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Comparison of monocultures and a mixed culture of three Chlorellaceae strains to optimize biomass production and biochemical content in microalgae grown in a greenhouse

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Abstract

Light and temperature are important environmental conditions affecting microalgal growth in outdoor culture. It is essential to evaluate microalgae strains growing under outdoor conditions where they are subjected to variable environmental parameters. The present study investigated three Chlorellaceae strains (*Micractinium* sp. MACC-728, *Chlorella sorokiniana* MACC-438, and *C. sorokiniana* MACC-452) and a mixed culture combining these three strains. Cultures were grown in 2-L bioreactors in a greenhouse over 3 months to assess the effects of high temperature and light on their growth, macromolecule content, and antioxidant and plant-stimulating bioactivities. The most influential environmental parameters on growth were average air temperature and the sum of photosynthetically active radiation, followed by maximum air temperature. The most affected growth parameter was daily change in cell number. *Chlorella sorokiniana* MACC-438 produced the lowest biomass and was most affected by the high temperature and light conditions. *Micractinium* sp. produced the highest biomass and was least affected, suggesting it was the most suitable strain for outdoor cultivation. The mixed Chlorellaceae culture performed well in biomass production, exceeding *C. sorokiniana* monocultures but significantly underyielding in lipid content. Antioxidant activity and the root-stimulating activity varied with strain and culture age. *Micractinium* sp. had the highest but most variable antioxidant and plant-stimulating activity. Bioactivity in the mixed culture was more consistent, remaining high regardless of culture age and environmental conditions. Thus, mixed cultures of productive strains could be a useful strategy to ensure stable and high-quality biomass production in outdoor cultivation with fluctuating environmental conditions.

Keywords Antioxidant activity · Biostimulants · *Chlorella sorokiniana* · Light · *Micractinium* sp. · Temperature

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Introduction

Microalgae contain lipids that provide a renewable feedstock for biodiesel production. Other valuable microalgal compounds include proteins, carbohydrates, pigments, vitamins, and polyunsaturated fatty acids which are useful resources for animal and human nutrition. Microalgae also synthesize an array of secondary metabolites with biological activities which are of interest to the pharmaceutical and health food sectors (de Morais et al. 2015; Chew et al. 2017; Barsanti and Gualtieri 2018) and phytohormones that can be used as plant biostimulants in agriculture (Stirk and van Staden 2020). However, the cost of producing and harvesting sufficient amounts of biomass is a major hurdle in developing economically viable biotechnology applications (Brennan and Owende 2010; Borowitzka and Vonshak 2017). Much research has focused on making feedstock production more cost-effective. This encompasses the selection of suitable microalgae species and strains with the desired attributes such as fast growth, robustness, and a high content of the target compound (usually lipid content), genetic modifications (Beacham et al. 2017) and improving culture techniques by manipulating physical conditions (Rodolfi et al. 2009; Aremu et al. 2016). This research has mostly been conducted in controlled laboratory conditions in small-scale photobioreactors where it is possible to maintain monocultures and control the physical environmental conditions (Newby et al. 2016; Borowitzka and Vonshak 2017). However, in order to produce sufficient microalgae feedstock for commercial production, cultures need to be scaled up to outdoor ponds and large photobioreactors (Newby et al. 2016). The cost of regulating environmental conditions to optimal levels is not feasible in outdoor setups, and thus, cultures are exposed to a wide and fluctuating range of suboptimal and supraoptimal environmental factors. This reduces biomass accumulation and productivity. Culture characteristics specific to large-scale production need to be considered and should be tested in outdoor conditions at an early stage of strain selection and evaluation (Borowitzka and Vonshak 2017; Dahlin et al. 2018).

Temperature affects enzymatic activity and thus directly influences microalgal metabolism. At low temperatures, the growth rate is slow, and as enzymatic activity increases exponentially with increasing temperatures, there is a linear correlation between growth rates and temperature until the optimum temperature for growth is reached. Growth rates decline sharply thereafter, and cell mortality increases at higher temperatures, especially when the cells are exposed for longer time periods, as enzymes and other cellular components are inactivated and/or denatured (Butterwick et al. 2005; Ras et al. 2013; Grimaud et al. 2017). Each microalgae species has its own thermal range with growth rates varying between species and strains (Butterwick et al. 2005; Grimaud et al. 2017). Most species can function in a wide range of temperatures, generally between 15 and 30 °C but with optimal temperatures between 20 and 25 °C (Ras et al. 2013). The lethal temperature is usually only slightly higher than the optimum temperature (Borowitzka 2016a). Thus, good temperature tolerance suited to the temperature range at the production site is a key characteristic when selecting strains for outdoor cultivation.

