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Evaluating the performance of freshwater macroalgae in the bioremediation of nutrient-enriched water in temperate environments

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Abstract

Algal bioremediation can significantly improve the quality of wastewater by assimilating nutrients. However, the efficiency and stability of this approach depends on identifying suitable algae based on their biomass productivity and ability to outcompete less desirable algae. Here, we compare the productivity and competitive ability of three taxa of filamentous macroalgae under the seasonal light and temperature conditions experienced in temperate environments, including extremes of heat and cold. Specific growth rate was greatest for the tropical isolate of *Oedogonium* under summer conditions (36–40%; $P < 0.05$); however, it had lower growth under cooler (autumn, winter) conditions than the temperate algae of *Stigeoclonium* and *Hyalotheca*. Overall, *Stigeoclonium* and *Hyalotheca* had the most stable production across all treatments. A 5-week competition experiment found that each algae grew fastest in monoculture compared with bi-culture and poly-culture treatments. While all three genera showed a considerable level of competitive dominance depending on algae composition and environmental conditions, no single genus outperformed all others under all conditions. *Oedogonium* was dominant in warmer conditions, *Stigeoclonium* in cooler conditions (> 90% for both) and, in its absence, *Hyalotheca* also dominate over *Oedogonium*. Our results suggest that rather than finding an optimal taxon for all four seasons, the best decision for maximising stable biomass production will require either seasonal rotation of algae, or bi-cultures of the most dominant ones. Further, prioritising competition over production when selecting freshwater algae for wastewater bioremediation is likely to prove the most successful strategy.

Keywords Algae · Biomass · Competition · Growth · Species dominance · *Oedogonium*

Introduction

Humans are altering virtually every environment at an unprecedented rate and extent, and we now represent the Earth's most important biotic selective force (Holdren and Ehrlich 1974; Vitousek et al. 1997; Palumbi 2001). In 2008, more than half of the world's population lived in urban areas for the first time (Martine and Marshall 2007) posing major challenges to the safe, reliable and sustainable use of natural resources. Nowhere are these pressures felt more acutely than in coastal areas (Rabalais et al. 2009; Pelling and Blackburn 2014). Today, approximately 3 billion people, about half of the world's population, live within 200 km of a coastline (Wright et al. 2019) with that figure projected to double by 2025 (Creel 2003). Based on these projections, the global impacts of urban growth will necessitate significant policy changes in how coasts are developed, and environmental impacts managed.

One of the most pressing challenges is how to meet the growing waste management needs of coastal cities (Lu et al. 2012; Pelling and Blackburn 2014). Estuaries and coasts adjacent to cities are the receiving environments of aquatic pollution from urban run-off, industrial effluent, and treated sewage, which can erode the biological integrity of coastal marine ecosystems and the services they provide (Kimor 1992; Smith

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2003; Rabalais et al. 2009; y Royo et al. 2009). Managing wastewaters before they enter marine ecosystems to reduce contaminant and nutrient inputs is thus a major objective of water management.

Traditionally, governments have focused primarily on engineered solutions in wastewater treatment plants, but these are becoming progressively more difficult to implement because of the increasing costs of upgrading infrastructure (Grigg 2012). International initiatives around water governance aimed at improving water quality have advocated a separate approach (e.g. the United Nation's 2030 Sustainable Development Goals (Colglazier 2015)), whereby wastewaters are viewed as potential resources that can be used by other industries. One alternative approach has been the application of industrial ecology to reduce pollution impacts by making waste products accessible as biomass with a focus on its use as an energy resource (Demirbas 2010; Nzihou and Lifset 2010; Cole et al. 2014a).

The cultivation of macroalgal biomass has several advantages over engineered solutions for treating wastewaters as it uses wastewater as a nutrient source (Roberts et al. 2013; Cole et al. 2014a) and in doing so, reduces both waste and the energy typically required for engineered solutions (Wett et al. 2007; Cole et al. 2016a; Lawton et al. 2017). In addition, bio-products from macroalgal biomass may offset treatment costs, improving economics (Cole et al. 2017).

A common challenge in integrating macroalgal culture into wastewater treatment is to identify an appropriate algae for monoculture that will provide low contamination and stable rates of production (Priyadarshani et al. 2012), resulting in little change in output benefits (i.e. biomass quantity) over time (Lawton et al. 2013). Many wastewater sources are freshwater, requiring a focus on suitable freshwater algal species. Common traits selected for in the industrial production of algal biomass include high growth rate and consequently biomass productivity per unit area (Goldman and Ryther 1975), consistent biochemical properties, robustness to live in challenging environments with diverse pollutants, the ability to utilise multiple nutrient sources (Shukla et al. 2019) and competitive dominance to prevent open cultures from becoming overgrown by non-desirable species that could compromise product purity and value (Lawton et al. 2013). Based on cultivation experiments in tropical environments, freshwater macroalgal species from the genus *Oedogonium* fulfill the requirements of high growth rates and competitive dominance (Lawton et al. 2013; Zhu et al. 2015; Neveux et al. 2016). However, the capacity of *Oedogonium*, or other freshwater macroalgal species, to perform similarly in terms of growth and competitive dominance in wastewater in temperate environments remains untested.

Here, we evaluated the performance of freshwater macroalgae for the bioremediation of nitrogen waste under conditions representative of seasonal averages and extremes in temperate south eastern Australia. We compared specific growth rate, biomass productivity and biochemical composition of three genera that occur in temperate environments (Day et al. 1995) under environmental conditions representative of hypothetical local culture systems. Algae were selected based on either their local abundance, presence in urban aquatic environments and/or high growth rate. We compared the performance of each genus as a monoculture, in bi-cultures and in a poly-culture, under seasonal conditions (temperature and light), to determine whether the paradigm of monospecific cultivation of freshwater macroalgae can be maintained in large, open culture systems in temperate regions.

Methods

Taxa descriptions

The experiments used three freshwater macroalgae from the genera *Stigeoclonium*, *Hyalotheca* and *Oedogonium*. *Stigeoclonium* is a branched algae (cell diameter 3–10 μm) and is typically attached to rocks, leaves or stems in lakes, mountains and lowland streams or urban creeks. *Hyalotheca* is an unbranched algae (cell diameter 5–15 μm) found in semi-eutrophic streams, often loosely attached. Finally, *Oedogonium* is an unbranched algae (cell diameter 18–32 μm) found freely floating or attached to rocks, wood or aquatic plants. Representatives of these genera choke water ways during blooms and consequently are associated with high growth rates and competitive dominance (Entwisle et al. 1997). This last trait makes them ideally suited for this study. Genera were identified using taxonomic keys (Entwisle et al. 1997).

