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Reproductive sterility increases the capacity to exploit the green seaweed *Ulva rigida* for commercial applications

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ABSTRACT

Periodic biomass losses caused by reproduction have greatly limited the expansion of the land-based cultivation of the seaweed *Ulva*. In an attempt to resolve this, we obtained a sterile mutant (SM) of *U. rigida* by mutating a wild type (WT) strain using ultraviolet radiation (UVC). The SM grew five times faster than the WT with 40.0% and 30.9% higher nitrate and phosphate uptake rates. Crucially, the SM remained in the vegetative state throughout the 27-day trial, unlike the WT. The lipid content was more than double that of the WT with more monounsaturated, polyunsaturated, omega-3 and omega-6 fatty acids but with a 26.3% lower protein content. The swelling, water holding, and oil holding capacities were all lower than the WT. The rapidly growing SM showed distinct promise for application to carbon capture and wastewater bioremediation and for more conventional aquaculture practices. Despite some modified nutritional and functional properties, the SM still retained a desirable nutritional profile.

1. Introduction

The green seaweed *Ulva* is an increasingly important food [45], feed [1,13], and biofuel feedstock [6], as well playing a role in the delivery of crucial wastewater and CO_2 remediation services [2,11]. Interest in *Ulva* as a source of bioproducts and bioprocesses has driven concomitant efforts to optimize *Ulva* cultivation [5,7,27]. *Ulva* species should, in theory, be ideal cultivation candidates given their cosmopolitan distribution [31], very high growth rates [6], and broad environmental tolerances [35]. However, previous studies (for example, [3,8,14,46]) have experienced difficulty in maintaining *Ulva* species in the vegetative state. The formation and release of reproductive cells effectively terminates *Ulva* growth and leads to a disintegration of part or all of the thallus, dramatically reducing *Ulva* productivity [46].

There is thus an imperative to develop robust *Ulva* strains exhibiting traits of either reduced or absent reproduction. Several strain/trait optimization methods are currently available for seaweeds, the simplest being the selection of strains that preferentially express somatic growth over gamete production from the existing wild populations [57]. For example, fast growing clones of *Eucheuma* [16] and *Gigartina exasperata* [59] have been discovered and distributed to farmers. In terms of *Ulva* species, a sterile *U. pertusa* mutant was discovered at Omura Bay, Japan in 1973, which underwent vegetative development without sexual reproduction and could be maintained under axenic laboratory conditions [29,41,42]. The sterile strain also had a higher photosynthetic efficiency and growth rate, and also had a higher total free amino acid and total carbon and nitrogen content.

Although it is straightforward to look for robust strains from wild populations, the process can be time and labour intensive as it requires the collection of a wide range of ecotypes. Instead, mutagenesis and mutant strain screening can be used to avoid the process. Mutagenesis has successfully been employed for seaweeds [20,44], with physical mutagens such as ultraviolet light (UV) widely used [9]. These studies demonstrate that developing sterile, or at least low level reproductive strains is technically achievable and would be beneficial for long-term cultivation.

To date, sterile strains of *U. rigida* (a ubiquitous green seaweed implicated in green tide events) have not been reported. This study aimed to develop a sterile strain that would demonstrate high growth potential. To achieve that, varying doses of UV radiation were employed to mutate a wild strain of *U. rigida*. In addition, patterns in growth, nutrient uptake, and chemical composition were examined to shed light on whether the sterile strain can be used for bioremediation and food purposes.

2. Materials and methods

2.1. Plant collection

Vegetative *U. rigida* fronds of 50–60 mm in length were collected from the low intertidal zone of Cullercoats Bay, Tyne and Wear, UK

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(55.03°N, 1.43°W) after a spring tide in August 2013. The fronds were placed in a plastic bag and transported to the laboratory within 1 h where they were gently rinsed with 1 mm filtered natural seawater to remove any sediment, epiphytes or small grazers.

2.2. Mutation of Ulva gametes and isolation of sterile mutants

Gametes were obtained by inducing reproduction from 2.5 mm discs of *U. rigida* thallus [26]. One hundred discs were rinsed with autoclaved seawater and transferred to a flask containing 200 ml of autoclaved seawater. The flask was held at 4 °C in darkness for 6 h and then transferred to an 18 °C incubator with 80 μmol photons m^{-2} s^{-1} light intensity and 16L:8D photoperiod. Gametes were released three days later, attracted to a point source of white light (gametes being phototactic) and collected by pipette. Gametes were further concentrated by centrifugation (1000 *g*, 5 min) and then transferred to 85 mm diameter Petri dishes with the final density of 2×10^7 gametes per dish. Penicillin G and streptomycin sulfate (final concentrations of 1000 and 250 mg l^{-1} respectively) were added to restrict bacterial growth. Six dishes were exposed to UV lamps (40 W, 254 nm) for 18, 36, 54, 72, 90 and 108 s (one dish per treatment). These exposures were chosen as no gametes survived when exposed for >120 s in preliminary experiments (data not shown). Afterwards, the dishes were placed in darkness at 18 °C for 24 h to allow gamete settlement. Germlings from each dish were detached (112,486 individuals in total) and transferred to six tanks (13 l) when they reached the length of 1 cm. The cultures were aerated with a flow rate of 100 l min^{-1} to suspend the germlings. The seawater was enriched with 1000 μmol l^{-1} nitrate and 50 μmol l^{-1} phosphate and was exchanged every 3–4 days to avoid nutrient limitation. The culture temperature was 18 ± 1 °C and light intensity was 80 μmol photons m^{-2} s^{-1} with a 16L:8D photoperiod. Over the following four months any thalli showing reproductive features—the colour of thalli changing from green to yellowish—were removed and those that maintained the vegetative state were cultivated further. Thalli that failed to demonstrate any reproductive tendencies were identified as sterile. Two sterile individuals were obtained, one of which was used in this study.