Polyculture is a potential approach to managing outdoor cultures to maintain stable biomass productivity which is essential in commercial production (Newby et al. 2016). In natural environments with multispecies assemblages, there is a positive correlation between species diversity, stability of the community, and biomass production (Shurin et al. 2014; Newby et al. 2016). As each microalgae species has specific environmental optima, microalgae populations within the community will fluctuate temporally and spatially according to environmental changes (Newby et al. 2016). Thus, if one species population declines in response to environmental conditions, another species will fill the niche (Schabhüttl et al. 2013; Newby et al. 2016). Although no one species will dominate over an extended period, this leads to a more stable biomass production over time (Newby et al. 2016).

Selection of suitable combinations of complimentary microalgae species with desirable and complimentary traits is essential when making up the consortium of microalgae for large-scale outdoor production. This includes their growth under a particular range of environmental conditions, efficient utilization of available resources (e.g., light and nutrients), and no allelopathy (Newby et al. 2016). Another option is incorporating microalgae strains with growth-promoting compounds such as phytohormones or quorum sensing signaling molecules which may be released into the media, thus influencing the other species in the culture (Borowitzka 2016b). Provided suitable and complimentary strains are selected, higher species diversity enhances crop stability and increases productivity as at least one species will be suited to the prevailing environmental conditions (Newby et al. 2016). There is a positive correlation between species diversity and biomass production (Stockenreiter et al. 2012; Schabhüttl et al. 2013; Shurin et al. 2014).

Another consideration is the selection of microalgae species with a suitable biochemical profile so that multiple metabolites can be extracted from the biomass. A biorefinery approach couples the extraction of lipids from the biomass for biofuel production with other high value products. This expands the market for microalgae products, allowing for maximum exploitation of the biomass (Chew et al. 2017). For example, microalgae have good antioxidant activity as they contain bioactive compounds such as chlorophyll,

carotenoids (e.g., lutein, zeaxanthin, and astaxanthin), tocopherol, phenols, peptides, specific amino acids, and polysaccharides (Goiris et al. 2012; Choochote et al. 2014; de Morais et al. 2015) and may provide valuable compounds for the food and pharmaceutical industries. Another possible application for microalgae biomass is in agriculture as biodegradable biofertilizers, biostimulants, and biopesticides and to improve the organic matter and water holding capacity of the soil (Barsanti and Gualtieri 2018). Microalgae are potential biostimulants and biopesticides as they contain plant growth-promoting compounds such as phytohormones (Stirk and van Staden 2020) and extracellular polymeric substances and other secondary metabolites with phytopathogenic activity (Costa et al. 2019). However, changes in the external culture conditions can induce changes in the microalga's metabolism, either stimulating or reducing the biosynthesis of specific compounds (Goiris et al. 2012; de Morais et al. 2015; Rossi and De Philippis 2016; Barsanti and Gualtieri 2018) and it is thus important to assess the biochemical profiles of microalgae species when grown in outdoor systems.

The aim of the present study was to compare three Chlorellaceae strains grown in a greenhouse in a series of experiments run over 3 months to assess the effects of variable environmental parameters, specifically high temperature and light, on their growth, biochemical content, and bioactivities. In addition, growth was compared in a mixed culture comprising the three investigated microalgae strains to determine if a mixed culture of productive strains could lead to more stable biomass production and quality in non-regulated culture conditions.

Materials and methods

Experimental strains and feedstock cultures

Based on previous laboratory experiments, three axenic microalgae strains with high growth rates, good protein and lipid productivity, and plant-biostimulating activity but with slightly different temperature optimum were selected from the Mosonmagyaróvár Algal Culture Collection (MACC), namely *Micractinium* sp. MACC-728 and two *Chlorella sorokiniana* strains (MACC-438 and MACC-452). The identity of each algal strain was confirmed by both 16S rDNA and 18S rDNA amplicon sequencing reactions (Wu et al. 2001). The three strains were inoculated from agar cultures into two 500-mL flasks containing 250 mL complete Tamiya liquid medium (Kuznjecov and Vladimirova 1964). The cultures were grown at 25 ± 2 °C in a 12:12-h light:dark photoperiod and illuminated from below with 130 µmol photons $m^{-2}s^{-1}$ light intensity and aerated for 12 h during the light phase with 20 L h^{-1} 1.5% CO_2-enriched sterile air (1.33 vvm). After 7 days, the suspension cultures were used to inoculate six 500-mL flasks per strain, each containing 250 mL N-limited Tamiya liquid medium (140 mg L^{-1} N; 20% N). The cultures were maintained in the described conditions for a further 7 days. Culture density was calculated by dry weight measurement (Stirk et al. 2020). These cultures were used as inoculum for the greenhouse experiments.