Culture methods

Stock samples from the three genera were collected from Townsville, Queensland, (*Oedogonium*); ponds in North Melbourne (*Stigeoclonium* and *Hyalotheca*) and from Melbourne Water's waste treatment facility in Werribee, Victoria (*Stigeoclonium* and *Hyalotheca*), Australia. *Oedogonium intermedium* was selected as it has a distribution across Australia, including SE Australia and Victoria (Day et al. 1995). However, the tropical isolate was used as a comparative reference for one that performs well under tropical (high light, high temperature) environments (Lawton et al. 2013). A total of 101 filaments were isolated from the North Melbourne ponds, cleaned using filtered and UV-sterilised fresh dechlorinated water and identified to genus level.

From these isolates, 27 were selected and grown in Petri dishes in a culture cabinet with a 12-h photoperiod (3.7 mol photons m^{-2} day^{-1}), after which they were placed into 1-L culture bottles to create stock cultures of each genus

for 3 weeks. Cultures were provided with continuous aeration and culture water enriched (0.1 g L^{-1}) with F2 growth medium (Algaboost) in all stages of the growth. The water volume was topped up with enriched culture water to 1 L when needed to maintain equal water volume conditions across all bottles.

Selection experiment

Water temperature and light intensity were tested at levels representative of conditions across the seasons (as described below) to assess their effect on growth rates and productivity for each of the three genera. For each genus, cultures were grown in 1-L culture bottles under one of six combinations of temperature and light using a stocking biomass of 0.5 g of fresh weight per litre (g FW L^{-1}) taken from all previous biomass stock cultures. Each treatment was replicated four times for each genus (Fig. 1) and the experiment repeated three times ($n = 72$ cultures per genus). Macroalgae were aerated to promote vertical movement, which increases areal productivity (Gonen et al. 1993; Hurd 2000).

Light conditions were based on seasonal daily mean irradiance information of three recording stations near Melbourne (Melbourne airport, Werribee and Essendon). Temperatures for cultivation were based on seasonal daily mean air temperatures for Melbourne: 20 °C (summer), 15 °C (autumn/spring), 10 °C (winter). In addition, 5 and 25 °C were chosen to simulate the extreme temperatures experienced during winter and summer that can persist for extended periods.

The six temperature (mean ± SD) and light conditions in the cabinets were: 19.8 °C ± 0.7 and 3.7 mol photons m^{-2} day^{-1} (summer), 15.3 °C ± 0.6 and 1.7 mol photons m^{-2} day^{-1} (autumn), 15.0 °C ± 0.4 and 2.53 mol photons m^{-2} day^{-1} (spring), 8.8 °C ± 0.9 and 0.66 mol photons m^{-2} day^{-1} (winter), 4.8 °C ± 0.3 and 0.66 mol photons m^{-2} day^{-1} (average minimum/cold snap) and 25.1 °C ± 0.5 and 3.7 mol photons m^{-2} day^{-1} (average maximum/heat wave).

Cultures were harvested and weighed after 1 week. After harvesting and subsequent measurements, the experiment was repeated, resulting in three replicate experiments conducted over three consecutive weeks. Harvested samples were dried in a desiccator at 65 °C for 24 h to obtain their fresh weight:dry weight ratio (FW:DW). The ash content was obtained by combusting a 500-mg subsample of dried biomass (when it was available) at 550 °C in a muffle furnace until weight stabilised.

For each harvest, we obtained the specific growth rate (SGR), the dry weight productivity (DW), the ash free dry weight (AFDW) and consequently the ash free dry weight productivity (g AFDW m^{-2} day^{-1}). SGR provides information on the relative growth rates of individuals within the culture. SGR (% day^{-1}) was calculated using the equation $\mathrm{Ln}(B_f/B_i)/\mathrm{T} \times 100$, where B_f and B_i are the final and initial algal biomass (g), respectively, and T is the number of days in culture (Lawton et al. 2014). DW productivity informs about the total biomass yield and was calculated using the following equation DW Yield = ([$(B_f - B_i)$/FW:DW]) / A/T), where FW:DW is the fresh weight to dry weight ratio, B_f and B_i are the final and initial algal biomass (g), A is the volume (m^3) of our culture tanks and T is the number of days in culture. AFDW productivity takes into account changes in ash content across conditions by measuring of the amount of organic biomass (without ash) produced per unit area and was calculated using the equation $P = ([(B_f - B_i)/\mathrm{FW:DW}] \times 1\text{-Ash}) / \mathrm{A/T}$, where FW:DW is the fresh weight to dry weight ratio, B_f and B_i are the final and initial algal biomasses (g), ash is the proportional ash content of the dried

