) and Multidimensional scaling plot (MDS) (Fig. 3**B**) of the FA dataset showed that low salinity exposure strongly impacts the FA profile of *Ulva australis*, with 22/25 FAs significantly affected by the salinity variable at both timepoints (Fig. S2, One-Way ANOVA, FDR < 0.05). All unsaturated FA were found in higher amount at low salinity (7.5 ppt), as well as saturated FAs (SFAs) with chain length of C15, C17 and C22. C18 and C20 SFAs accumulated in similar quantities across treatments, and C14 and C16 SFAs were impacted by salinity in only one of the two timepoints (Table S2). Interestingly, the timepoint and salinity*timepoint interaction described above was confirmed to be due to a strong accumulation of FAs in end of day at 15 ppt, which clusters with 7.5 ppt FA profiles (Fig. 2**A** and Fig. 3**A**). In *Ulva lacinulata*, no FAs were found differentially accumulated in the end of night samples, and only 6/25 were found significantly impacted by salinity in the end of day samples (One-Way ANOVA, FDR < 0.05, Table S2).

To further characterise the impact of a low salinity treatment, we compared 7.5 ppt with the euhaline environment (35 ppt), and calculated the mean $\log_2$ fold change between the two salinities (combining End of Day and End of Night, given that timepoint has no impact on accumulation at those salinities, PERMANOVA with Species, Salinity and Timepoint as independent variables, $p > 0.05$, Fig. 3**C,** Table S3). Comparing 7.5 vs 35 ppt confirmed that all monounsaturated and polyunsaturated fatty acids are over-accumulated in *Ulva australis* at 7.5 ppt, together with 4/7 saturated fatty acids (Two-Way ANOVA, FDR < 0.05), median $\log_2$ fold change of all FAs at 7.5 versus 35 ppt = 1.46. In contrast, in *Ulva lacinulata*, only six fatty acids were significantly impacted by low salinity (16:1n-7, 18:1n-7, 18:1n-9, 20:3n-6, 15:0 and 22:0), with a median $\log_2$ fold change for all FAs of 0.33.

Table 1
PERMANOVA results highlighting the variables that impact the fatty acid profile of *Ulva*. Species = *Ulva lacinulata* and *Ulva australis*, Salinity = 7.5, 15, 25 & 35 ppt, Timepoint = End of Day or End of Night. Df = Degrees of freedom, R^2 = R-squared value which represents the percentage of variance explained by the variables, F = F-ratio.

Variable	Df	Sum of Squares	R^2	F	p-value
Species	1	390.18	0.22	57.41	**<0.001**
Salinity	3	472.39	0.27	23.17	**<0.001**
Timepoint	1	48.92	0.03	7.20	**<0.001**
Species*Salinity	3	217.57	0.12	10.67	**<0.001**
Species*Timepoint	1	11.06	0.01	1.63	0.1701
Salinity*Timepoint	3	188.17	0.11	9.23	**<0.001**
Species*Salinity*Timepoint	3	66.09	0.04	3.24	**0.0056**
Residuals	56	380.63	0.21		