Greenhouse experiments

The experiments were conducted in a greenhouse at the Széchenyi István University, Mosonmagyaróvár, Hungary (47° 52′ N; 17° 16′ E). The microalgae were grown in 700-mm-long × 80-mm-diameter glass tubes with rounded bottoms. Each tube held 2 L suspension culture. The tubes were completely closed with a glass head with three openings. These housed a 650-mm-long glass tube which reached to the bottom of the culture tube and provided aeration for the suspension. The other two outlets were for releasing the air and sampling the suspension (Online resource 1). Cultures were aerated with 200 L h^{-1} air enriched with 1% CO_2 (1.65 vvm) between 7 am and 7 pm.

Incoming radiation, sunshine duration, and air temperature values in the greenhouse were monitored continuously and recorded every 5 min for the duration of the experiments. The following meteorological elements were used to characterize the environmental conditions in the greenhouse—average air temperature, minimum air temperature, maximum air temperature, daily mean of sunshine duration, sum of photosynthetically active radiation (PAR), and maximum of PAR. Temperature elements were quantified using the temperature sensor EcoStation BTP-06/SP/hun/v1.1 (Boreas Ltd., Érd, Hungary), and radiation elements were measured using the radiation intensity and sunshine duration sensor EcoStation BIS-06/PAR/hun/v1.2 (Boreas Ltd., Érd, Hungary).

A series of growth experiments were conducted where the three microalgae strains were grown in monoculture with a starting density of 30 mg L^{-1} DW. For the mixed Chlorellaceae culture, the inoculum comprised of 10 mg L^{-1} DW of each of the three strains. The cultures were grown in N-limited Tamiya nutrient medium (20% N) with six culture tubes per strain, providing 12 L suspension upon harvesting. Experiments ran for 5-day and 10-day duration. Each experiment was run three times over a period of 13 weeks in spring and summer (14 April—6 July 2018; Table 1). N-limited Tamiya medium (20% N) provides sufficient nitrogen in the first few days of the growth but becomes limiting by 10 days of culture (Ördög et al. 2012). Activity in the mung bean bioassay was generally higher in older *Chlorella* cultures (where N is limiting) (Stirk et al. 2020) and thus 20% N Tamiya medium was used for the suspension inoculum

Table 1 Dates and environmental variables of the microalgae growth experiments conducted in a greenhouse during spring and summer 2018 at the Széchenyi István University, Mosonmagyaróvár, Hungary

Experiment	Date (2018)	Meteorological variables					
		Average air temperature (°C)	Minimum air temperature (°C)	Maximum air temperature (°C)	Daily mean sunshine duration (h)	Sum of PAR (µmol photons m^{-2} s^{-1})	Maximum PAR (µmol photons m^{-2} s^{-1})
Micractinium sp. MACC-728							
5 day experiment							
1	19–24 April	26.7	15.7	47.4	5.2	9.19	1152
2	10–15 May	26.4	18.0	47.3	5.4	9.32	1263
3	21–26 June	25.2	17.3	49.7	5.4	8.11	1527
10 day experiment							
1	18–28 May	27.2	16.5	48.6	5.7	10.80	1516
2	8–18 June	29.1	18.2	50.9	4.3	10.03	1344
3	26 June–6 July	27.7	16.5	50.6	5.9	10.73	1582
C. sorokiniana MACC-438							
5 day experiment							
1	14–19 April	23.2	14.0	43.0	4.8	7.23	1100
2	4–9 May	26.5	17.4	45.8	5.8	10.84	1414
3	15–20 June	29.7	21.0	48.1	4.3	12.37	1336
10 day experiment							
1	14 – 24 May	24.6	13.9	47.4	5.5	8.77	1391
2	4 – 14 June	29.8	20.3	50.9	4.4	9.93	1341
3	18 – 28 June	27.3	17.3	51.0	5.4	10.32	1527
C. sorokiniana MACC-452							
5 day experiment							
1	18–23 April	26.0	14.0	47.4	5.1	9.94	1152
2	9–14 May	27.2	18.9	47.3	5.7	10.55	1394
3	27 June–2 July	25.9	16.5	42.9	5.3	8.80	1582
10 day experiment							
1	15–25 May	25.2	13.9	48.1	5.8	9.32	1391
2	5–15 June	28.9	18.2	50.9	4.2	9.20	1256
3	25 June–5 July	27.1	16.5	50.2	5.8	10.41	1582
Mixed Chlorellaceae culture							
5 day experiment							
1	20–25 April	26.6	15.7	47.4	5.3	8.59	1383
2	11–16 May	24.7	13.9	46.9	4.3	7.68	1250
3	22–27 June	24.5	17.3	42.7	6.1	8.85	1527
10 day experiment							
1	10–20 April	23.5	13.6	45.8	5.3	8.06	1336
2	22 May–1 June	28.6	18.4	51.2	4.0	10.17	1516
3	11–21 June	29.2	18.2	50.6	4.0	10.74	1344