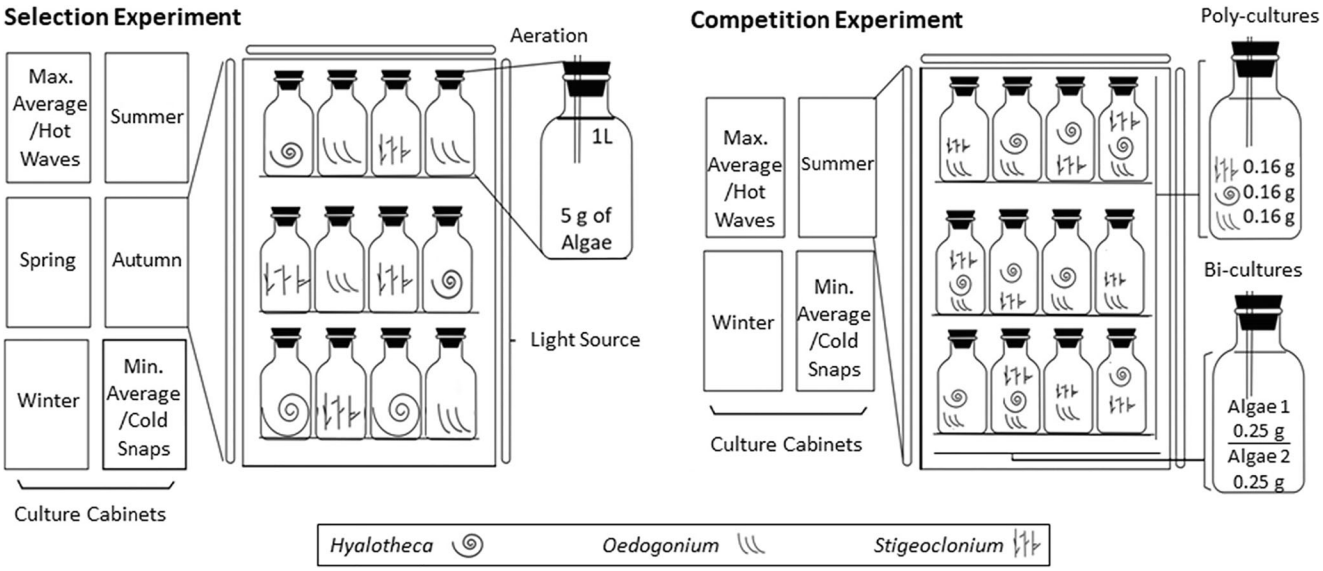


Fig. 1 Schematic diagram of experimental setup. Randomised distribution of bottles and quantities of each algae in the cabinets used in both the selection and the competition experiments.

biomass, A is the volume (m^3) of our culture tanks and T is the number of days in culture.

Biomass samples of the stock cultures were analysed for elemental components (Ultimate analysis, OEA Laboratories, UK). To evaluate the potential of each algae for applications to bioenergy, we calculated the HHV (higher heating value) for each sample. This data reflects the energy stored within the biomass and is based on its elemental composition. The formula used to calculate the HHV was as follows: HHV (MJ kg^{-1}) $= 0.3491 \times C + 1.1783 \times H + 0.1005 \times S - 0.1034 \times O - 0.0151 \times N - 0.0211 \times$ ash, where C, H, S, O, N and ash are the carbon, hydrogen, sulphur, oxygen, nitrogen and ash percentages of the dried algae, respectively (Channiwala and Parikh 2002).

Competition experiment

To investigate the competitiveness of the three algal genera in open culture, three replicates of all possible bi-cultures and one poly-culture combination of all three algae were grown (Fig. 1) with equal inoculation rates (bi-culture (50:50) or poly-culture, (33:33:33); summing up to 0.5 g FW L^{-1}) and under one of the following four temperature and light combinations (mean ± SD), 5.2 °C ± 0.4 and 0.66 mol photons m^{-2} day^{-1} (cold snap); 8.7 °C ± 0.2 and 0.66 mol photons m^{-2} day^{-1} (winter); 19.7 °C ± 0.6 and 3.7 mol photons m^{-2} day^{-1} (summer); and 25.16 °C ± 0.6 and 3.7 mol photons m^{-2} day^{-1} (heatwave). Average pH in the culture media was measured every week, resulting in a mean of 7.7 ± 0.2.

Replicates were harvested and weighed after 1 week, and a 0.4-g FW biomass sample was taken from each replicate to calculate percentage algae composition (see description below), and then each replicate was reset to a starting total algal biomass of 0.5 g FW L^{-1}. This was done because the culture bottles become space limiting without thinning. Individual FW:DW ratios and ash contents were calculated for each replicate as described above. This procedure was repeated weekly for 5 weeks, the media were topped up when needed and the algae ratios were not reset after each week.

The biomass sample extracted (0.4 g FW), for every replicate, was suspended in 200 mL dechlorinated water and images of 8 replicate subsamples (example found in Online Resource; Fig. 6) were taken using a dissecting microscope (LEICA DM LB) with an Olympus DP26 camera (a total of 1920 images for the whole experiment) and used to estimate the proportion of each alga in the bi-cultures and poly-culture. This was done by placing a 100-point grid included in the microscope ocular randomly over each image and calculating the proportional algae composition by summing the number of grid points directly overlying each algal filament.

For each replicate, specific growth rates (SGR) were calculated for all algae. To evaluate SGR under competition, we modified the previous formula. B_f and B_i are the final and initial biomasses of the target algae within each culture. B_f was calculated by multiplying the total final FW biomass of each replicate by the proportional composition of the target algae in that replicate. In week 1, B_i was calculated as half (bi-cultures) or one-third (poly-culture) of the total initial biomass stocked. In weeks 2, 3, 4 and 5, B_i was calculated by multiplying the resulting (i.e. the previous week's) total FW biomass by the current proportional composition of the target algae in each replicate.

Statistical analyses

A linear mixed effects model was used to analyse the parameters of growth and productivity of the selection experiment through a 3-factor randomised complete block ANOVA. The three main effects were taxa, treatment and their interaction (all fixed factors, with 3, 6 and 18 levels, respectively), with week as a random blocking factor. The response variables were Ash, FW:DW, SGR, DW and AFDW. Prior to analysis, DW, AFDW and FW:DW ratio were log+1, log and fourth-root transformed, respectively, to homogenise variances.

As the differences between monocultures and bi/poly-cultures were also important, three two-way ANOVAs tested for differences in SGR of genus grown separately against their combinations with the other two genera. When in combination, SGR was estimated as the dominance percentage of the selected genus during their first week. Since only four temperatures were used in the competition experiment (5, 10, 20 and 25 °C), the two additional treatments from the selection experiment were excluded from this analysis. The main effects were temperature, algae combination and their interaction (with 4, 4 and 16 levels, respectively). The response variable was the specific growth rate. Tukey's honestly significant difference (HSD) test was used to compare means among the treatments and taxa. All statistical analyses were performed using the REML platform in JMP v12.0.1. (SAS Institute, Inc., USA).

Results

Selection experiment

There were significant differences among treatments in ash content, SGR and DW and among genera in DW and FW:DW but these effects were not independent (Table 1).

SGR was highest in warmer conditions (max. average and summer) (30–40% day^{-1}; $P < 0.05$) with *Oedogonium* having a higher SGR (40% day^{-1}; $P < 0.05$) than both *Stigeoclonium* (34% day^{-1}; $P < 0.05$) and *Hyalotheca* (32% day^{-1}; $P < 0.05$) (Fig.2a). There was a large and significant decline in SGR in cooler conditions (winter, min. average) and a reversal in

Table 1 Results of the linear mixed effects model testing for the influence that the taxa and treatments have over the production indicators. No *F* and *P* values are reported for those factors that include the randomised block term (week), as there are no tests for these. Values in italics are significant at $P < 0.05$.