The data indicates that short term low salinity treatment results in higher accumulation of virtually all FAs in *Ulva australis*, and only has a marginal impact in *Ulva lacinulata*. Such results will affect the nutritional properties of *Ulva australis* biomass exposed for a short term to low salinity, particularly 7.5 ppt. To estimate more precisely those, we calculated saturated/unsaturated FA ratios, the amount of Omega 3 (ω-3) FA, as well as the Omega 6/Omega 3 (ω-6 / ω-3) ratio of the FA dataset, with both timepoints merged (Fig. 3**D**). A figure including both timepoints as separate variables is available in Fig. S3. The saturated/unsaturated FA ratio decreased as salinity decreased, from 1.7 ± 0.23 at 35 ppt to 0.74 ± 0.12 at 7.5 ppt (Two-Way ANOVA with Tukey's test on salinity, $p < 0.05$) in *Ulva australis*. In *Ulva lacinulata*, the difference was also significant, albeit to a lesser extent (decreased from 3.03 ± 0.7 at 35 ppt to 2.21 ± 0.6 at 7.5 ppt, Two-Way ANOVA with Tukey's test on salinity, $p < 0.05$). The ratio of saturated over unsaturated FAs was consistently higher in *Ulva lacinulata* compared with *Ulva australis*, for each salinities. ω-3 FAs also varied significantly with salinity, with an increase in ω-3 s as salinity decreases, from an average of 1.34 ± 0.29 mg.g DW^{-1} at 35 ppt to 4.56 ± 0.85 mg.g DW^{-1} at 7.5 ppt in *Ulva australis*. In *Ulva lacinulata*, ω-3 content was overall lower than in *Ulva australis*, and was only significantly affected by salinity at 15 ppt versus 35 ppt. Finally, Omega 6/Omega 3 (ω-6/ ω-3) ratio decreased slightly in *Ulva australis* grown in low salinity, from 0.3 ± 0.02 at 35 ppt to 0.22 ± 0.1 at 7.5 ppt, and was lower at 25 versus 7.5 and 35 ppt in *Ulva lacinulata*. The difference between species was significant across all salinities.

3.3. Lipidome analysis of Ulva biomass exposed to low salinity

We performed a similar analysis on the polar lipidome profile from the same samples by LC-MS, for the analysis of lipid extracts obtained using the biomass from *Ulva lacinulata* and the second *Ulva australis* individual. We identified 268 polar lipids belonging to four different lipid classes within the dataset (69 Galactolipids, 94 Phospholipids, 30 Sulfolipids and 75 Betaine lipids, Table S4). Only one lipid was absent in one of the two species, the SQMG(16:4), and all other lipids were represented across species in at least one experimental condition.

The PERMANOVA (Table 2), clustered heatmap and MDS (Fig. 4**A** and Fig. 4**B**) analyses of the polar lipids dataset showed similar results as the fatty acid one. Species, salinity and timepoint were all significant factors contributing to the variance in the dataset, and interactions between algae species and salinity as well as salinity and timepoint were also significant. Notably, species was the most important factor explaining the variation in the dataset, with a R^2 of 0.27, followed by salinity ($R^2 = 0.17$).

We found that salinity strongly affects the lipidome profile of *Ulva australis,* and to a lesser extent that of *Ulva lacinulata* (Table S2, Fig. S4). At both timepoints, ~90 % of betaine lipids and ~64 % of galactolipids were affected by salinity in *Ulva australis*, while 24 % and 52 % of phospholipids were differentially accumulated at the end of day and end of night, respectively (One-Way ANOVA, FDR < 0.05). Finally, we found no difference in sulfolipids content in *Ulva australis*. In *Ulva lacinulata*, ~15 % of betaine lipids, ~40 % of galactolipids and ~13 % of phospholipids were impacted by salinity at both timepoints (One-Way ANOVA, FDR < 0.05), and 4/30 sulfolipids were found differentially accumulated at the end of night. The difference between end of day and end of night samples was found to be largely due to the higher accumulation of some lipids at the end of day at 15 ppt, similar to the results of the fatty acids dataset.

As for FAs, we then focused on low salinity (7.5 ppt) versus normal salinity (35 ppt) to calculate a $\log_2$ fold change (end of day +end of night, as timepoint has no impact on accumulation at those salinities, PERMANOVA with species, salinity and timepoint as independent variables, $p > 0.05$). Under this analysis, we found 59 % of all lipids to be differentially accumulated in *Ulva australis* at 7.5 ppt. 94.7 % of betaine lipids (71/75) showed different amounts between 7.5 and 35 ppt (Two-