2.3. Growth measurement

Five wild type (WT) and three sterile mutant (SM) thalli (one thallus per 500 ml conical flask) were grown at 18 ± 1 °C with a 16L:8D photoperiod and light intensity of 80 μmol photons m^{-2} s^{-1} using a combination of warm and cold fluorescent tubes. The temperature and light conditions used here were previously determined as optimum for *U. rigida* growth [21]. The cultures were aerated with a flow rate of 100 l min^{-1}. The seawater was enriched with 500 μmol l^{-1} nitrate and 25 μmol l^{-1} phosphate and was renewed every day to avoid nutrient limitation. The initial fresh weight for each thallus was ~0.3 g, equal to a stocking density of 0.6 g l^{-1}. The lower quantity of the SM thalli was due to the limited availability of the sterile material. Fresh masses of WT and SM thalli were recorded every three days for 18 or 27 days. The 27-day culture only applied to the SM strain as the WT thalli were lost after day 18 due to reproduction events. The SM was transferred to a 13 l tank on day 9 and then reduced to the stocking density of 1.8 g l^{-1} on day 18 to avoid/reduce self-shading. The stocking density for the WT strain was not reduced given the lower growth rate. The fresh mass was determined after gently blotting each thallus with tissue paper. Specific growth rates (SGR) were calculated using the formula: SGR (%) = [ln (M_2 / M_1)] / t × 100, where M_2 is the final mass, M_1 is the initial mass and t is the number of culture days. The mean SGR over 18 days was based on the initial biomass and the biomass reached by day 18.

2.4. Reproduction assessment

Discs of 7 mm diameter from WT and SM strains were placed in 500 ml flasks (three flasks for each strain, each flask containing 25 discs) and cultured under the same conditions as per the growth trial. This experiment was conducted for 27 days. Reproductive discs were recognized by their colour change; formation of reproductive cells in *Ulva* is accompanied by a change in colour from green (vegetative state) to yellowish (reproductive state) and then to white (after gamete release). This was verified microscopically. Sporulation formation in more than half of the disc area was considered equivalent to complete sporulation. Reproduction rate was expressed as the ratio of reproductive discs to all discs in a flask.

2.5. Nutrient uptake rate measurement

Fresh thalli of the WT and SM strains were cultivated in 13 l tanks with a stocking density of 0.5 g l^{-1} for 12 days. The culture conditions were the same as per the growth experiment. The thalli were cultured in seawater enriched with 500 μmol N and 25 μmol P for three days prior to the experiment. The change in nutrient levels in the seawater was monitored daily and the daily consumed quantities of nitrate and phosphate were replenished after the measurements. The seawater was renewed every three days. Nitrate and phosphate uptake rates by thalli on days 3, 6, 9, and 12 were estimated from the decrease of NO_3^- and PO_4^{3-} in the culture medium over a given time interval (24 h). Only the data on day 3 are presented as both strains were fully vegetative by day 3. Nitrate was measured by a rapid spectrophotometer method [12] and phosphate by the phosphomolybdenum blue colorimetry method [43].

2.6. Dry mass

The thalli in the cultures of Section 2.5 were harvested at the end of the experiment, and then oven dried at 50 °C until consistent mass was obtained (for 24 h). As shown in Fig. 1, the WT growth rate decreased with culture time and was close to zero by day 12, suggesting that biomass would not increase after day 12. To obtain a maximum biomass for biochemical composition analysis, we harvested the thalli on day 12. The tissues were ground to a powder, sieved and placed into tubes within a desiccator pending analyses of chemical composition and functional properties.

2.7. Biochemical composition

Triplicate samples were run for all biochemical and functional analyses for both strains.

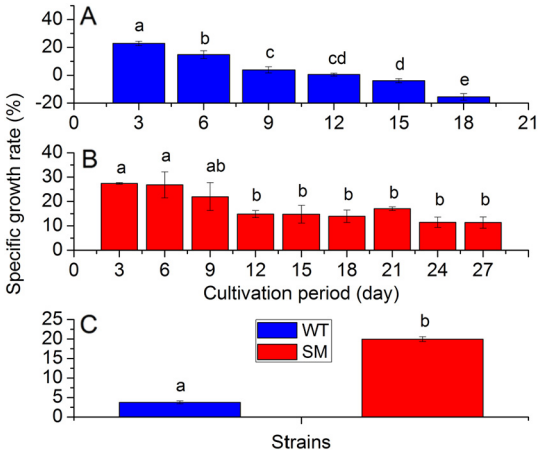

Fig. 1. The specific growth rates of wild type (WT) and sterile mutant (SM) strains of *Ulva rigida*: A) WT, B) SM, and C) mean specific growth rates of WT and SM over an 18-day cultivation. The error bars indicate the standard deviations (n = 3 or 5, 3 for the sterile strain and 5 for the wild strain). Bars sharing the same letter were not significantly different from each other over culture periods (A, B) or strains (C).

2.7.1. Protein content

Total protein was determined by the Kjeldahl method. The crude protein was calculated using nitrogen content multiplied by 5.45 based on the mean value of three *Ulva* species [55], and total nitrogen was measured using an Elementar Vario Macro Cube (Elementar, Hanau Germany). Data were expressed as percentage of dry mass.