Source	MS	DF	*F*	*P*
SGR				
Taxa	144.099	2	1.0891	0.4192
Treatment	4644.46	5	293.0871	*< 0.0001*
Taxa*Treatment	167.697	10	5.1687	*0.0009*
Week	96.5981	2	–	–
Taxa*Week	132.315	4	–	–
Treatment*Week	15.8467	10	–	–
Taxa*Treatment*Week	32.4445	20	–	–
FW:DW				
Taxa	2.5525	2	76.0704	*0.0007*
Treatment	0.05713	5	1.4375	0.2918
Taxa*Treatment	0.07768	10	2.5434	*0.0362*
Week	0.00495	2	–	–
Taxa*Week	0.03355	4	–	–
Treatment*Week	0.03974	10	–	–
Taxa*Treatment*Week	0.03054	20	–	–
DW				
Taxa	1.28669	2	9.1860	*0.0320*
Treatment	4.22108	5	311.7872	*< 0.0001*
Taxa*Treatment	0.16668	10	5.5390	*0.0006*
Week	0.01003	2	–	–
Taxa*Week	0.14007	4	–	–
Treatment*Week	0.01354	10	–	–
Taxa*Treatment*Week	0.03009	20	–	–
Ash				
Taxa	0.00043	2	2.7021	0.1809
Treatment	0.00253	5	40.7772	*< 0.0001*
Taxa*Treatment	0.0001	10	2.786	*0.0245*
Week	3.25E−05	2	–	–
Taxa*Week	0.00016	4	–	–
Treatment*Week	6.21E−05	10	–	–
Taxa*Treatment*Week	3.65E−05	20	–	–

hierarchy with *Stigeoclonium* (18% day^{-1}; $P < 0.05$) and *Hyalotheca* (13% day^{-1}; $P < 0.05$) having a higher SGR than *Oedogonium* (5% day^{-1}; $P < 0.05$).

Oedogonium had a higher FW:DW ratio, accumulating twice the amount of water of temperate genera when grown under cooler conditions, including spring (Fig.2b 11.2 ± 3.2; $P < 0.05$ for *Oedogonium*; 5.7 ± 0.1; $P < 0.05$ for *Stigeoclonium*). This is reflected in productivity and supports strong seasonality and consequentially the dominance of *Oedogonium* in warmer temperatures, and *Stigeoclonium* and *Hyalotheca* in cooler temperatures.

There was large variation in DW productivity between genera and seasons due to the combined effects of SGR and FW:DW (Fig.2c). All taxa had a similar DW between 7 and 11 g m^{-2} day^{-1} under warmer conditions (max. average, summer). However, there was a dramatic reduction in SGR for *Oedogonium* from ~ 10 g m^{-2} day^{-1} ($P < 0.05$) in summer to a less than a half of that rate under temperate temperatures (spring, autumn) (~ 2–3% day^{-1}; $P < 0.05$) and reaching minimum levels under cooler conditions (winter, min. average) (< 1% day^{-1}; $P < 0.05$). The SGR for *Stigeoclonium* and *Hyalotheca* was more than twice that of *Oedogonium* in cooler conditions (Fig.2c).

The ash content of all three genera was low at < 10% (Fig. 2d; $P < 0.05$). Ash content generally ranged between 4 and 8% ($P < 0.05$), with *Oedogonium* reaching ~ 8% in temperate conditions (spring, autumn) and *Stigeoclonium* and *Hyalotheca* ~ 6% in winter (Fig. 2d).

The biochemical profiles of the three algae were similar with comparable profiles for ash and CHONPS, and consequently HHV (Table 2). In terms of nutrient assimilation, all algae had a similar proportion of nitrogen (4.24–4.4%) and phosphorous (0.39–0.53%). In terms of energy potential *Hyalotheca* had the highest carbon content (46%) and higher heating value (HHV > 20 MJ kg^{-1}) followed by *Stigeoclonium* (6.74% C, 19.63 MJ kg^{-1}) and *Oedogonium* (6.69% C, 18.66 MJ kg^{-1}).

Competition experiment

There were large differences in specific growth rate depending upon whether algae were cultured as a monoculture, or in mixed culture as either a bi-culture or a poly-culture. SGR was strongly affected by the temperate and light conditions applied (two-factor ANOVAs, Table 3; Fig. 3).

The highest SGRs were found under warmer conditions (max. average, summer). Under these conditions, monocultures generally had the highest SGRs for each alga and were between 2 and 20% greater than bi- and poly-cultures in warmer conditions (Fig. 3a and b). *Oedogonium* had the highest SGR under these conditions ~ 5% greater than *Stigeoclonium* and *Hyalotheca* (Fig. 3a). In bi-cultures those containing *Oedogonium* generally had a higher SGR (Fig.3a and b)) than bi-cultures of only *Hyalotheca* and *Stigeoclonium*. *Oedogonium* had the highest growth rate in poly-cultures of all three algae in summer conditions and that difference was amplified under extremely hot conditions.

Specific growth rates decreased for all algae and cultures (mono, bi- and poly) in cooler conditions (winter, min. average) (Fig. 3c and d) and in general, all cultures containing *Stigeoclonium* or *Hyalotheca*, as a monoculture or bi-culture, outperformed those containing *Oedogonium*. In contrast to the warmer months and consistent with the results above, monocultures of *Stigeoclonium* and *Hyalotheca* (Fig. 3c and d) had a much higher SGR than *Oedogonium*. Bi-cultures containing