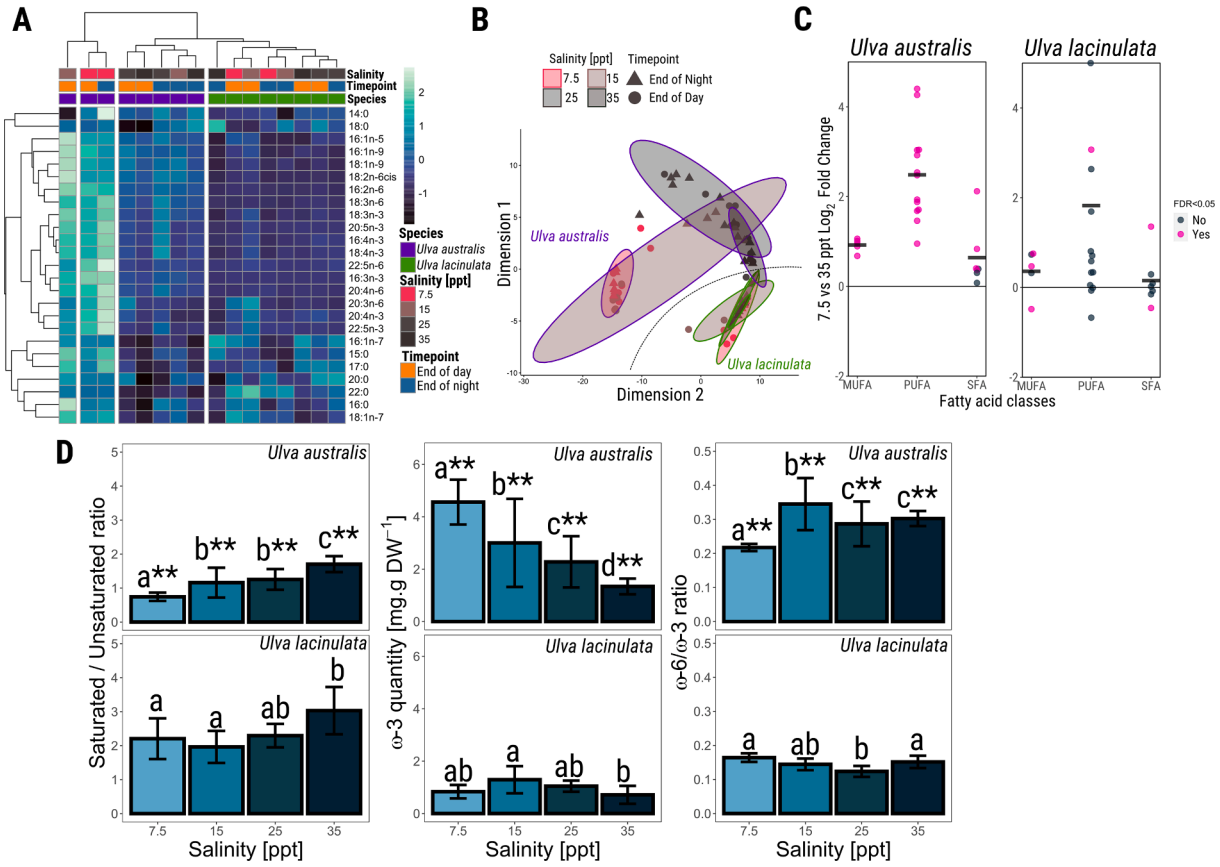

Fig. 3. Fatty Acid profile of *Ulva* biomass exposed to different salinities. A) Clustered heatmap based on salinity, timepoint and species variables. B) Multi-dimensional Scaling Plot (MDS) of the fatty acid profile of all samples. C) Log_2 fold change of FA quantity at low salinity (7.5 ppt) versus euhaline environment (35 ppt). Each dot represents a fatty acid, the colour of the dots indicates whether the difference is significant (Two-Way ANOVA, FDR < 0.05). Horizontal bars represent median values for each FA class. D) Left: Saturated/Unsaturated FA ratios, Middle: Omega 3 quantity, Right: Omega 6/Omega 3 ratio. Top: *Ulva australis*, Bottom: *Ulva lacinulata*. Data represent mean ± s.d, $n = 6$, with the timepoints merged. Letters indicate significant groups within a species as per a Two-Way ANOVA with Tukey's test. Asterisks represent significant differences between species at each salinities (Two-Way ANOVA, **: p < 0.001).