2.7.2. Amino acids analysis

Amino acid composition was determined by high-performance liquid chromatography (Ultimate 3000, USA)-tandem mass spectrometry (API 3200 Q-TRAP, USA) (HPLC-MS/MS) [54]. *Ulva* powder (100 mg) was transferred to an ampoule and hydrolysed with 4 ml of 6 N hydrochloric acid at 110 °C for 24 h. Fifty microliters of this solution was dried under nitrogen and then re-dissolved in 200 μl of deionized water. Eighty microliters of borate buffer (0.1 M, pH 8.8) was added to 40 μl of sample solution and mixed. The solution was derivatised with 40 μl of diethyl ethoxymethylene-malonate at 55 °C for 15 min. Samples were transferred to insert vials and 20 μl were injected into the HPLC. The HPLC system consisted of a SRD-3600 Solvent Rack with analytical 6-channel vacuum degasser, a DGP-3600A pump, WPS-3000TSL analytical autosampler, and a tcc-3200 column compartment. Chromatographic separations were performed on an MSLab HP-C18 column (150 × 4.6 mm, 5 μm). The mobile phase consisted of water (A) and acetonitrile (B). The solvent was delivered to the column at a flow rate of 0.8 ml min^{-1} as follows: 0–0.1 min from A–B (95:5) to A–B (95:5); 1–8 min from A–B (95:5) to A–B (40:60); 8–8.1 min from A–B (40:60) to A–B (0:100); 8.1–10 min from A–B (0:100) to A–B (0:100); 10–10.1 min from A–B (0:100) to A–B (95:5); 10.1–15 min from A–B (95:5) to A–B (95:5).

The conditions for MS-MS detection were optimized to obtain the highest signal intensity and were as follows: mode: positive-ion mode; ion spray voltage: 5500 V; nebulizer gas pressure: 55 psi; curtain gas pressure: 20 psi; collision gas pressure: medium; turbo gas temperature: 500 °C; entrance potential: 10 V; collision cell exit potential: 2 V. Nitrogen gas was used as the collision gas in the multiple reaction monitoring (SRM) mode. The data were obtained using Analyst software version 1.5.1 (Applied Biosystems). Amino acid standard solution (Aladdin, China) was injected for the system's calibration and amino acid quantification.

2.7.3. Lipid extraction

Lipid was extracted by a modified Folch method [19]. Homogenized samples (3 g) were extracted with 60 ml chloroform:methanol (2:1) solution. After vortexing for 20 min at room temperature, 12 ml of NaCl (0.88%) was added to aid stratification. Samples were centrifuged for 5 min at 1000 *g* and the upper phase removed. Sixty milliliters of methanol:water (1:1) was used to rinse the tubes. After centrifuging and removal of the upper phase once more, the bottom phase was dried under a steady nitrogen stream. When the chloroform was completely evaporated, the crude lipid was weighed. Results were expressed as percentage of dry mass.

2.7.4. Fatty acid analysis

Fatty acid methyl esters (FAME) were prepared based on extracted lipid from Section 2.7.3 according to Joseph and Ackman [28]. A mixture of 100 mg of fat matter and 1.5 ml 0.5 N methanolic NaOH (2 g NaOH in 100 ml methanol) was heated at 100 °C for 5 min. After reaching room temperature, 2 ml of boron trifluoride 12% in methanol was added to the solution, which was then heated at 100 °C for 30 min. After cooling for 5 min at room temperature, 1 ml of isooctane and 5 ml of saturated NaCl solution were added. The solution was centrifuged for 5 min at 859 *g* and the upper layer was transferred to a new tube. One milliliter of isooctane was added to the first solution and the procedure was repeated. The two layers of isooctane were dried under a steady stream of nitrogen and the residue was re-dissolved in 1 ml of hexane. FAMEs were analysed using a gas chromatograph (Shimadzu, GC-2014, Kyoto, Japan) fitted with an Agilent CP-SIL 88 fused silica capillary column (100 m × 0.25 mm ID × 0.2 μm film thickness). Purified helium was used as a carrier gas with a head pressure of 210 kPa and a column flow of 1 ml min^{-1}. A split injection system was used with an auto injector (Shimadzu, AOC-20i) with a split injection ratio of 50.0 and an injector temperature of 255 °C. FAME peaks were detected by flame ionisation detection at 260 °C. Samples of 1 μl were injected at an initial column temperature of 70 °C, held for 1 min. The temperature was then raised at 5 °C min^{-1} to 100 °C, held for 2 min, and then increased at 10 °C min^{-1} to 160 °C, and held for 61 min. Finally, the temperature was increased to 240 °C at a rate of 5 °C min^{-1}, and held for 21 min, thus giving a final gradient of 113 min total runtime. Peaks were identified by comparing the chromatograms to a commercial 52 FAME standard (GLC463, Nu-Chek Prep Inc., Elysian, MN, USA). Quantification was based on peak areas of individual identified and non-identified FAME, expressed as a percentage of the total peak areas for quantified FAME.

2.7.5. Ash content

Ash was determined by incinerating samples in a muffle furnace at 550 °C for 24 h. The total ash content was expressed as percentage of dry mass.

2.7.6. Carbohydrate

Carbohydrate was estimated by subtracting the total protein, lipid, and ash content from the total dry mass [45].

2.8. Functional properties

2.8.1. Swelling capacity

The swelling capacity (SWC) was calculated using a slightly modified method of bed volume after equilibrating in excess solvent [61]. Triplicate samples of *Ulva* powder (0.2 g) were placed into 10 ml measuring cylinders. Ten milliliters of distilled water was added, immediately vigorously mixed and incubated for 18 h at 37 °C. The swelling volume was recorded and SWC was expressed as ml of swollen sample per gram of sample.

2.8.2. Water holding capacity

The water holding capacity (WHC) was analysed by the centrifugation method [61]. Triplicate samples of *Ulva* powder (0.2 g) were placed into pre-weighed centrifuge tubes. Five milliliters of distilled water was added and mixed vigorously. The dispersion was centrifuged for 25 min at 3000 *g* after incubating at 37 °C for 1 h. The supernatant was removed and the residue was oven dried at 50 °C for 25 min. The WHC was expressed as grams of water bound per gram of sample.