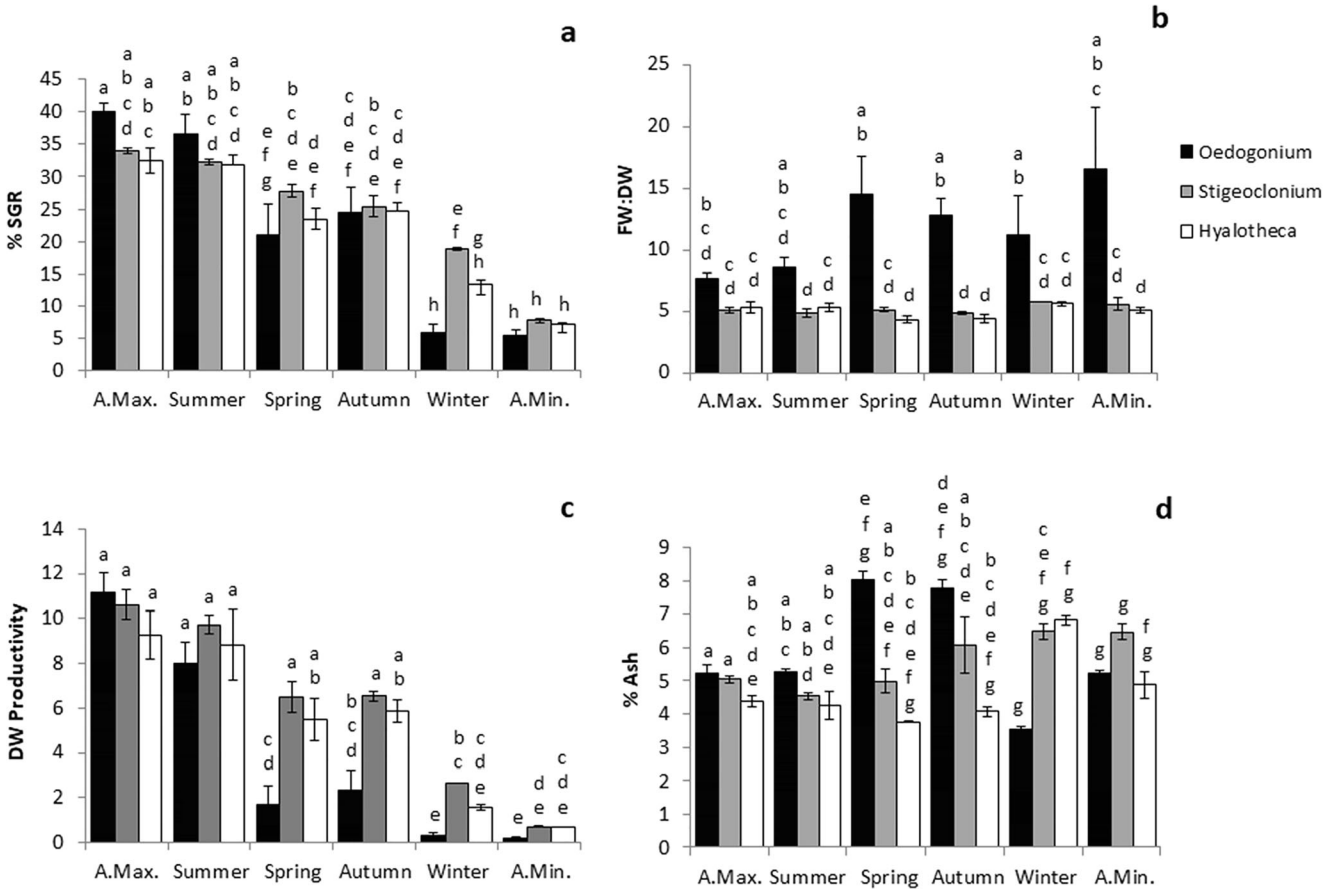


Fig. 2 Productivity growth rates, FW:DW ratios and ash contents of macroalgae cultures. Mean (±S.E.) specific growth rate (SGR, % day) (a); FW:DW ratio (b); dry weight productivity (g m^{-2} day^{-1}) (c) and ash content (d) of the three macroalgae grown under six combinations of light and temperature. Standard errors are calculated as the variation in means between the 3 weeks of the experiment ($n = 3$). Letters above bars represent the results of Tukey HSD tests of the taxa by treatment interaction from each linear model in Table 1. For each panel, columns not connected by the same letter are significantly different.

Stigeoclonium and *Hyalotheca* generally had a lower SGR than monocultures for each alga (Fig. 3c and d); however, the poly-culture had a comparable, or greater, SGR for algae cultured under these cooler conditions (winter, min. average) (Fig. 3c and d).

There were large and compelling changes in the proportion of genera over time for all cultures and conditions. All replicates were initially stocked with an equal portion of biomass of genera as either a bi-culture (50:50) or poly-culture, (33:33:33) with all replicates being dominated (> 95%) by a single genus after 5 weeks (Fig. 4e). The proportional increase in genera was affected by time and after week 3, the dominance of one algae exceeded 70% in all treatments (Fig. 4c) and this was more than 90% by week 5 (Fig. 4e). The dominance of a genus was consistent with results above where *Oedogonium* was competitively dominant under warmer and high irradiance conditions (max. average, summer) for bi-cultures (> 95% week 5) and the poly-cultures (> 95% week 5). Similarly, *Stigeoclonium* was dominant under colder conditions (winter, min. average). Notably, *Stigeoclonium* was also dominant over *Hyalotheca* as a bi-culture (> 98% in week 5). *Hyalotheca*, on the other hand, was competitively dominant over *Oedogonium* under colder conditions (winter, min. average) (> 80% in week 2 and > 95% in week 5).

Table 2 Ash, ultimate analysis (weight %, on dry basis) and higher heating value (HHV expressed as MJ/kg, on a dry basis) of biomass from three freshwater macroalgae. Values means (± S.E.), $n = 3$, biomass was sampled from culture stocks at the end of the experiment.

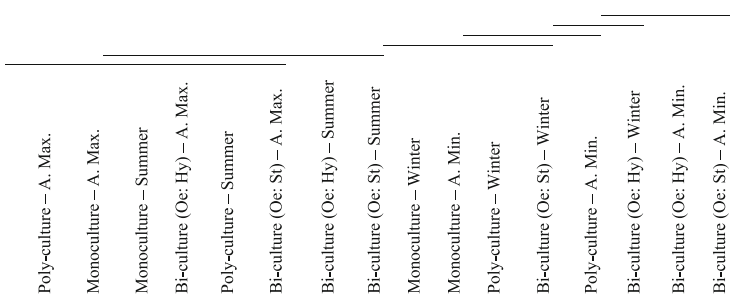
Algae	Ash	C	H	O	N	P	S	HHV
Oedogonium	8.72 (0.14)	43.67 (0.05)	6.69 (0.01)	41.61 (0.05)	4.4 (0.01)	0.39 (0.001)	0.19 (0.01)	18.66 (0.03)
Stigeoclonium	9.6 (0.09)	44.81 (0.09)	6.74 (0.02)	35.92 (0.16)	4.24 (0.03)	0.47 (0.004)	0.22 (0.002)	19.63 (0.005)
Hyalotheca	6.73 (0.29)	46.34 (0.06)	6.85 (0.01)	37.13 (0.35)	4.35 (0.13)	0.53 (0.02)	0.18 (0.01)	20.22 (0.03)

Table 3 Results of three 2-factor randomised complete block ANOVAs of the algae combinations selected for the experiment on specific growth rate (SGR). Values in bold are significant at $p < 0.05$. Tukey HSD test included for each alga. Samples are ordered from high to low SGR. The ones not connected by the same line among a single genus are significantly different.