Table 2
PERMANOVA results highlighting the variables that impact the lipid profile of *Ulva*. Species = *Ulva lacinulata* and *Ulva australis*, Salinity = 7.5, 15, 25 & 35 ppt, Timepoint = End of Day or End of Night. Df = Degrees of freedom, R^2 = R-squared value which represents the percentage of variance explained by the variables, F = pseudo F-statistic.

Variable	Df	Sum of Squares	R^2	F	p-value
Species	1	3428.17	0.27	32.46	**<0.001**
Salinity	3	2122.27	0.17	6.70	**<0.001**
Timepoint	1	288.56	0.02	2.73	**0.02**
Species*Salinity	3	1964.10	0.16	6.20	**<0.001**
Species*Timepoint	1	155.43	0.01	1.47	0.17
Salinity*Timepoint	3	768.90	0.06	2.43	**0.003**
Species*Salinity*Timepoint	3	489.33	0.04	1.54	0.08
Residuals	32	3379.24	0.27		

Way ANOVA, FDR < 0.05, Fig. 4**C,** Table S3). Of those, 67 were present in higher quantity at 7.5 ppt. The galactolipids results showed similar trend to that of betaine lipids, with 82.6 % (57/69) significantly affected by low salinity, 51 of which present in higher quantity at 7.5 ppt. Phospholipids on the other hand were less impacted, with 32 % (30/94) significantly different, and 28 of those more highly accumulated at 7.5 ppt. Finally, sulfolipids did not show any significant differences.

In contrast, *Ulva lacinulata*'s response to low salinity was markedly different than that of *Ulva australis*. Only 26.5 % of all lipids were found differentially accumulated in 7.5 vs 35 ppt: 7/75 (9 %) of betaine lipids, 32/69 (46 %) of galactolipids, 32/94 (34 %) of phospholipids and no sulfolipids showed significant differences. Importantly, while most of the differentially accumulated phospholipids were present in higher quantity at 7.5 ppt in *Ulva australis*, the pattern was opposite in *Ulva lacinulata*. Altogether, the lipidome analysis revealed a marginal impact of low-salinity treatment in *Ulva lacinulata* (median log_2 fold change of all lipids at 7.5 ppt vs 35 ppt = -0.14). In *Ulva australis* however, we found a general over-accumulation of most lipid classes outside of sulfolipids (Log_2 fold change = 1.19), highlighting profound differences between species, both in terms of quantitative lipid composition and in response to the salinity treatment.

3.4. Low salinity leads to a decrease in mineral content, but FA and lipid changes are maintained when only organic matter content is considered

The analyses described above were normalised based on units of dry weight (DW), and we found a general increase in FAs/lipid content in *Ulva australis* biomass exposed to low salinities for a seven-day period. These findings could be due to two physiological processes: 1) FAs/lipids specifically over-accumulate at low salinity or 2) the mineral content of the biomass decreases at low salinity, leading to a proportional increase in organic content per unit of dry weight. Option 2 would also lead to a higher amount of FAs/lipids per unit of dry weight, but would not represent a physiological response specific to lipid metabolism. To estimate the contributions of both options to the results described above, we measured the proportion of ash (and therefore of organic matter content) per unit of dry weight in the same *Ulva lacinulata* and *Ulva australis* individuals following the different salinity treatments. We