2.8.3. Oil holding capacity

The oil holding capacity (OHC) was measured according to Yaich et al. [61]. Triplicate samples of *Ulva* powder (0.5 g) were dispersed in 5 ml of food grade corn oil in centrifuge tubes. The mixtures were stirred and incubated at 37 °C for 1 h, followed by centrifugation at 3000 *g* for 25 min. The oil supernatant was transferred to a 10 ml measuring cylinder and measured. The OHC was expressed as grams of oil held by 1 g of sample (dry weight). The density of the corn oil was 0.92 g ml^{-1}.

2.9. Statistical analyses

Results were expressed as means of replicates ± standard deviation. All data are analysed using the software SPSS v.21.

The SGRs of both the wild and mutant strains at each culture time were normally distributed (Shapiro-Wilk, $P > 0.05$) and had equal variances (wild: Levene's test, $F = 0.743$, $P > 0.05$; mutant: Levene's test, $F = 2.257$, $P > 0.05$). The effect of culture time on SGR was assessed using repeated measures ANOVA with Least-Significant Difference (LSD) for *post hoc* investigation. The mean SGR of each strain over the first 18-day culture was also normally distributed with

equal variances (Levene's test, $F = 2.208$, $P > 0.05$); therefore a *t*-test was conducted.

The reproduction rate of the WT strain at all culture times was also normally distributed with equal variances (Levene's test, $F = 3.300$, $P > 0.05$); a repeated ANOVA with LSD was conducted.

Data on nutrient uptake, protein, lipid, ash, carbohydrate, and functional properties of both strains were normally distributed (Shapiro-Wilk, $P > 0.05$) with equal variances (Levene's test, $F < 6.750$, $P > 0.05$); *t*-tests were conducted. A confidence interval of 95% was set for all tests.

All amino acids in the WT strain and total amino acids in the SM strain were normally distributed apart from the total essential amino acids (Shapiro-Wilk, $P = 0.010$). The variances of the two samples for all amino acids could be considered equal (Levene's test, $F < 6.750$, $P > 0.05$) except for arginine (Levene's test, $F = 11.144$, $P = 0.029$) and aspartic acid (Levene's test, $F = 11.046$, $P = 0.029$). A one-way multivariate ANOVA (MANOVA) was used to compare the differences in amino acid content as MANOVA is reasonably robust to violations of normal distribution and homogeneity of variance, particularly when the group sizes are equal [47]. Bonferroni adjustment was used to reduce the chance of Type I error. A new alpha level of 0.0024 (0.05 divided by 21) was set since there were 21 dependent variables. All fatty acids in the WT strain and polyunsaturated fatty acids (PUFA) and n6-fatty acids in the SM strain were normally distributed except for C18:2n6 (Shapiro-Wilk, $P = 0.011$) and C18:2n3 (Shapiro-Wilk, $P = 0.040$). The variances for all fatty acids could be considered equal (Levene's test, $F < 6.750$, $P > 0.05$) except for C20 (Levene's test, $F = 13.398$, $P = 0.022$) and C20:4n6 (Levene's test, $F = 13.408$, $P = 0.022$). A one-way MANOVA was used to compare the differences in fatty acid content between the two strains; a Bonferroni adjusted alpha level of 0.0014 (0.05 divided by 37) was set since there were 37 dependent variables.

3. Results and discussion

3.1. Growth and reproduction

The WT SGR differed significantly over culture times (Fig. 1; $F = 197.212$, df = 5, 20, $P < 0.001$), reaching 22.8 ± 1.7% during the first three days but declining to almost zero by day 12 (LSD, $P < 0.001$; Fig. 1A) and subsequently losing biomass with the SGR dropping to −15.6 ± 2.4% during the last three days (Fig. 1A). The SM SGR also differed significantly over culture times ($F = 9.872$, df = 8, 16, $P < 0.001$). In contrast to the WT, the SM maintained a positive SGR over the course of the 27-day culture, albeit at a reduced rate with time (LSD, $P < 0.01$), with the SGR on the last three days still relatively high (11.4 ± 2.3%; Fig. 1B). There were significant differences in mean SGR between the WT and SM strains over the first 18 days (Fig. 1C), with the SM mean SGR five times faster than the WT (independent samples *t*-test, $t = -48.173$, df = 6, $P < 0.001$).

The WT growth decline with time was consistent with other published *Ulva* research [3]. The decrease in growth may be due to a switch to reproduction, which effectively terminates vegetative growth and leads to a pronounced disintegration of part, or all of the thallus [6,22]. The WT reproduction rate differed significantly over culture times ($F = 545.701$, df = 8, 16, $P < 0.001$). There was no reproductive activity during the first six days whereas 18.7 ± 2.3% of discs became fertile by day nine (Fig. 2). The reproduction rate increased with time (LSD, $P < 0.01$) reaching 100% by day 21. In contrast, all SM discs remained in the vegetative state throughout the 27-day trial. As the SM discs did not experience reproductive biomass loss, the resulting fronds grew to >50 cm in length (Fig. 3). In addition, the decline of growth in both strains with culture time could be due to decreased light availability through self-shading. The biomass was only harvested at the end of the experiment with a possibility of increased self-shading and thus decreased light availability. A positive relationship between light and biomass productivity in *U. ohnoi* has been demonstrated [40].

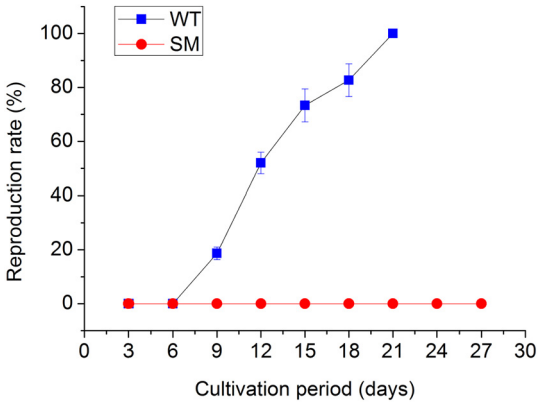

Fig. 2. Reproduction rates of the wild type (WT) and sterile mutant (SM) strains of *Ulva rigida* over a 27-day cultivation. The error bars indicate the standard deviations (n = 3).