Oedogonium

Source	MS	DF	F	P
Algae combination	593.143	3	51.9864	**<.0001**
Treatment	6613.033	3	579.6034	**<.0001**
S. combination*Treatment	73.094	9	6.4064	**<.0001**

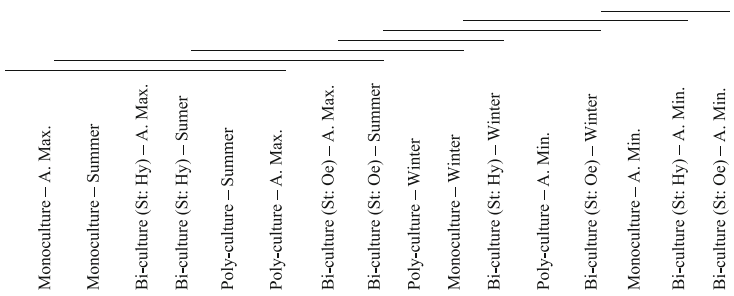
Tukey HSD



Stigeoclonium

Source	MS	DF	F	P
Algae combination	131.998	3	15.0033	**<.0001**
Treatment	1164.865	3	132.4027	**<.0001**
S. combination*Treatment	28.053	9	3.1886	**0.0073**

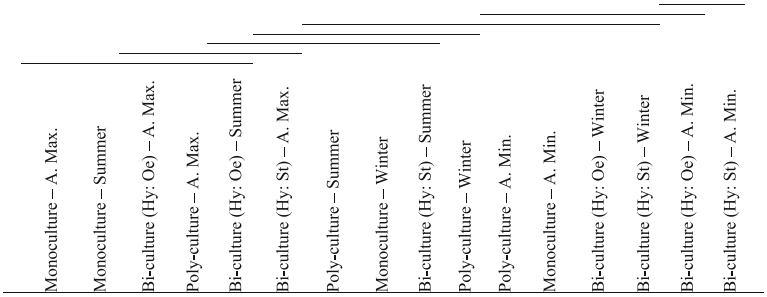
Tukey HSD



Hyalotheca

Source	MS	DF	F	P
Algae combination	351.3	3	27.9331	**<.0001**
Treatment	1426.421	3	113.4195	**<.0001**
S. combination*Treatment	49.203	9	3.913	**0.0020**