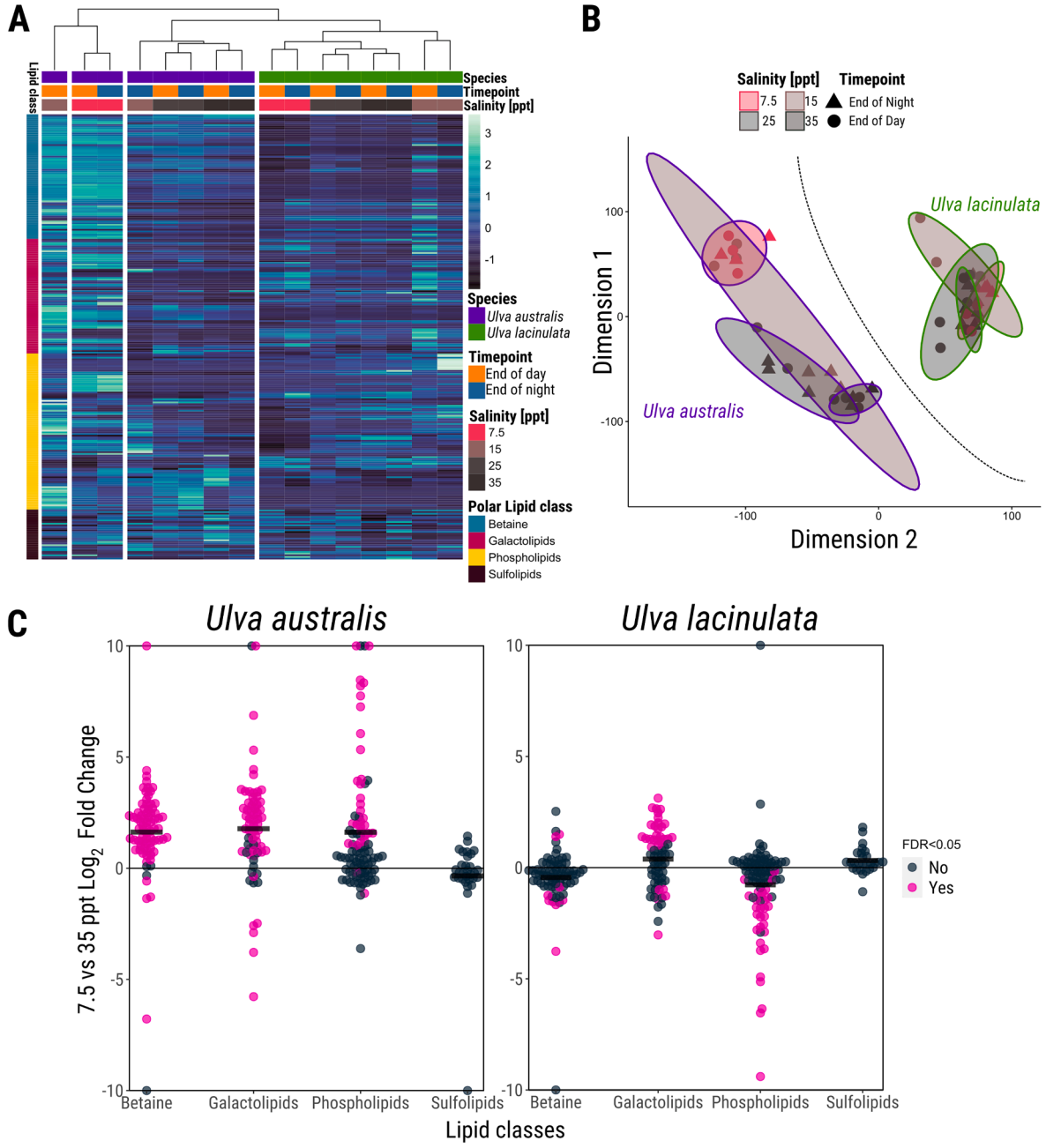

Fig. 4. Polar lipid profiles of *Ulva* biomass exposed to different salinities. A) Clustered heatmap based on salinity, timepoint and species variables. B) Multidimensional Scaling Plot (MDS) of the lipid profile of all samples. C) Log_2 fold change of lipid quantities at low salinity (7.5 ppt) versus euhaline environment (35 ppt). Each dot represents a lipid, the colour of the dots indicates whether the difference is significant (Two-Way ANOVA, FDR < 0.05). Horizontal bars represent median values for each lipid class.

