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The effect of salinity on the biomass productivity, protein and lipid composition of a freshwater macroalga

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ABSTRACT

A critical knowledge gap in the production of macroalgae for protein (animal feed) and lipid (bioenergy) is the ability of target species to grow in saline groundwater and thereby avoid competition with traditional crops. We assessed the effect of increased salinity (0.11 ppt–3 ppt) on the growth of 5 strains of the freshwater macroalga *Oedogonium* in laboratory cultures and subsequently on the productivity and biochemical composition in outdoor cultures under ambient conditions. Growth and biomass productivity decreased with increasing salinity in both experiments across all strains. However, in contrast to biomass productivity, protein content increased with increasing salinity and consequently, protein productivity (0.2–0.6 g DW m^{-2} day^{-1}) did not decrease markedly as salinity increased. Salinity had inconsistent effects on the lipid content among the strains, with the content of 2 strains increasing 3 to 4-fold under the 3 ppt treatment compared to 0.11 ppt. However, lipid productivity decreased with increasing salinity for 4 of the 5 strains. Similarly, biomass energy values increased with increasing salinity across all strains while bioenergy productivity decreased. These findings demonstrate that *Oedogonium* grown in salinities of up to 3 ppt maintains its productivity as a source of protein, potentially for animal feed, but not for bioenergy.

1. Introduction

Salinization of soils and groundwater is a significant global problem, with more than 800 million hectares of land, or more than 6% of the worlds' total land area, affected by salinity [32]. Nearly 20% (45 million hectares) of all irrigated land and 2% (32 million hectares) of dryland agricultural land are salt affected [32], and more than 50% of all arable lands are expected to be affected by salinity by the year 2050 [51]. Although some commercially important plants and crops such as barley, cotton and wheat can tolerate high salinity, a potential use of this salt affected land and groundwater is to cultivate macroalgae for biomass applications.

The use of saline groundwater to cultivate algae has been a focus of research and development for biomass applications [12,40,49,50]. However, successful cultivation of algae in these areas is dependent on the tolerance of strains to higher salinities. Most research on this topic has focused on selecting salt-tolerant strains of microalgae for biofuel production [40,50]. In a different but analogous approach, research has also been conducted on the cultivation of the marine macroalga *Gracilaria chilensis* for hydrocolloid production in dryland salinity areas where the salinity of the water in evaporation basins (20–40 parts per thousand (ppt)) is close to that of seawater (35 ppt) [12]. This is possible because many marine macroalgae can tolerate a wide range of salinities [25]. For example, *Porphyra umbilicalis* grows well in salinities ranging from 7 to 52 ppt [25], while *Chaetomorpha indica* and *Ulva ohnoi* can grow in salinities ranging from 5 to 45 ppt [13]. However, saline groundwater supplies often have lower salinities than those tolerated by marine macroalgae (e.g. <5 ppt, [50]). Consequently, the only macroalgae suitable for cultivation in these waters are salt-tolerant freshwater species that, similar to microalgae, are capable of tolerating or adapting to the changes in salinity that would occur in open culture systems due to evaporation and rainfall.

The freshwater macroalgal genus *Oedogonium* has recently been identified as a target for biomass applications due to its high productivity, favorable biochemical composition, cosmopolitan distribution and competitive dominance over other algal species in open culture systems [9,27,34,55]. *Oedogonium* has been cultivated in water sources with very different chemical compositions. Successful production has been achieved in water rich in heavy metals and metalloids [41,42] and water with high alkalinity [10]. Moreover, growth rates among 11 *Oedogonium* strains differ under a range of temperature treatments with some having better tolerance to lower temperatures [28]. The broad environmental distribution of *Oedogonium*, its ability to grow across water sources with a range of chemical compositions and its among-strain variability in response to temperature support the potential for strains to vary in their tolerance to other environmental parameters such as salinity. However, the salinity tolerance of *Oedogonium*, and more generally the tolerance of freshwater macroalgae, is not well understood. Identification of salt-tolerant strains of *Oedogonium*

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would enable production of this alga utilizing saline groundwater across a broad range of sites, including those unsuitable for agriculture.

Two key factors determine the suitability of algae for biomass applications, areal productivity (the amount of dried ash-free biomass per unit area (m^2) per unit time (day)) [17,36] and biochemical composition. The proportion of protein in the biomass is a key parameter for animal feed applications [6] and the proportion of lipids in the biomass is a key parameter for the thermochemical production of biocrude, a promising pathway to biofuels from macroalgae [15,43,44]. The energy potential of the biomass is important for both applications. However, the effect of increased salinity on the biochemical composition of freshwater macroalgae is fundamentally unknown. Therefore, the objective of this study was to assess the salinity tolerance of multiple *Oedogonium* strains and determine the effect of increased salinity on the productivity and biochemical composition of the biomass. To achieve this objective we assessed the effect of increased salinity over a period of 3 weeks on the growth of 5 strains of *Oedogonium* in small-scale laboratory cultures, and subsequently on the productivity and biochemical composition of the same 5 strains of *Oedogonium* in outdoor cultures under ambient conditions.

2. Methods

2.1. Sample collection and isolation

Tolerance to salinity was assessed in 5 genetically distinct strains of *Oedogonium* — Tar1, Tar3, Tsv1, Tsv2 and Riv6 [28,29]. *Oedogonium* is a cosmopolitan genus of filamentous freshwater green macroalgae that is a common component of freshwater ecosystems. It is a genus of unbranched, uniseriate algae made up of small cylindrical cells. Strains were originally isolated from samples of freshwater macroalgae collected from naturally occurring water bodies, irrigation channels and wetland areas in 3 distinct geographic regions of Australia–Riverina (35°S, 145°E: "Riv"), Tarong (26°S, 151°E: "Tar") and Townsville (19°S, 146°E: "Tsv"). Detailed collection information and methods for species identification are provided in Lawton et al. [28] for strains Tar1, Tar4, Tsv1, Tsv2 and Lawton et al. [29] for Riv6. Following isolation, strains were maintained in nutrient-enriched autoclaved freshwater (MAF growth medium, Manutech Pty Ltd, 13.4% N, 1.4% P; 0.05 g L^{-1}) in a temperature controlled laboratory under low light (12:12 light:dark cycle, 50 μmol photons PAR m^{-2} s^{-1}, 23 °C) for at least 1 year and were well acclimated to culture conditions. All strains are maintained in culture collections at James Cook University, Townsville, Australia.