High and sustained growth rates are desired in aquaculture. The SM mean SGR over 18 days was 20.0 ± 0.6% – five times faster than the WT. The mean SGR over the 27-day trial was 17.4 ± 0.3%, exceeding values reported by Gordillo et al. [63] (1–6% in a 10-day lab culture of *U. rigida*) and Viaroli et al. [64] (2.5–8.1% in a two-week outdoor culture of *U. rigida*).

3.2. Nutrient uptake

Apart from the beneficial effects of sterility, this quick growth (Figs. 1 & S1) could also be related to an enhanced capacity for nutrient uptake, which if true, would indicate that SM could be an effective strain for nutrient capture. This assumption was supported by the nutrient uptake data. Nitrate uptake by the SM was 171.6 ± 12.1 μmol g FW^{-1} day^{-1}, which was 40.0% higher than the WT (Fig. 4A) (independent samples *t*-test, $t = -5.337$, df = 4, $P = 0.006$). The phosphorus uptake of the SM was 5.7 ± 0.4 μmol g FW^{-1} day^{-1}, significantly higher (30.9%) than the WT (4.4 ± 0.5 μmol g FW^{-1} day^{-1}; independent samples *t*-test, $t = -3.506$, df = 4, $P = 0.025$, Fig. 4B).

Nitrogen and phosphorus are essential nutrients that support growth, with rapidly proliferating cells having a large nutrient demand. The nitrate uptake capacity of the SM was greater when compared with other *Ulva* species, *e.g.* 133.7 and 109.7 μmol g FW^{-1} day^{-1} in *U. fenestrata* and *U. intestinalis* respectively [4], and ~70 μmol g DW^{-1} day^{-1} in *U. lactuca* [3], making the SM a potentially efficient biofilter for wastewater bioremediation, as a biofilter to combat eutrophication, or as an extractive component within an integrated multi-trophic aquaculture system. The phosphate uptake capacity of the SM exceeded the 0.637 ± 0.266 and 0.15 μmol g FW^{-1} day^{-1} of *U.*

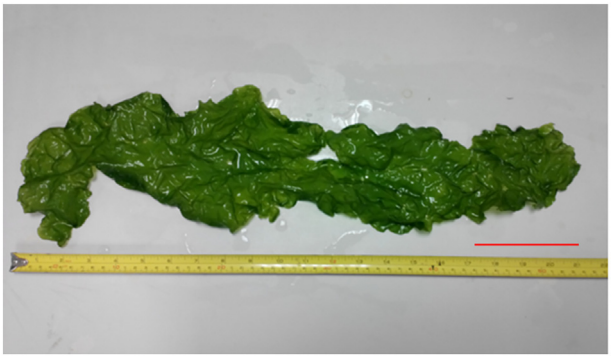

Fig. 3. The sterile mutant strain of *Ulva rigida* obtained by mutating a wild type strain. Scale bar = 10 cm.

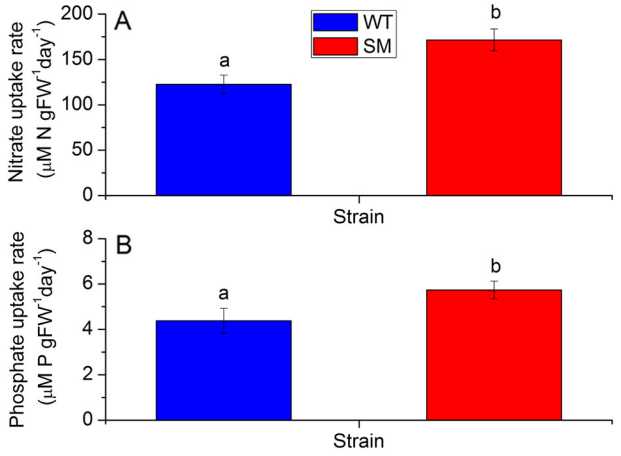

Fig. 4. Nutrient uptake of the wild type (WT) and sterile mutant (SM) strains of *Ulva rigida* on day 3 over 12 days of cultivation; A) nitrate, and B) phosphate. The error bars indicate the standard deviations (n = 3). Bars sharing the same letter were not significantly different from each other.

rotundata [24] and *U. pertusa* [53] respectively, the 1.4–1.6 μmol g^{-1} FW day^{-1} (based on the ratio of FW:DW = 5:1) for *U. intestinalis* [39], and was comparable to the 1.01–5.04 and 3.42–6.54 μmol g FW^{-1} day^{-1} for *U. fenestrata* and *U. intestinalis* respectively [4].

The N:P uptake ratio (29.9) in the SM was higher than the 3.7–4.4 for *U. linza*, 4.5–5.1 for *U. prolifera* [36], 9.4 for *U. rotundata* [24], and 16.6–16.8 for *U. intestinalis* [4], and was comparable to the 11.8–36.0 for *U. fenestrata* [4].

3.3. Chemical composition and functional properties

The protein content of the WT accounted for 24.1 ± 0.8% of the biomass, which was 58.8% higher than the SM (Fig. 5; independent samples *t*-test, $t = 8.224$, df = 4, $P = 0.001$). Although the SM had a faster nitrate uptake rate, the higher growth rate (Fig. S1) could dilute the assimilated nitrogen, resulting in a lower protein content. In addition, nitrate limitation occurred in the SM but not in the WT towards the end of the culture (Fig. S2), which could also contribute to the lower protein content. Løvlie [34] also reported a lower protein content in a fast-growing mutant of *U. mutabilis*. In contrast, the total lipid content of the SM was more than double that of the WT with the latter only 5.4 ± 0.4% of dry mass (independent samples *t*-test, $t = -19.052$, df = 4, $P < 0.001$). The SM ash content (34.0 ± 1.1%) was also higher than the WT (31.4 ± 1.2%) (independent samples *t*-test, $t = -2.812$, df = 4, $P = 0.048$). There were no significant differences between strains for carbohydrate content.