Tukey HSD



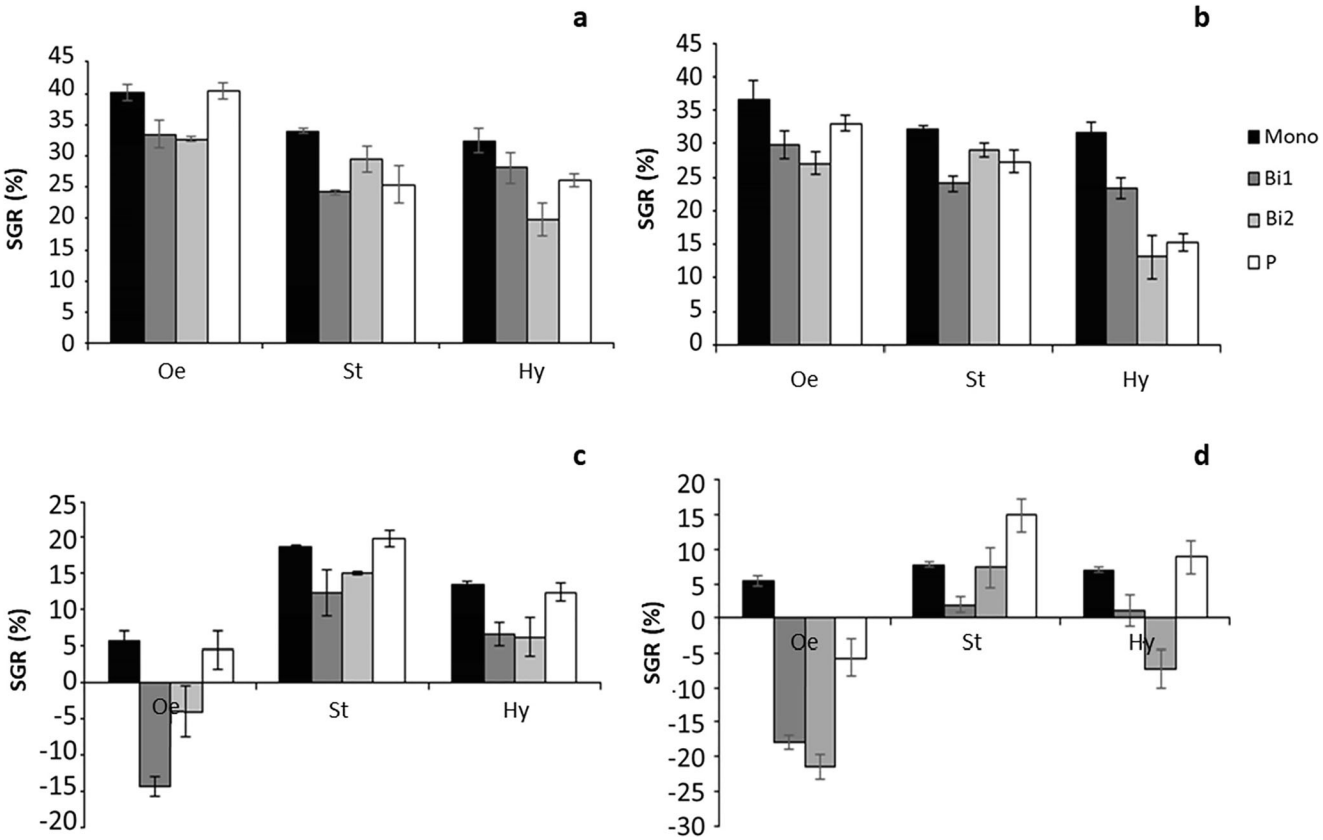


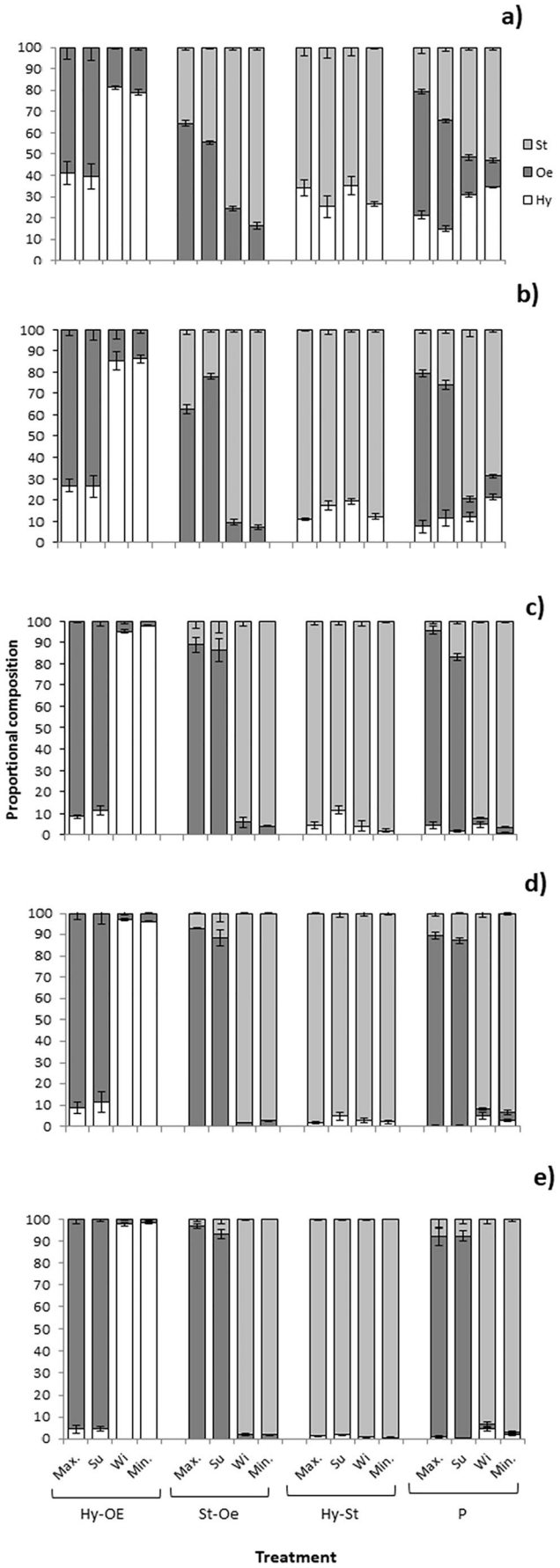
Fig. 3 SGR productivity of cultures in selection and competition experiments. Mean (±S.E.) total specific growth rate productivity (%) of monocultures (average of three runs of the selection experiment), bi-cultures (Bi 1 and 2) and poly-culture (P) combinations (first week of competition experiment) of three macroalgae growth under 4 temperature-light combinations: (a) Average maximum (hot waves), (b) summer, (c) winter and (d) average minimum (cold snaps). The bi-culture combinations in case of *Oedogonium* (Oe) are as follows: Bi1 *Oedogonium-Hyalotheca* (Oe-Hy); Bi2 *Oedogonium-Stigeoclonium* (Oe-St). In case of *Stigeoclonium* (St): Bi1 *Stigeoclonium-Oedogonium* (St-Oe); Bi2 *Stigeoclonium-Hyalotheca* (Hy-St). And in case of *Hyalotheca*: Bi1 *Hyalotheca-Oedogonium* (Hy-Oe); Bi2 *Hyalotheca-Stigeoclonium* (Hy-St)

Discussion

This study has demonstrated large and predictable variability in the production of macroalgae driven by specific conditions in temperate latitudes for the target algae. High irradiance and warmer temperatures favoured *Oedogonium*, whereas low irradiance and colder temperature favoured *Stigeoclonium*, which had twice the growth rate of *Oedogonium* under those conditions. In addition, prolonged exposure to colder periods reduced growth in all three algae, with the effect being most pronounced for *Oedogonium*. Our results also showed that under preferred conditions, for example high irradiance and temperature for *Oedogonium*, the most competitive algae in bi-cultures and poly-cultures exceed 70% of biomass after only 3 weeks and is functionally a monoculture after 5 weeks.

As an overall objective to optimise the production of biomass, identifying a single suitable genus to grow across all seasons is the most desirable scenario, as it reduces complexities in growing, collection and post-harvest processing. While all three algae are suitable for monoculture under warm temperatures based on their productivity, this alone does not support their selection. *Oedogonium* has been identified as suitable for year-round production in the tropical areas (Cole et al. 2018). However, under the temperate conditions tested, this is not the case. Both *Stigeoclonium* and *Hyalotheca* were suitable in cooler conditions based on productivity, which exceeded 9 g DW m^{-2} day^{-1} for both algae. However, *Stigeoclonium* outcompeted both *Oedogonium* and *Hyalotheca* in cooler conditions. As such, *Stigeoclonium* is the preferred candidate if a single species monoculture is desired in temperate locations. In contrast, if culture rotation is possible then the combination of *Oedogonium* and *Stigeoclonium* cultivated across the year would optimise biomass productivity on a per unit area basis of infrastructure. In our case, a monoculture of *Oedogonium* and *Stigeoclonium* would produce 14.1 and 23.9 t per hectare annually, respectively, while a mixed culture would reach 24.4 t ha^{-1} $year^{-1}$, showing a substantial improvement. However, if these values can be reached in realistic culture conditions at scale will require further testing.

Previous studies have delved into the importance of temperature and light conditions on algal growth (Fortes and



◀ **Fig. 4** Proportional compositions of mixed cultures. Mean (±S.E.) proportional algae composition of bi-cultures and poly-culture combinations of three macroalgae grown under 4 temperature-light combinations (average maximum (hot waves), summer, winter, average minimum (cold snaps)) in (a) week 1, (b) week 2, (c) week 3, (d) week 4 and (e) week 5 of the competition experiment. Algae abbreviations follow Fig. 2.