2.2. Laboratory salinity tolerance experiment

Laboratory growth trials were conducted to determine the salinity tolerance of 5 strains of *Oedogonium*. Laboratory trials enabled us to test a greater number of treatments than outdoor experiments, providing greater resolution on the point of salinity tolerance. These trials were conducted on each strain under eleven salinity treatments plus a freshwater control. Thirty-six filaments of each strain were cut to a standardized length of 6 mm. Three filaments from each strain were then grown using each of eleven different salinity treatments ranging from 0.5 ppt (parts per thousand, equal to percentage/10) to 3 ppt increasing in 0.25 ppt increments (Fig. 1) for a period of 7 days. This upper limit of 3 ppt was chosen based on the results of a pilot trial (Appendix 1) and falls within the range of saline groundwater (up to 5 ppt; [50]). These values equate to approximately 0.3% to 8.6% of seawater salinity and many groundwaters are commonly up to 10% of the salinity of seawater (35 ppt, 3.5%, 3500 mg L^{-1} or ppm). Nutrient enriched (MAF growth medium, Manutech Pty Ltd., 0.05 g L^{-1}) dechlorinated freshwater was used as a control and had a salinity of 0.11 ppt. Salinity treatments were created by adding NaCl to the dechlorinated nutrient enriched freshwater until desired salinities were reached. Each individual filament was maintained in a sterile 60 mm Petri dish in culture cabinets at 24.5 °C with 12 hour light: 12 hour dark cycles and a light level of 50 μmol photons PAR m^{-2} s^{-1}. These conditions correspond to the middle temperature treatment used in a previous growth experiment with these strains [28] and are comparable to ambient summer conditions in the majority of regions where samples were originally collected. Each replicate was photographed under a dissecting microscope (Olympus model SZ61) at the start and end of the 7 day period and the 2-dimensional surface area of filaments was determined using ImageJ [47]. Specific growth rates (SGR) were calculated for each individual replicate of each strain under each treatment using the equation SGR (% day^{-1}) = $\mathrm{Ln}(B_f/B_i)/T * 100$, where B_f and B_i are the final and initial surface areas (mm^2) and T is the number of days in culture. This entire protocol was repeated a further 2 times to give a total of 3 replicate weeks of growth data. In the second and third week of the experiment, new filaments were cut from the biomass grown in each replicate during the previous week of the experiment and then placed into new, independent Petri dishes. Permutational analysis of variance (PERMANOVA) was used to analyze the effects of strain, salinity (fixed factors) and week (random factor) on the specific growth rate of isolates. Analyses were conducted in Primer v6 (Primer-E Ltd., UK) using Bray–Curtis dissimilarities on fourth root transformed data and 9999 unrestricted permutations of raw data [1].

2.3. Outdoor salinity tolerance experiment

To determine the salinity tolerance of each strain under intensive cultivation conditions, outdoor growth trials were conducted on all 5 strains under 3 salinity treatments — 1 ppt, 2 ppt, 3 ppt and a control treatment of nutrient enriched (MAF growth medium, Manutech Pty Ltd., 0.05 g L^{-1}, 0.11 ppt) dechlorinated freshwater. Salinity treatments were created by adding NaCl to the nutrient enriched dechlorinated freshwater until desired salinities were reached. Stock cultures of each strain were grown in the control treatment of nutrient-enriched dechlorinated freshwater in 5 L plastic buckets in a greenhouse with ambient natural light at the Marine and Aquaculture Research Facility Unit, James Cook University. Buckets were placed in a water bath with continuous flow to minimize large temperature fluctuations. Average water temperature was 24.2 °C (±2.5 S.D.) and cultures received an average photosynthetically active radiation of 97.4 mol photons m^{-2} $week^{-1}$ (±24.5 S.D.). Cultures were provided with aeration by a continuous stream of air entering the cultures through multiple inlets around the base of the buckets. All experimental replicates were maintained under identical conditions. Stock cultures were maintained in the experimental culture system for a period of at least 3 weeks prior to the start of each experiment to allow acclimation to the culture system and ensure that all strains were pre-exposed to identical conditions. Biomass was transferred from stock cultures into the relevant salinity treatments. Four replicate cultures of each strain were grown under each treatment. Cultures were stocked at a rate of 0.5 g fresh weight (FW) L^{-1} and harvested and weighed after 7 days. Following harvesting, the same biomass was restocked into each replicate and stocking density was reset back to 0.5 g FW L^{-1} by removing excess biomass in each culture. The experiment was run for a total of 3 weeks, providing for 3 harvests with the final week 3 samples used for biochemical analyses (see below).