found that in both species, ash content tends to decrease as salinity decreases, from 27.7 to 22.2 % and from 29.6 to 13.8 % in *Ulva australis* and *Ulva lacinulata*, respectively (Fig. S5**A**). The difference was however not significant between 7.5 and 35 ppt in *UIva australis* (One-Way ANOVA with Tukey's test, $p = 0.18$). Nonetheless, these results indicate that a lower amount of mineral (and therefore higher amount of organic matter) per unit of dry weight could explain some of the higher accumulation of FAs/lipids observed at low salinity. We then compared the FAs/lipids significantly impacted by salinity when normalised by dry weight or by organic matter content (Fig. S5**B**). In *Ulva australis*, 96 % of FAs/lipids showed similar statistical results under both normalisations, while in *Ulva lacinulata* the overlap ranged from 92 % for end of day to 78 % for end of night samples. Therefore, we conclude that the differences in FA/lipid content observed here are not due to a general increase in organic matter per unit of dry weight, but to a specific physiological response leading to a higher accumulation of those metabolites at low salinity.

4. Discussion

4.1. Seven days at low salinity negatively affects biomass accumulation in Ulva

In this study, we explored whether short-term low salinity treatments

could impact the growth and nutritional properties of two species of *Ulva*, specifically their fatty acid and polar lipid profiles. First, regarding the impact of low salinity on growth, we found that low salinity (7.5 ppt) leads to a decrease of almost 50 % of growth rates when compared with euhaline conditions for both *Ulva* species. Deleterious impact of low-salinity on growth was previously documented, particularly when salinity falls below 15 ppt. Indeed, Angell et al. (2015) and Lu et al. (2006) showed a significant decrease in growth rate below 15–20 ppt in *Ulva ohnoi, Ulva australis* and *Ulva lactuca*. Importantly, the response to the low salinity treatment was similar between the two species when biomass accumulation is considered, likely indicating a similar inhibitory mechanism for euhaline species. The slower growth rates observed here at those salinities likely have metabolic implications and could to lead to variations in the nutritional content of *Ulva* biomass exposed to low salinity. The precise links between low salinity and its impact on growth remains to be investigated in detail, but it was shown that in *Ulva prolifera*, photosynthetic rates decreased at low salinity, together with increased signs of oxidative stress (Luo & Liu, 2011; Xiao et al., 2016). In addition, *Ulva australis* exposed to low salinity for 24 h was shown to accumulate higher amount of photosynthetic pigments (Kakinuma et al., 2006). Therefore, exposure to low salinity impacts *Ulva* growth and likely its metabolism, which could be leveraged to modify metabolite content in selected species and strains for tailored industrial applications. However, using low salinity (7.5 ppt) to modify *Ulva's* biomass composition would be best used shortly before harvest, to mitigate the loss of biomass yield associated with the environmental change.

We showed in addition that organic matter content tends to increase as salinity decreases in both species. While high mineral content can be useful for some applications such as for daily diet intake, it concomitantly dilutes the valuable organic matter contained within seaweed biomass (such as proteins, fats, pigments, fibres, carbohydrates etc.). Therefore, decreasing mineral content via short-term low salinity treatment could lead to higher nutritional benefits of *Ulva* consumption.