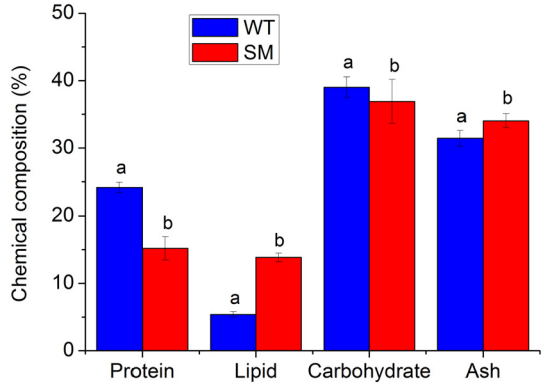

Fig. 5. Bulk chemical composition of the wild type (WT) and sterile mutant (SM) strains of *Ulva rigida*. The error bars indicate the standard deviations (n = 3). Bars sharing the same letter were not significantly different from each other.

The 13.8% lipid content of the SM was high compared with the WT and higher still when compared with other *Ulva* species; 2.5–3.5% in *U. clathrata* [48] and 7.9% in *U. lactuca* [61]. The most abundant fatty acid in both strains was C16:0 (Table 1). There was also a high proportion of C18 unsaturated fatty acids. The SM had significantly more C12:0, C14:0, C17:1 (*cis*-9), C18:0, C18:2n6, C18:3n3, C18:3n6, C20:3n6, C20:4n6, C22:5n3 and C22:4n6 fatty acids than the WT, which translated to significantly more monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), omega-3 fatty acids (n3-FA), and omega-6 fatty acids (n6-FA), which could have greater benefits for health and nutrition applications [32,52]. The ratio of n6 to n3-FA was higher in the SM but the value was still below the maximum World Health Organization recommended value of 10, indicating that the SM strain is an efficient food resource to reduce n6-FA/n3-FA.

Among the amino acids, glutamic acid and alanine were the most abundant in both strains. Aspartic acid and arginine were the second highest group for the WT while valine and leucine were the next highest for the SM. There was more histidine, serine, glutamic acid, isoleucine, total amino acids, and non-essential amino acids (NEAA) in the WT compared to the SM strain (Table 2). Angell et al. [65] defined three internal nitrogen states in *U. ohnoi*: nitrogen-limited (<1.2% N), metabolic (1.2–2.6% N), and luxury (>2.6%). All amino acids and growth increase with rising nitrogen content in the nitrogen-limited state. Growth rate

Table 1
Fatty acid profiles (% of total fatty acids) of wild type (WT) and sterile mutant (SM) strains of *Ulva rigida*. Data are the means ± standard deviation (n = 3). df, F, and *P* values were from the MANOVA. Bonferroni adjustment with new alpha level of 0.0014 was used to reduce the chance of Type I error. The fatty acids with significant differences between the strains are in bold. SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids. n6-FA = total omega-6 fatty acids, n3-FA = total omega-3 fatty acids.

Fatty acid	Wild type	Sterile mutant	df	F	*P* value
C10:0	0.02 ± 0.00	0.02 ± 0.00	1	1.000	0.374
C12:0	**0.04 ± 0.00**	**0.01 ± 0.00**	**1**	**519.501**	**<0.001**
C14:0	**0.87 ± 0.03**	**0.29 ± 0.03**	**1**	**1056.034**	**<0.001**
C14:1 (*cis* 9)	0.06 ± 0.05	0.06 ± 0.02	1	0.045	0.842
C15:0	0.07 ± 0.00	0.04 ± 0.00	1	40.500	0.003
C16:0	20.99 ± 1.58	15.93 ± 1.70	1	14.231	0.020
C16:1 (*trans* 9)	0.01 ± 0.00	0.02 ± 0.01	1	0.500	0.519
C16:1 (*cis* 9)	1.21 ± 0.13	0.67 ± 0.10	1	32.564	0.005
C17:0	0.04 ± 0.01	0.02 ± 0.00	1	12.000	0.026
C17:1 (*cis* 9)	**0.13 ± 0.02**	**0.05 ± 0.00**	**1**	**84.500**	**0.001**
C18:0	**0.31 ± 0.01**	**0.19 ± 0.01**	**1**	**342.250**	**<0.001**
C18:1 (*cis* 9)	0.44 ± 0.08	0.33 ± 0.06	1	4.070	0.114
C18:1 (*cis* 11)	10.21 ± 0.43	8.18 ± 0.29	1	46.061	0.002
C18:1 (*cis* 12)	0.04 ± 0.01	0.02 ± 0.00	1	12.000	0.026
C18:2n6	**1.63 ± 0.13**	**9.78 ± 0.51**	**1**	**717.533**	**<0.001**
C18:2n3	0.01 ± 0.00	0.02 ± 0.01	1	12.500	0.024
C20:0	0.06 ± 0.01	0.04 ± 0.00	1	25.000	0.007
C18:3n6	**0.26 ± 0.01**	**1.17 ± 0.12**	**1**	**166.156**	**<0.001**
C18:3n3	**12.42 ± 0.17**	**16.75 ± 0.77**	**1**	**89.938**	**0.001**
C20:2n6	0.01 ± 0.00	0.05 ± 0.01	1	32.000	0.005
C22:0	1.05 ± 0.29	0.47 ± 0.05	1	12.101	0.025
C20:3n6	**0.14 ± 0.01**	**0.51 ± 0.06**	**1**	**136.800**	**<0.001**
C22:1 (*cis* 13)	0.09 ± 0.01	0.07 ± 0.01	1	16.200	0.016
C20:4n6	**0.24 ± 0.01**	**2.09 ± 0.17**	**1**	**361.140**	**<0.001**
C22:2n6	0.02 ± 0.01	0.00 ± 0.00	1	2.000	0.230
C20:5n3	1.04 ± 0.02	1.05 ± 0.05	1	0.211	0.670
C24:0	0.09 ± 0.03	0.02 ± 0.00	1	16.531	0.015
C24:1 (*cis* 15)	0.01 ± 0.00	0.01 ± 0.00	1	4.500	0.101
C22:4n6	**0.06 ± 0.00**	**0.44 ± 0.02**	**1**	**627.200**	**<0.001**
C22:5n3	**2.57 ± 0.15**	**0.86 ± 0.11**	**1**	**255.001**	**<0.001**
Unidentified	45.78 ± 2.11	40.78 ± 1.88	1	9.544	0.037
SFA	23.55 ± 1.76	17.04 ± 1.76	1	20.358	0.011
MUFA	12.22 ± 0.65	9.39 ± 0.19	1	52.852	0.002
PUFA	**18.44 ± 0.36**	**32.79 ± 0.22**	**1**	**3506.722**	**<0.001**
n6-FA	2.38 ± 0.14	14.08 ± 0.60	1	26.430	0.007
n3-FA	**16.03 ± 0.35**	**18.69 ± 0.82**	**1**	**1078.298**	**<0.001**
n6/n3	**0.15 ± 0.01**	**0.76 ± 0.06**	**1**	**240.890**	**<0.001**