At each harvest point a sample was taken from the excess biomass of each replicate, spun to remove excess water and weighed to determine the FW. Samples were then dried in an oven at 65 °C for at least 24 h and then reweighed to determine the fresh weight:dry weight (FW:DW) ratio for each individual replicate for each week of growth. The ash content of each replicate was quantified by combusting a 500 mg subsample of dried biomass at 550 °C in a muffle furnace until constant weight was reached. Ash-free dry weight (AFDW) productivity (g AFDW m^{-2} day^{-1}) was calculated for each replicate using the

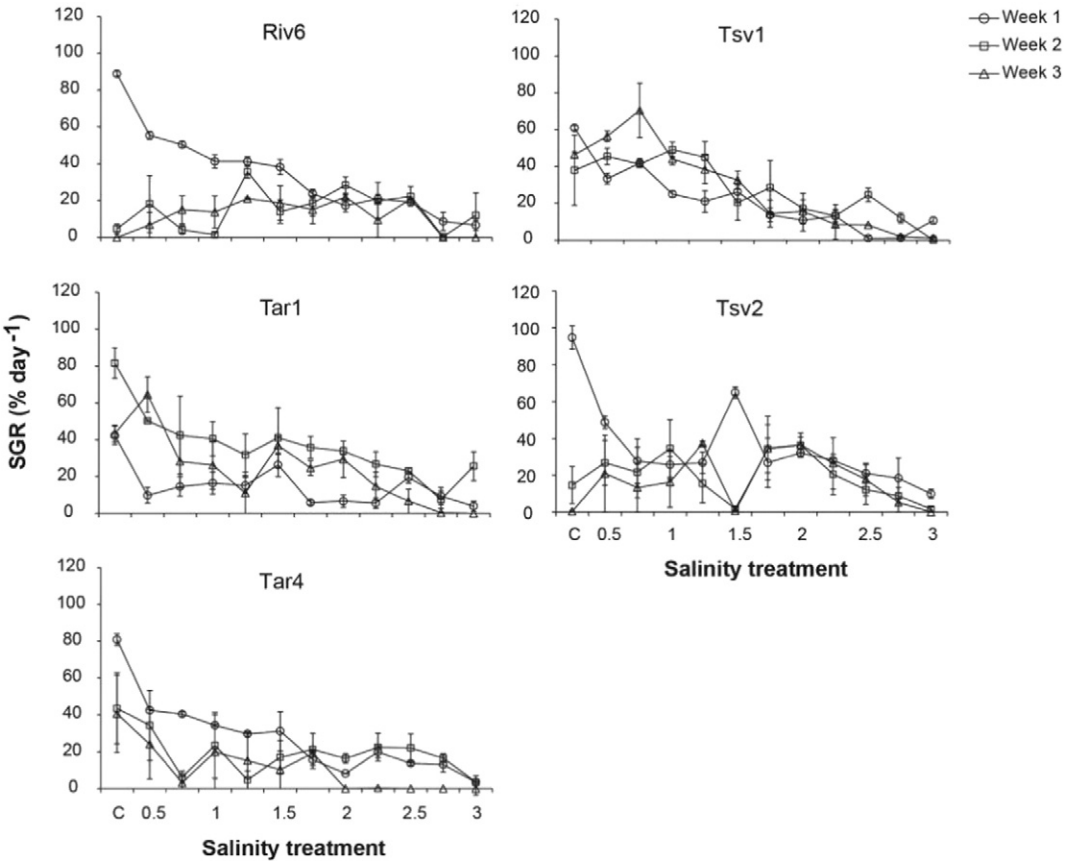

Fig. 1. Mean (±S.E.) specific growth rate (SGR) of 5 strains of *Oedogonium* (Tar1, Tar4, Tsv1, Tsv2, Riv6) under 11 salinity treatments (0.5–3 ppt) and a control (C) of nutrient enriched dechlorinated water in weeks 1, 2 and 3 of the laboratory experiment.

equation $P = \{[(B_f - B_i)/FW{:}DW] * (1 - ash)\}/A/T$, where B_f and B_i are the final and initial algal biomasses (g), *FW:DW* is the fresh weight to dry weight ratio, *ash* is the proportional ash content of the dried biomass, *A* is the area (m^2) of culture tanks and *T* is the number of days in culture. PERMANOVA was used to analyze the effects of strain and salinity (both fixed effects) on the SGR, AFDW productivity, and ash content of replicates in the final week of the experiment only. Analyses were conducted in Primer v6 (Primer-E Ltd., UK) using Bray–Curtis dissimilarities on fourth root transformed data and 9999 unrestricted permutations of raw data [1].

2.4. Effect of salinity on biochemical composition and energy content

Biomass samples from each replicate from week 3 of the outdoor experiment were analyzed for carbon, hydrogen, oxygen, nitrogen and sulfur (ultimate analysis) and total lipid, protein and carbohydrate content. Ultimate analysis was outsourced to OEA labs (http://www.oealabs.com/), while % oxygen was calculated as $\%O = 100 - \sum(C, H, N, S, ash)$ where C, H, N, S, ash are expressed as a percentage of the total mass. Total lipid content (% DW) was determined as described in Gosch et al. [18], while protein was calculated based on the ultimate analysis of nitrogen content (% DW) of the biomass multiplied with a protein to nitrogen factor of ×4.7 [33], and carbohydrate was calculated by difference as $100 - \sum(\text{lipid, protein, ash})$ where lipids, proteins and ash are expressed as a percentage of the total weight. The carbohydrate, protein and lipid productivities (g DW m^{-2} day^{-1}) of each strain were then calculated for each treatment by multiplying the AFDW productivity of each replicate from week 3 of the outdoor experiment by its carbohydrate, protein or lipid content (% DW).

To quantify the suitability of the biomass as a potential energy feedstock, the higher heating value (HHV) was calculated for each sample. The HHV is based on the elemental composition of the biomass and is a measure of the amount of energy stored within. The HHV was calculated using the equation $HHV\ (\text{MJ kg}^{-1}) = 0.3491 * C + 1.1783 * H + 0.1005 * S - 0.1034 * O - 0.0151 * N - 0.0211 * ash$, where *C*, *H*, *S*, *O*, *N* and *ash* are the carbon, hydrogen, sulfur, oxygen, nitrogen and ash mass percentages of the algae on a dry basis [7]. The sulfur content of all replicates was <0.05% and outside the limits of detection and therefore was not included in calculations of HHV. The energy productivity (MJ m^{-2} day^{-1}) of each strain was then calculated for each treatment by multiplying the AFDW productivity (converted to kg AFDW m^{-2} day^{-1}) of each replicate from week 3 of the outdoor experiment by its HHV (MJ m^{-2} day^{-1}).