4.2. Profound genotype and Genotype X Environment interactions in Ulva spp's response to low salinity

Foliose *Ulva* species show relatively subtle morphological differences (Malta et al., 1999) despite large genetic differences (Fort et al., 2021). This indicates that morphologically similar sea lettuce individuals have the potential to display strong variation in their growth rate as well as metabolic compositions, as exemplified in previous studies (Fort et al., 2019; Fort et al., 2020; Lawton et al., 2021). Such large genetic variations could also lead to significant Genotype × Environment (GxE) interactions when subjected to different environmental conditions. Here, we found that the two *Ulva* species, when grown under standard euhaline conditions, display strong differences both in terms of growth patterns (tissue expansion particularly), but also in their fatty acids and lipid profiles. Both species can be readily differentiated by clustering analyses at 35 ppt (Monteiro et al., 2022) (Fig. 3 and Fig. 4). This genotype effect becomes more pronounced when exposed to low salinities, with little differences between salinities in *Ulva lacinulata,* in stark contrast to the profound impact of salinity observed in *Ulva australis*. Those results demonstrate a strong GxE interaction in the response to salinity of *Ulva spp*. The underlying mechanisms of such interactions need to be further investigated to precisely characterise the cellular, molecular and metabolic changes triggered by low salinity treatments. Nonetheless, the data indicates that carefully selecting species for mass cultivation is of paramount importance for the valorisation of *Ulva* as a viable commercial food source.

4.3. Increase in fatty acids and lipid composition in Ulva via low salinity treatments

Following the salinity trials, we investigated the fatty acids and lipid content of the *Ulva* biomass produced. We focused on fatty acids and lipids as they represent an important target for improvement in the sea lettuce. Indeed, *Ulva* is naturally low in both types of fats but its lipid pool contains relatively high amount of unsaturated fatty acids, particularly ω-3 PUFAs (Monteiro et al., 2022). Inclusion of high ω-3 FAs as well as low ω-6/ω-3 ratios in diet have been linked with many health promoting effects, such as anti-inflammation activity, reduced cardiovascular diseases or improved neurological development (Shahidi & Ambigaipalan, 2018). However, not only is the western diet is deficient in ω-3 with poor ω-3/ω-6 ratios (Simopoulos, 2011), but ω-3 supply from fish is viewed as unsustainable due to over-fishing as well as concerns regarding fish farming intensification in coastal areas (Adarme-Vega et al., 2014; Belton et al., 2020). Therefore, finding alternative sources of ω-3 PUFAs is of timely importance.

First, we collected the biomass at two timepoints, at the End of Day and the End of Night, to investigate whether some fats could display diurnal patterns of accumulation/consumption akin to that of carbohydrates (Fort et al., 2019). For example, starch is strongly accumulated during the day and consumed during the night, to provide (in part), the carbon necessary for growth and maintenance at night. This is of importance not only for better understanding the primary metabolism of *Ulva spp*, but also to dictate the most optimal harvest time of the biomass. For instance, if the main purpose of growing the sea lettuce is to produce carbohydrates, then the optimal harvest time is be the end of the day, where carbon reserves are at their highest. In this dataset, we found no evidence of large day/night accumulation differences of FAs and lipids outside of 15 ppt in *Ulva australis*. 15 ppt appears as an outlier in the analyses, with 15 ppt end of day samples resembling that of 7.5 ppt ones. This indicate that 15 ppt likely represents a salinity at which some diurnal turnover of FAs and lipids exists, perhaps to sustain the relatively high growth rates maintained at this salinity (Fig. 1). Altogether, our dataset shows that for maximising the FA/lipid content of *Ulva*, the time of harvest doesn't appear important if the biomass is grown at low (7.5 ppt) or euhaline (35 ppt) salinities.

We found a strong accumulation of most types of fatty acids in *Ulva australis* when grown at low salinity for seven days. Specifically, total ω-3 FAs increased by a factor of 3 when grown at 7.5 ppt versus 35 ppt (up to 4.5 mg.g DW^{-1}), with EPA (20:5n-3) increasing 5.5 fold. Linoleic Acid (18:2n-6) and α-linoleic acid (18:3n-3), two essential fatty acids for human diet also increased significantly at 7.5 ppt. Therefore, we show here that by carefully selecting species and subjecting them to a low salinity treatment it is possible to significantly stimulate PUFA and ω-3 content in *Ulva*, rendering *Ulva* biomass an attractive alternative source of valuable fatty acids.