Lüning 1980; Wiencke and Fischer 1990; Butterwick et al. 2005; Bruhn et al. 2011; Yun et al. 2014). While high productivity has always been a preferred trait of freshwater species (Cole et al. 2018; Ge et al. 2018), seasonality adds an element of uncertainty (Cole et al. 2018). Consistent and stable biomass production by an algal monoculture is essential to attain accurate energy values or use the algae towards specific products (e.g. biomass for animal feed; (Garcia-Vaquero and Hayes 2016)). Consequently, the ubiquity of multiple suitable macroalgae for culture in municipal and aquaculture wastewaters (Hughes et al. 2012) poses an interesting dilemma regarding the management and productivity of cultivation systems. There may be 'strength in diversity' when considering production and productivity in temperate environments given by different traits present in different groups.

The productivities measured in this study compare favourably with those documented for diverse species in temperate and tropical regions. Optimum culture conditions in this study produced DW values of 10 to 12 g m^{-2} day^{-1}, matching those documented for *Oedogonium* (10.7 g m^{-2} day^{-1}), and exceeding those recorded for other tropical genera of freshwater macroalgae as monocultures, or as mixed cultures (e.g. *Cladophora*, 3.4 g m^{-2} day^{-1} (Neveux et al. 2015) and *Microspora*, *Ulothrix*, *Rhizoclonium* and *Oedogonium*, 5.3–5.5 g m^{-2} day^{-1} (Wilkie and Mulbry 2002)) under laboratory conditions.

The productivities in this study are within the range determined for marine macroalgae under laboratory conditions when considered on an ash free dry weight (AFDW) basis (see Online Resource, Table 4 & Fig. 5). Ash was below 9% for all three algae, whereas ash content in marine genera generally exceeds 25% due to salt content, for example *Ulva* (31%), *Derbesia* (35%) or *Chaetomorpha* (37%) (Neveux et al. 2014).

Notably, freshwater macroalgae are substantially more productive than some terrestrial plants like corn, rice or hay (Bruhn et al. 2011; Yun et al. 2014). In our case, the productivity of *Oedogonium*, *Hyalotheca* and *Stigeoclonium* ranged from 7 to 11 g m^{-2} day^{-1} depending on the algae and conditions tested, whereas some terrestrial plants such as peanuts, wheat or soybeans produce up to 5 g m^{-2} day^{-1} (Yun et al. 2014). This opens the door co-production, of equally valuable, if not more valuable, cultures. The use of non-arable land and wastewater for growth (Cole et al. 2014b) coupled with their higher productivities per area (Yun et al. 2014) makes domesticated freshwater macroalgae a feasible biomass source co-

culture to terrestrial crops in addition to their obvious application in the treatment of nutrient-rich wastewaters, in particular municipal wastewater (Cole et al. 2016b; Neveux et al. 2016).

Previous experiments have demonstrated the bioenergy potential of *Oedogonium* (Hossain et al. 2008) with comparisons with terrestrial plant crops (Yun et al. 2014). While the biochemical composition of freshwater macroalgae depends on growth conditions (Bruhn et al. 2011; Balina et al. 2017), *Stigeoclonium* and *Hyalotheca* cultivated under comparative conditions in this study are on par with the High Heating values of *Oedogonium* (19.63, 20.22 and 18.66 MJ kg^{-1}, respectively). These values are 1.5–1.7 times higher than the value for wood (HHV = 11.86 MJ kg^{-1}) (Yun et al. 2014). While other aquatic photosynthetic groups such as freshwater microalgae have comparative values (HHV = 18.66 MJ kg^{-1}), freshwater filamentous macroalgae require a significantly lower energy input for harvesting and consequently have an improved net energy balance (Yun et al. 2014). The characteristics of our three species in terms of captured energy support, thus, the seasonal rotation of multi-species to maintain overall bioenergy potential.

While cultivation of mixed communities may result in more robust operations (Clarens et al. 2010), we propose two options based on competitive dominance rather than productivity for the implementation of bioremediation for the temperate conditions and species trialled. The first option would alternate dominant monocultures over time; *Oedogonium* in the warmer months and *Stigeoclonium* in the cooler months. Logistical problems may arise maintaining algal inoculates off-season. The second option would establish a bi-culture, of the same two algae, that would naturally vary in its composition in response to environmental conditions. A major concern in this second approach would be whether natural residual populations of each algae would provide enough biological reservoirs to compete for space after adverse seasons. Correspondingly, further research delving into their resilience over time to produce biomass and enable bioremediation at appropriate scale is required. Likewise, since only two temperate algae were tested in this study, future experiments must also consider other, more suitable possibilities for temperate freshwater bioremediation.

Conclusions

Viable freshwater macroalgal species for wastewater bioremediation must have the capacity to consistently produce biomass through time and compete with other algal species that may arise in relatively open culture systems. This study compared the influence of both traits and biochemical composition of two temperate and one tropical macroalgae to evaluate their suitability for monoculture in industrial wastewaters. All three genera of algae were suitable for culture in wastewater in temperate conditions; however, culture conditions which reflected the seasonality of temperate environments, altered outcomes. *Oedogonium* dominated during hotter conditions, *Stigeoclonium* dominated in cooler conditions, while all three algae were negatively affected by extremely cold periods. Due to its competitive dominance in cold conditions, *Stigeoclonium* is most suitable for monoculture throughout the year. *Oedogonium*, *Hyalotheca* and *Stigeoclonium* had comparable bioenergy values and productivity to several terrestrial crops.

This study has enhanced our understanding of how to select freshwater macroalgae for intensive cultivation in temperate environments. Consistent production throughout the year requires selection of a species with reasonable production and competitivity ability in cooler periods coupled with high productivity at warmer times. *Oedogonium* and *Stigeoclonium* were competitively dominant in hot and cold conditions, respectively, and outcompeted the other algae within weeks, making them ideal for commercial production in their respective most productive seasons. Our findings demonstrate that the use of macroalgae for wastewater remediation in temperate settings entails challenges distinct from those in the tropics. Season-dependent selection of macroalgae, with emphasis on species with high resilience and the possibility of bi-cultures, or alternating monocultures, will be required.

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