PERMANOVA was used to analyze the effects of strain and salinity (both fixed effects) on carbohydrate content, protein content, lipid content, HHV, carbohydrate productivity, protein productivity, lipid productivity, and energy productivity of replicates. Analyses were conducted in Primer v6 (Primer-E Ltd., UK) using Bray–Curtis dissimilarities on fourth root transformed data and 9999 unrestricted permutations of raw data [1]. Pairwise correlations between the mean SGR, AFDW productivity, ash content, carbohydrate content, protein content, lipid content and HHV for each strain under each salinity treatment in the outdoor experiment were assessed using SPSS vs 20. Pairwise correlations between the mean SGR of each strain (salinity treatments of 1, 2 and 3 ppt and control treatment only) in week 3 of the laboratory experiment and the mean SGR and AFDW productivity of each strain in week 3 of the outdoor experiment were also assessed to determine whether the performance of individual strains in the laboratory experiment could predict their performance in the outdoor experiment.

3. Results

3.1. Laboratory salinity tolerance experiment

Average specific growth rates (SGRs) in laboratory cultures showed a decreasing trend as salinity increased (Fig. 1). Growth rates varied significantly between strains, however the growth of strains relative to each other also varied between weeks as evidenced by a significant week x strain interaction effect (PERMANOVA, Pseudo$F_{8,360} = 0.58$, $p < 0.001$). In week 1 of the experiment strains Tsv2 and Riv6 had the highest growth rates across most salinity treatments, but in week 3, strains Tsv1 and Tar1 had the highest growth rates in salinity treatments up to 1.5 ppt. There was also a significant week × salinity × strain interaction effect (PERMANOVA, Pseudo$F_{88,360} = 1.35$, $p = 0.03$), reflecting the variable effect of salinity on the growth of individual filaments over the course of the experiment (Fig. 1). Overall, strain Tsv2 was the most tolerant to high salinity treatments, maintaining SGRs above 20% day^{-1} for almost all treatments up to and including 2.25 ppt across the 3 week experiment. Strain Tar4 was the least tolerant to higher salinity treatments, with all replicates dying in salinity treatments above 1.75 ppt by the end of the experiment.

3.2. Outdoor salinity tolerance experiment

SGRs and AFDW productivities in the outdoor experiment significantly decreased with increasing salinity across all 5 strains (Table 1, Fig. 2A and B). However, all strains were able to maintain growth and productivity under the 3 ppt salinity treatment throughout the 3 week experiment and no single strain stood out as being the most tolerant to high salinities. SGRs in the control treatment were 16.7% day^{-1} (±3.7 S.E.) across all strains, almost double those in the 3 ppt treatment of 9.6% day^{-1} (±2.1 S.E.). Similarly, AFDW productivities in the control treatment were 4.7 g m^{-2} day^{-1} (±1.0 S.E.) across all strains, almost double those in the 3 ppt treatment of 2.3 g m^{-2} day^{-1} (±0.5 S.E.).

Table 1
Results of permutational analyses of variance (PERMANOVAs) testing the effects of strain (St) and salinity (Sa) on specific growth rates (SGR), ash-free dry weight (AFDW) productivity, ash content, carbohydrate content, protein content, lipid content and higher heating values (HHV) of 5 strains of *Oedogonium* in the final week of the outdoor experiment. Pseudo F (F) and P values are presented.

Variable	Effect	df	F	P
SGR	St	4	0.5	0.733
	Sa	3	28.4	<0.001
	St × Sa	12	0.4	0.945
	Res	60		
AFDW Productivity	St	4	0.8	0.521
	Sa	3	32.8	<0.001
	St × Sa	12	0.6	0.837
	Res	60		
Ash	St	4	16.1	<0.001
	Sa	3	12.6	<0.001
	St × Sa	12	9.5	<0.001
	Res	60		
Carbohydrate	St	4	7.4	<0.001
	Sa	3	80.4	<0.001
	St × Sa	12	4.8	<0.001
	Res	57		
Protein	St	4	4.2	0.005
	Sa	3	54.8	<0.001
	St × Sa	12	2.1	0.036
	Res	57		
Lipid	St	4	13.8	<0.001
	Sa	3	11.2	<0.001
	St × Sa	12	2.4	0.011
	Res	59		
HHV	St	4	21.0	<0.001
	Sa	3	23.6	<0.001
	St × Sa	12	1.5	0.170
	Res	57		