Next, we studied the polar lipidome of the same samples to investigate if a similar effect was present. We found a higher accumulation of polar lipids at 7.5 ppt in *Ulva australis* versus 35 ppt (overall $\log_2$ fold change = 1.19, representing a ~2.3 fold increase), compared with little overall differences in *Ulva lacinulata*. Interestingly, the salinity effect in *Ulva australis* was only apparent in some classes of polar lipids, whereby galactolipids, betaine lipids and some phospholipids were significantly impacted. In contrast, none of the 30 sulfolipids found in the dataset displayed any differences. Such tight regulation of sulfolipids amount in *Ulva* is fundamentally interesting. Sulfolipids, particularly Sulfoquinovosyldiacylglycerols (SQDGs) are found in the membranes of thylakoids. They represent an essential component in Photosystem II assembly, together with Mono- and Di-Galactosyldiacylglycerols (MGDG and DGDG), as well as phosphatidylglycerol (PG) (Jones, 2007). SQDGs and PG are the only two anionic lipids present in chloroplasts, and sulfolipids can replace phospholipids under phosphate limitation in plants and marine organisms (Benning et al., 1993; Van Mooy et al., 2009). This system is thought to allow to maintain anionic lipid homeostasis in chloroplasts, ensuring PSII integrity even under nutrient limitation (Kobayashi, 2016). In addition to its role in chloroplasts, SQDG was shown to function as a source of internal sulfur (S) in *Chlamydomonas* under S starvation (Sugimoto et al., 2010). Strikingly, none of the PGs and SQDGs in our dataset displayed any changes when exposed to low

salinity, even in a salinity environment where growth is stalled. These results indicate that chloroplast integrity likely remains stable in the range of salinities investigated here, and that nutrient uptake is sufficient, even at 7.5 ppt. Therefore, lower salinity is unlikely to modify sulfolipid and PG content in *Ulva*, and modulating those lipids will likely require different growth conditions where nutrients, particularly phosphorus or sulfur, are limiting.

Finally, polar lipids from seaweeds and microalage have been found to possess a range of potential bioactivities, from anti-inflammatory and anti-oxidant to anti-proliferative activities (Banskota et al., 2013; da Costa et al., 2021). For example, MGDGs and DGDGs, as well as betaine lipids such as Diacylglyceryltrimethylhomoserines (DGTS) isolated from marine microalgae were found to possess strong anti-inflamatory activities (Banskota et al., 2013). In addition, glycolipids such as DGDGs and SQDGs isolated from marine sources were associated with anti-proliferation activity against colon cancer cells (Hossain et al., 2005). In *Ulva australis* exposed to low salinity, we found that virtually all of those lipid species were accumulating in higher quantities (Fig. 4**C**), indicating that the biomass produced could become a valuable source of bioactive lipids.

5. Conclusions

In this study, we report the use of a seven-day low salinity treatment to modify the fatty acid and lipid content of two species of *Ulva*. We found that in *Ulva australis*, and not in *Ulva lacinulata*, such treatment has a profound impact on the profile of the biomass. Most notably, higher amounts of omega-3 PUFAs as well as possible bioactive lipids were found following the low salinity treatment in *Ulva australis*. This indicates that *Ulva* biomass can be readily valorised as a food source by selecting specific species and using short-term modifications of their environmental conditions prior to harvest. Such methodology could become an important factor to increase the marketability of seaweeds as healthy nutritional product. Finally, while we focused on fatty acids and polar lipid accumulation in this study, it is likely that other valuable compounds such as total fat content, proteins, carbohydrates or fibres vary in response to a low salinity treatment. Those would need to be investigated in more detail in future experiments to explore the complete nutritional qualities of *Ulva* biomass exposed to low salinity, and their benefits as food source.

CRediT authorship contribution statement

Antoine Fort: Conceptualization, Formal analysis, Writing – original draft. **João P. Monteiro:** Methodology, Formal analysis, Writing – review & editing. **Clara Simon:** Formal analysis. **M. Rosário Domingues:** Supervision, Funding acquisition, Writing – review & editing. **Ronan Sulpice:** Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

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

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137865.

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