Table 2
Amino acids profile (g 100 g^{-1} DW) in wild type (WT) and sterile mutant (SM) strains of *Ulva rigida*. Data are the means ± standard deviation (n = 3). df, F, and *P* values were from the MANOVA. Bonferroni adjustment with a new alpha level of 0.0024 was used to reduce the chance of Type I error. The amino acids with significant difference between two strains are in bold. EAA = essential amino acids, NEAA = non-essential amino acids.

Amino acids	Wild type	Sterile mutant	df	F	*P* value
Histidine	**0.36 ± 0.01**	**0.22 ± 0.02**	**1**	**83.248**	**0.001**
Serine	**1.27 ± 0.08**	**0.85 ± 0.04**	**1**	**64.233**	**0.001**
Arginine	1.57 ± 0.20	0.93 ± 0.03	1	29.797	0.005
Glycine	0.83 ± 0.10	0.77 ± 0.06	1	0.918	0.392
Aspartic acid	1.94 ± 0.24	1.01 ± 0.04	1	42.525	0.003
Alanine	1.98 ± 0.11	1.47 ± 0.11	1	30.452	0.005
Glutamic acid	**2.21 ± 0.05**	**1.41 ± 0.11**	**1**	**120.476**	**<0.001**
Threonine	1.15 ± 0.15	0.89 ± 0.09	1	6.305	0.066
Proline	0.96 ± 0.10	0.66 ± 0.04	1	24.223	0.008
Lysine	1.24 ± 0.11	0.79 ± 0.09	1	28.358	0.006
Tyrosine	0.58 ± 0.05	0.46 ± 0.03	1	12.000	0.026
Methionine	0.42 ± 0.04	0.38 ± 0.04	1	1.194	0.336
Valine	1.39 ± 0.05	1.12 ± 0.10	1	18.229	0.013
Isoleucine	**0.81 ± 0.02**	**0.65 ± 0.03**	**1**	**52.285**	**0.002**
Phenylalanine	1.17 ± 0.13	1.09 ± 0.09	1	0.726	0.442
Cysteine	0.18 ± 0.02	0.15 ± 0.01	1	6.487	0.064
Leucine	1.12 ± 0.06	1.10 ± 0.09	1	0.135	0.732
Total	**19.18 ± 0.54**	**13.93 ± 0.55**	**1**	**3790.801**	**<0.001**
EAA	9.22 ± 0.49	7.17 ± 0.49	1	18.732	0.007
NEAA	**9.95 ± 0.17**	**6.76 ± 0.05**	**1**	**869.035**	**<0.001**
EAA:NEAA	0.93 ± 0.05	1.06 ± 0.06	1	8.176	0.046

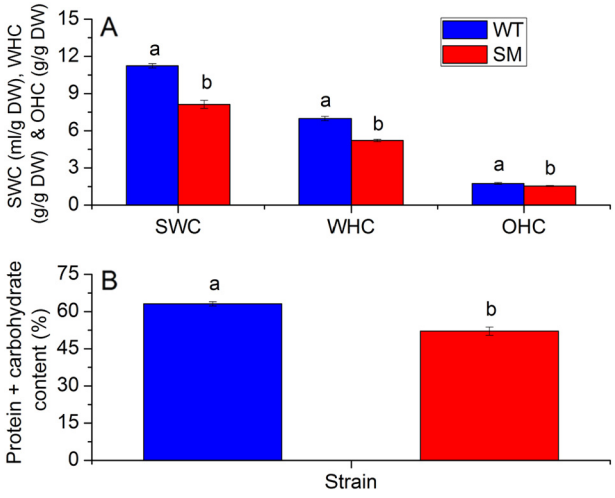

Fig. 6. Functional properties (A) and content of protein plus carbohydrate (B) in wild type (WT) and sterile mutant (SM) strains. The error bars indicate the standard deviations (n = 3). SWC = swelling capacity, WHC = water holding capacity, and OHC = oil holding capacity. Bars sharing the same letter were not significantly different from each other.

does not change and all amino acids still increase with rising nitrogen content in the metabolic state. The luxury state was in which growth rate decreases and only specific amino acids (glutamic acid, glutamine, and arginine) have a large increase. Meanwhile, the nitrogen-limited content for *U. intestinalis* and *U. fenestrata* were 2.5% and 3.2% respectively [4], suggesting species specific differences. In the present study, the internal nitrogen content in the WT and SM strains were 4.34 ± 0.14% and 2.79 ± 0.32%. Based on the nutrient consumption (Fig. S2) and growth data (Fig. S1), the SM experienced nitrogen limitation while the WT may have been in the state of luxury. The high content of glutamic acid in the WT would also support its nitrogen state (Angell et al., 2014).