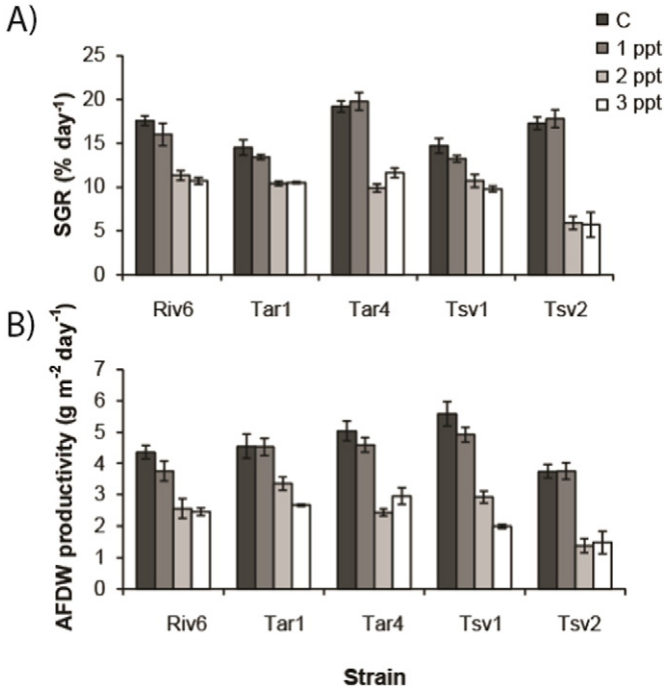

Fig. 2. Mean (±S.E.) A) specific growth rate (SGR, % day^{-1}) and B) ash-free dry weight productivity (g AFDW m^{-2} day^{-1}) of 5 strains of *Oedogonium* (Riv6, Tar1, Tar4, Tsv1, Tsv2) in week 3 of the outdoor experiment under 3 salinity treatments (1–3 ppt) and a control (C) of nutrient enriched dechlorinated water.

There was little variation in AFDW productivities across all strains, with AFDW productivities highest for strain Tsv1 (3.7 g m^{-2} day^{-1} ± 0.3 S.E.) and lowest for strain Tsv2 (3.3 g m^{-2} day^{-1} ± 0.3 S.E.) across all treatments. SGRs and AFDW productivities in the outdoor experiment were positively correlated with each other ($r = 0.805$, $p < 0.001$). There was a weak but significant positive correlation between the SGR of strains in the laboratory experiment and the AFDW productivity ($r = 0.554$, $p = 0.01$) of strains in the outdoor experiment (Appendix 2, Fig. A2). The SGR of strains in the laboratory experiment was not correlated with the SGR of strains in the outdoor experiment ($r = 0.195$, $p = 0.41$).

3.3. Effect of salinity on biochemical composition

Ash contents varied significantly among salinity treatments, however, the effect of salinity was not consistent among strains (Tables 1 & 2). Ash content more than doubled in strains Tsv1 and Tsv2 in the 3 ppt treatment compared to the control. In contrast, ash content decreased with increasing salinity in strain Tar4, and showed no pattern in strains Riv6 and Tar1. Strain Tar4 had the lowest ash content (4.3% ±0.3 S.E.) and strain Tsv1 had the highest ash content (6.7% ±0.7 S.E.) across all salinity treatments.

There was a significant reduction in carbohydrate content with increasing salinity (Table 1, Fig. 3A). Carbohydrate content declined from 79.9% DW (±0.8 S.E.) in the control treatment to 65.8% DW (±1.5 S.E.) in the 3 ppt treatment across all strains. Carbohydrate content was negatively correlated with ash content ($r = -0.634$, $p < 0.001$), protein content ($r = -0.829$, $p < 0.001$), lipid content ($r = -0.686$, $p < 0.001$) and HHV ($r = -0.594$, $p < 0.001$), but was positively correlated with AFDW productivity ($r = 0.593$, $p < 0.001$) and SGR ($r = 0.585$, $p < 0.001$). There was also a significant reduction in carbohydrate productivity with increasing salinity (Table 3, Fig. 4A). Carbohydrate productivity ranged from 1.0 (±0.3 S.E.) to 4.7 (±0.4 S.E.) g DW m^{-2} day^{-1} across all strains and treatments and was highest in the control treatment and lowest in the 3 ppt treatment.

Table 2
Ash content (% DW) and ultimate (% of DW) analysis of 5 strains of *Oedogonium* (Riv6, Tar1, Tar4, Tsv1, Tsv2) at the end of the outdoor experiment under 3 salinity treatments (1–3 ppt) and a control of nutrient enriched water. Values are means (S.E.), N = 4 unless indicated. Data are reported on an "as received" basis.

Strain	Ash	C	H	O	N
Control					
Riv6	6.1 (0.1)	41.4 (0.3)	6.7 (0.1)	43.3 (0.4)	2.5 (0.1)
Tar1	4.0 (0.2)	42.5 (0.2)	6.8 (0.1)	43.8 (0.3)	2.9 (0.1)
Tar4	5.2 (0.8)	41.6 (0.3)	6.7 (0.1)	44.1 (0.7)	2.4 (0.1)
Tsv1	4.0 (0.6)	42.7 (0.2)	7.0 (0.0)	44.2 (0.6)	2.1 (0.1)
Tsv2	4.1 (0.2)	41.8 (0.4)	6.8 (0.1)	44.8 (1.0)	2.6 (0.4)
1 ppt					
Riv6	5.8 (0.4)	42.2 (0.1)	6.7 (0.1)	42.2 (0.6)	3.2 (0.2)
Tar1	3.3 (0.2)	43.5 (0.2)	7.0 (0.1)	43.5 (0.3)	2.7 (0.1)
Tar4	5.2 (0.8)	41.6 (0.4)	6.6 (0.2)	44.0 (0.9)	2.6 (0.1)
Tsv1	4.6 (0.4)	42.9 (0.1)	7.0 (0.0)	43.4 (0.3)	2.0 (0.2)
Tsv2	5.8 (0.5)	42.0 (0.5)	6.7 (0.1)	42.8 (0.4)	2.7 (0.4)
2 ppt					
Riv6	6.8 (0.8)	43.0 (0.1)	6.8 (0.1)	38.9 (0.7)	4.5 (0.1)
Tar1	3.5 (0.1)	45.2 (0.4)	7.2 (0.0)	40.9 (0.6)	3.2 (0.2)
Tar4	3.3 (0.5)	42.5 (0.2)	6.8 (0.2)	43.5 (0.9)	4.0 (0.0)
Tsv1	8.1 (0.7)	43.4 (0.2)	6.9 (0.1)	38.6 (2.0)	3.0 (0.8)
Tsv2	5.2 (0.2)	41.7* (0.4)	6.7* (0.1)	42.9* (0.5)	3.4* (0.3)
3 ppt					
Riv6	6.0 (0.0)	42.9 (0.2)	6.8 (0.0)	39.9 (0.3)	4.4 (0.1)
Tar1	6.6 (0.2)	45.0 (0.2)	7.2 (0.0)	37.2 (0.5)	3.9 (0.1)
Tar4	3.5 (0.8)	43.2 (0.8)	6.9 (0.0)	42.2 (1.0)	4.2 (0.9)
Tsv1	10.2 (0.6)	43.4† (0.3)	7.0† (0.1)	35.4† (0.3)	4.8† (0.3)
Tsv2	7.9 (0.2)	42.1 (0.4)	6.7 (0.0)	38.3 (0.6)	5.0 (0.1)

* N = 3.
† N = 2.

Table 3
Results of permutational analyses of variance (PERMANOVAs) testing the effects of strain (St) and salinity (Sa) on carbohydrate productivity, protein productivity, lipid productivity and energy productivity of 5 strains of *Oedogonium* in the final week of the outdoor experiment. Pseudo F (F) and P values are presented.

Variable	Effect	df	F	P
Carbohydrate productivity	St	4	14.4	<0.001
	Sa	3	93.0	<0.001
	St × Sa	12	3.0	0.003
	Res	56		
Protein productivity	St	4	16.1	<0.001
	Sa	3	10.6	<0.001
	St × Sa	12	3.2	0.003
	Res	57		
Lipid productivity	St	4	19.5	<0.001
	Sa	3	2.0	0.118
	St × Sa	12	3.1	0.003
	Res	59		
Energy productivity	St	4	2.0	0.108
	Sa	3	23.3	<0.001
	St × Sa	12	0.7	0.798
	Res	57		

Protein content increased significantly with increasing salinity (Table 1, Fig. 3B). Protein content across all strains in the 3 ppt treatment was 20.7% DW (±0.7 S.E.), almost double that in the control of 11.7% DW (±0.5 S.E.). Protein content was negatively correlated with AFDW productivity ($r = -0.604$, $p < 0.001$) and SGR ($r = -0.603$, $p < 0.001$). In contrast, salinity had a variable effect on protein productivity (Table 3, Fig. 4B) which ranged from 0.2 (±0.03 S.E.) to 0.6 (±0.05 S.E.) g DW m^{-2} day^{-1} across all strains and treatments and was similar or slightly lower in the 2 and 3 ppt treatments compared to the control.

Salinity had variable effects on lipid content among strains (Table 1, Fig. 3C). Lipid content was higher overall in strains Tar1 and Tsv1 and increased with increasing salinity, increasing from 3.6% DW (±0.9 S.E.) to 12.5% DW (±0.7 S.E.) for Tar1 and 4.7% DW (±1.9 S.E.) to 12.0% DW (±0.8 S.E.) for Tsv1 in the control treatment and 3 ppt treatment respectively. In contrast, the lipid content of strains Riv6, Tar4 and Tsv2 was similar, ranging from 3.3 (±0.4 S.E.) to 5.8 (±0.5 S.E.) % DW, with little difference in lipid content between salinity treatments for each of these strains. Lipid content was positively correlated with HHV ($r = 0.726$, $p < 0.001$) and negatively correlated with AFDW productivity ($r = -0.308$, $p = 0.006$). Salinity also had variable effects on lipid productivity among strains (Table 3, Fig. 4C). Lipid productivity decreased with increasing salinity in strains Riv6, Tar4 and Tsv2, increased with increasing salinity in strain Tar1, and was comparable across all treatments for strain Tsv1. Lipid productivity was higher overall in strains Tar1 and Tsv1 (0.3 g DW m^{-2} day^{-1}) compared to Riv6, Tar4 and Tsv2 (<0.15 g DW m^{-2} day^{-1}).

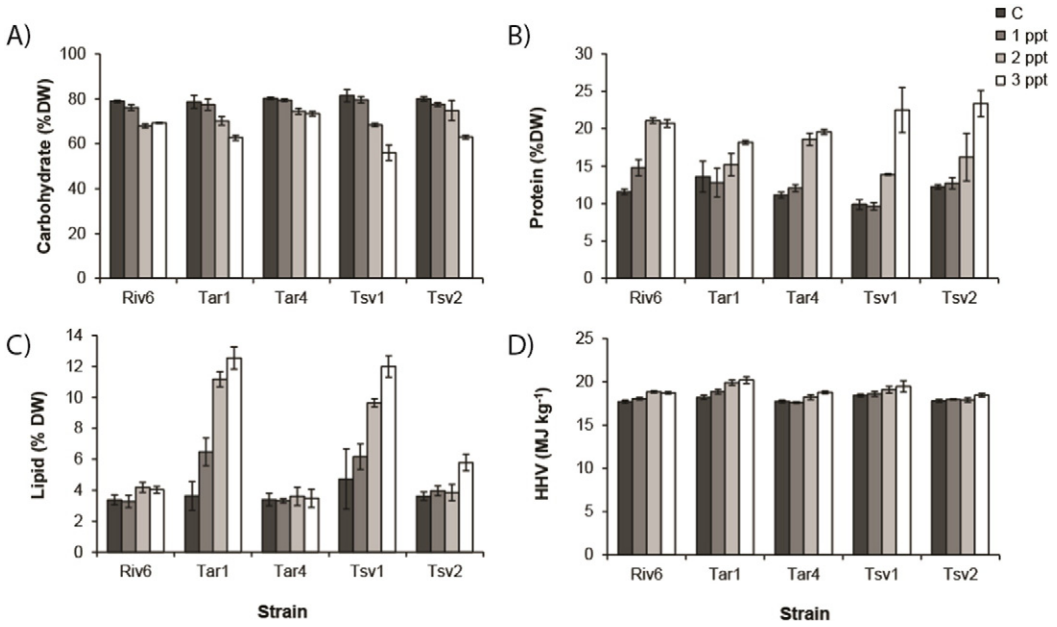

Fig. 3. Mean (±S.E.) A) carbohydrate content (% DW); B) protein content (% DW); C) lipid content (% DW) and D) higher heating value (HHV, MJ kg^{-1} DW) of 5 strains of *Oedogonium* (Riv6, Tar1, Tar4, Tsv1, Tsv2) in week 3 of the outdoor experiment under 3 salinity treatments (1–3 ppt) and a control (C) of nutrient enriched dechlorinated water.