Although there was less protein in the SM, there was a relatively high ratio of essential amino acids (EAA) to NEAA (1.06 ± 0.06), making the SM preferable in terms of EAA sources as the EAA:NEAA ratio of other *Ulva* species is usually below 1 [48,56].

The faster growing SM will have had a higher demand for phospholipid membrane synthesis due to cell division. Cell membranes have a high PUFA content to maintain membrane fluidity [62]. This would explain why the SM had both a higher overall lipid content (relative to protein) and an increased concentration of PUFA, as well as the higher metabolic demand for phosphorous. In addition, more energy will have been needed by the SM to maintain the higher rate of cell division, requiring a higher rate of transformation of fixed carbon into biomass. However, there was no statistical difference in carbohydrate content between strains, a crude proxy for photosynthetic performance. The reason could be that more of the ATP and NADPH produced during photosynthesis were used to synthesize lipid rather than carbohydrate and protein.

The rapid vegetative growth of the SM may have contributed to inadvertent nitrogen limitation towards the end of the culture trial (Fig. S2). When nitrogen is limited, the fixed carbons are channeled towards the synthesis of lipid rather than protein, as there are no chemical building blocks for making more (nitrogen containing) protein [18,37,51].

The swelling, and water- and oil-holding capacities of the SM were all significantly lower than the WT (SWC = 27.7% lower, Independent samples *t*-test, $t(4) = 30.946$, $P < 0.001$; WHC = 25.3% lower, $t = 15.399$, df = 4, $P < 0.001$; OHC = 11.5% lower; $t = 4.461$, df = 4, $P = 0.011$; Fig. 6A). Intake of food with a higher swelling capacity can enhance satiety and thereby lead to a reduced calorie intake [15]. Foods with a high SWC therefore have applications in adjunctive therapies for the treatment of obesity [15]. The WT SWC (11.2 ± 0.2 ml g^{-1} DW) was slightly lower than that of *U. lactuca* (13.0 ± 0.70 ml g^{-1} DW; [60]), but higher than that reported elsewhere for *U. lactuca* (0.3 ml g^{-1} DW; [61]) and also higher than commercial fibres from oat, wheat, apple, and bamboo (4.98–7.60 ml g^{-1} DW; [50]). The SWC is mainly influenced by the protein and fibre content as well as the particle size of fibre. In addition, the conditions of measurement, such as pH, temperature, and ionic strength and types of ions in solution, can also affect the SWC [17,61]. The water-holding capacity can modify the viscosity and texture of formulated food and a higher WHC can assist in avoiding syneresis. The WT WHC (6.98 ± 0.17 g g^{-1} DW) was lower than that reported by Wong and Cheung [60] (9.71 ± 0.11 g g^{-1} DW), comparable to Yaich et al. [61] (6.66 to 7.00 g g^{-1} DW), and higher than commercial fibres from oat, wheat, apple, and bamboo (3.69–6.89 g g^{-1} DW; [50]). According to Robertson and Eastwood [49], water can be associated with fibre either as bound or trapped water. The amount of bound water depends on chemical composition whereas trapped water refers to the fibre structure. The centrifugation method used here incorporates both fibre-water states. Protein conformations and the nature of the water binding sites in the protein molecules also play a role in water holding [10]. The total protein and carbohydrate content of the SM (52.1 ± 1.7%) was 17.5% lower than the WT (Fig. 6B), contributing to the SM's lower SWC and WHC. The WT (1.75 ± 0.07 g g^{-1} DW) and SM OHCs (1.54 ± 0.04 g g^{-1} DW) were higher than reported by Wong and Cheung [60] (0.65 ± 0.03 g g^{-1} DW). This may be due to different treatment temperatures. The OHC in the present study was determined at 37 °C, whereas Wong and Cheung [60] used 25 °C. Our results were comparable to Yaich et al. [61] (around 1.60 g g^{-1} DW at 40 °C). In general, proteins play a major role in fat absorption by hydrophobic bonding. This may result in a higher OHC in WT that has a higher protein content compared to the SM. Furthermore, the OHC of seaweeds is also related to particle size, overall charge density and the hydrophilic nature of fibre [30,58]. Due to their OHC, *Ulva* species can be used as stabilizers in formulated food products. Moreover, the OHC of both *Ulva* strains in the present study was significantly higher than commercial cellulose (0.98 ± 0.09 g g^{-1} DW; [23]), making them useful in reducing blood lipid level, obesity and coronary heart disease risk.

4. Conclusion

The results clearly demonstrate that this new sterile strain is a positive step towards increasing the long-term productivity of *Ulva* by

preventing reproductive events. Thus, this provides a platform to serve multiple market applications. However, the caveat should be added that using clonal strains does increase the risks of disease outbreaks. The rapid and sustained growth rate combined with high rates of nitrate and phosphate uptake suggest a strong carbon capture and wastewater bioremediation capacity. In addition, the enhanced MUFA, PUFA, and omega-3 fatty acids offer benefits for health food applications. More targeted molecular studies combined with further genetic optimization will likely deliver benefits beyond that achieved by physical mutagenesis; however, the complexities of modifying eukaryotic genomes prevent these approaches from making an immediate impact.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.algal.2017.03.008.

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