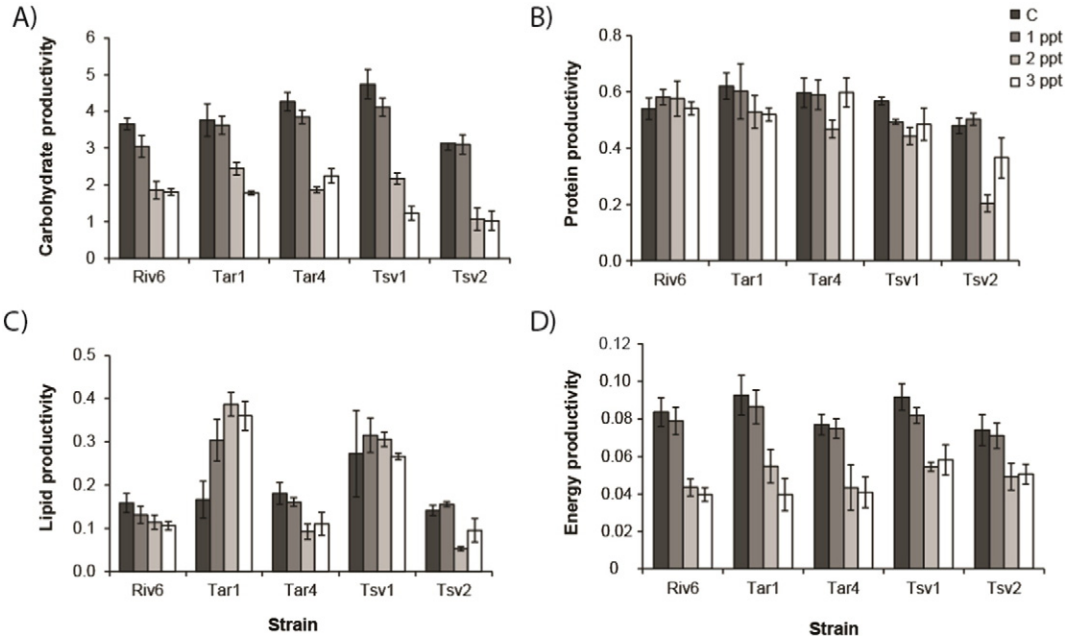

Fig. 4. Mean (±S.E.) A) Carbohydrate productivity (g DW m^{-2} day^{-1}); B) Protein productivity (g DW m^{-2} day^{-1}); C) lipid productivity (g DW m^{-2} day^{-1}); and D) energy productivity (MJ m^{-2} day^{-1}) of 5 strains of *Oedogonium* (Riv6, Tar1, Tar4, Tsv1, Tsv2) in week 3 of the outdoor experiment under 3 salinity treatments (1–3 ppt) and a control (C) of nutrient enriched dechlorinated water.

There was a small but significant increase in HHVs with increasing salinity across all strains (Table 1, Fig. 3D). HHVs were lowest under the control treatment (18.0 MJ kg^{-1} ± 0.1 S.E.) and highest under the 3 ppt treatment (19.1 MJ kg^{-1} ± 0.2 S.E.) across all strains. HHVs also varied significantly among strains (Table 1). Strain Tsv2 had the lowest HHV (18.0 MJ kg^{-1} ± 0.1 S.E.) and strain Tar1 had the highest HHV (19.3 MJ kg^{-1} ± 0.3 S.E.) across all treatments. In contrast, energy productivity decreased with increasing salinity across all 5 strains (Table 3, Fig. 4D). Energy productivity across all strains halved from 0.08 MJ m^{-2} day^{-1} (±0.004 S.E.) in the control treatment to 0.04 MJ m^{-2} day^{-1} (±0.003 S.E.) in the 3 ppt treatment.

There was a slight increase in carbon content, a noticeable increase in nitrogen content and a decline in oxygen content with increasing salinity across all 5 strains, while hydrogen content was similar across all salinity treatments and strains (Table 2).

4. Discussion

A critical knowledge gap in the production of macroalgae for biomass applications is the ability of target species to grow in saline groundwaters that are characteristic of marginal or non-arable land. Through a controlled small-scale laboratory experiment and an outdoor experiment under ambient conditions, we show that although growth and productivity decreased under increasing salinity, multiple strains of *Oedogonium* – a target freshwater macroalgal genus for biomass applications – were able to maintain growth at salinities of up to 3 ppt but died at salinities of 4 ppt and higher. These values equate to approximately 0.3% to 8.6% of seawater salinity and many groundwaters are commonly up to 10% of the salinity of seawater (35 ppt). Moreover, we show that increased salinity had significant positive effects on the biochemical composition of biomass, with higher protein contents and energy contents consistently recorded in biomass grown under higher salinity treatments (2 and 3 ppt) across all 5 *Oedogonium* strains. Two strains also had higher lipid contents at the highest salinity. However, biomass productivity was the most important driver of bioproduct potential. The large decreases in biomass productivity (3-fold decreases) under increased salinity were effectively offset by the large increases in protein content (2-fold increases). However, the decreases were not offset by the relatively small increases in lipid content (10%) or energy content (2%).

The decrease in growth and biomass productivity with increasing salinity was not unexpected as similar findings of decreased growth under salinity stress have been reported in a range of studies on both macro- and microalgae and marine and freshwater species (e.g., [5,14,22,38]). A reduction in growth and biomass productivity under salinity stress is most likely due to a redirection of available energy towards processes such as osmoregulation rather than cell growth and photosynthesis [2,8,25,48]. Photosynthesis, growth and survival of macroalgae are typically highest at those salinities that the algae are predominantly exposed to in their natural environment [25]. However, a broader range of salinities can be tolerated when light and temperature are closer to species specific optima [25]. Salinity tolerance may also be increased when nutrient availability, particularly nitrogen, is increased [8,13,35]. This interactive effect of other environmental parameters on the salinity tolerance of algae may explain the lower tolerance to salinity in the laboratory experiment compared to the outdoor experiment and the weak correlation between the two experiments. Consequently, it may be possible to improve the salinity tolerance from that measured in the current study if cultures of *Oedogonium* are maintained under tailored nutrient levels, and light and temperature regimes, or are originally isolated from more saline natural habitats.

Freshwater macroalgae have been proposed as an alternative animal feed source or supplement on the basis of the protein content and energy potential of the biomass [11,31,53]. The protein contents recorded here under increased salinity (13.9–23.4% DW in the 2 and 3 ppt treatments) are within the range reported in the literature for *Oedogonium* (7–44% DW, [11,31]) and are comparable to many traditional crops used as a source of protein for animal feeds (8–43% DW, [21,23,37,54]). Moreover, we found substantial increases in protein content and small increases in energy content (as measured by HHV) for the 5 strains of *Oedogonium* with increasing salinity, and importantly, protein productivity did not markedly decrease. These results demonstrate that the cultivation of *Oedogonium* in saline groundwater could provide a sustainable source of biomass for animal feed applications. The increases in protein content with increasing salinity are most likely due to the decoupling of the processes of nutrient acquisition from those of carbon fixation [4]. Net rates of photosynthesis and carbon fixation

in macroalgae are generally lower under hypo- or hypersaline conditions [25,39], while nitrogen uptake rates remain largely unaffected [26] or increase [46]. This results in an increased concentration of protein in the biomass relative to other components. In addition to affecting the total quantity of protein in the biomass, salinity can also affect the quality of protein (i.e. amino acid composition) [2,30]. More in depth analysis of the quality of protein in *Oedogonium* biomass cultivated under increased salinity will be necessary, however, to date the composition of amino acids in *Oedogonium* appears to be relatively hard-wired across environmental conditions, particularly the key essential amino acids of lysine (6.8–7.3% of protein) and methionine (1.6–1.9% of protein) [11]. Feedstocks with high concentrations of lysine are valuable as essential amino acids such as lysine are often supplemented to animal feeds [6]. Of particular interest for animal feed applications was strain Tsv1. When cultivated under the 3 ppt salinity treatment, this strain had a high protein content and a high HHV, highlighting the importance of strain selection in maximizing the biochemical composition of the biomass for target applications.

Macroalgae have also recently emerged as a promising feedstock for the production of bioenergy [20,34,43,52,55]. The lipid content of algal biomass is a critical component for biocrude yield, a promising pathway to biofuels from macroalgae [15,43,44]. The lipid content of strains Tar1 and Tsv1 increased noticeably with increasing salinity. Moreover, the lipid contents measured for Tar1 and Tsv1 grown under salinity treatments of 2 and 3 ppt (9.6–12.5% DW) are higher than those reported for *Oedogonium* (5.3–9.4% DW) grown in non-saline waters (i.e. 0%, current study and [33]), and comparable to those reported for species of marine macroalgae characterized as having a high lipid content (10–12% DW, [18]). To our knowledge, this is the first time that salinity has been reported to significantly affect the lipid content of macroalgae, as other factors (temperature and nutrients) are more commonly recognized as drivers [16,19,24]. However, in contrast to the increases in protein content under increased salinity, the increases in lipid content for strain Tsv1 were not large enough to offset the significant decreases in productivity with increasing salinity and, consequently, lipid productivity for all strains except Tar1 decreased with increasing salinity. Furthermore, energy productivity decreased with increasing salinity across all 5 strains. These results demonstrate that *Oedogonium* biomass cultivated in saline groundwater is less suited to the production of biocrude, or bioenergy more generally, based on the energy productivity per unit area.

5. Conclusions

Increased salinization is a significant global problem with more than 50% of all arable lands expected to be affected by salinity by the year 2050 [51]. Cultivation of macroalgae in saline groundwater would provide a sustainable source of biomass without impacting on agriculture. We have demonstrated that strains of the freshwater macroalgal genus *Oedogonium* can be grown at salinities of up to 3 ppt and that increased salinity can improve the suitability of biomass for animal feed applications through increases in protein and energy content, and for bioenergy through increases in lipid content. However, trade-offs between growth and lipid or energy content must be considered for overall production per unit area of land as lipid and energy productivity decrease with increasing salinity. In contrast, salinity had negligible effects on protein productivity, and therefore *Oedogonium* could be cultivated more successfully in saline groundwater for animal feed applications. The significant salinity by strain interaction for multiple biochemical characteristics highlights the importance of strain selection to maximize biomass and biochemical productivities from *Oedogonium* and supports further screening in natural saline environments to identify strains with a higher salinity tolerance. Alternatively, selective breeding of high performing strains such as Tsv1 under increased salinity may lead to heritable increases in biomass productivity and traits of interest [29,45].

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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.2015.09.001.

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