cultures and greenhouse experiments to ensure good bioactivity in the mung bean bioassay.

Growth was monitored throughout the experiments with 5–10 mL samples taken every 2–3 days to determine the DW, cell number, and cell area (size) as previously described (Stirk et al. 2020). Samples were fixed with Lugol's solution and the cell number and size were determined using an Olympus BX60 microscope (Japan) and the Olympus Stream Image Analysis Software (Olympus Soft Imaging Solutions GmbH). Daily dry matter production (mg L^{-1} day^{-1}), daily change in cell number (cell number L^{-1} day^{-1}), and daily change in cell size (µm^2 day^{-1}) were calculated from this data. Total suspension of the six tubes for each treatment was harvested at the end of the experiment on day 5 or day 10. The harvested suspension was centrifuged at 2150×*g* for 15 min at room temperature (SIGMS 6K15). The

supernatant-free biomass was freeze-dried (Christ Gamma 1–20, Germany) for 22 h at 0.035 mbar and stored at − 19 °C until required for the bioassays and chemical analysis.

Macromolecule quantification

Using the dried biomass, crude protein content was quantified using a standard Kjeldahl method. Lipid content was determined by hydrolysis with 3 M HCl at 95–100 °C for 1.5 h followed by sequential solvent elution using methanol, hexane, and diethyl ether as previously described (Ördög et al. 2012).

Antioxidant activity

Microalgae biomass (1.2 g DW) was extracted in 30 mL dichloromethane. The extracts were sonicated for 20 min with ice added to the water bath so that the temperature would not increase above 20 °C. The extracts were then shaken at 80 rpm for 2 h at 25 °C, left overnight at 25 °C, and then shaken for a further 30 min. The extracts were filtered using Whatman No. 1 filter paper and rinsed and dried in a flow of air. The resulting residue was weighed and suspended in 10 mg mL^{-1} methanol. Antioxidant activity was quantified using the diphenylpicrylhydrazyl (DPPH) free radical scavenging assay as previously described (Moyo et al. 2010). Ascorbic acid (AA) and butylated hydroxytoluene (BHT) were used as positive controls. The final concentration of the extracts and standards was 30 µM. The % free radical scavenging activity (% RSA) was determined by the decoloration of the DPPH solution over 30 min in the dark. This assay was repeated three times to give three technical replicates per sample.

Plant growth-stimulating activity

Plant-biostimulating activity of the biomass was measured using the mung bean rooting bioassay (Crouch and van Staden 1991). Mung beans (*Vigna radiata*) were germinated in moist vermiculite at 26 ± 1 °C in 16:8 h light:dark photoperiod and 120 µmol photons m^{-2} s^{-1} light intensity. Water extracts of the biomass were prepared at 3 mg mL^{-1}. The extracts were shaken at 80 rpm for 2 h at 25 °C and left overnight at 25 °C. On day 10, uniform mung bean cuttings (12-cm stem length) with two leaves were placed in the prepared microalgae solutions for 6 h, then rinsed and transferred to clean vials containing water. There were five cuttings per vial and four vials per solution (20 cuttings in total per extract). Distilled water was included as the control and indole-3-butyric acid (IBA) at 10^{-8}–10^{-3} M as a positive control. The cuttings were placed in the growth conditions described above. The number of roots was recorded 10 days after the pulse treatment.

Statistical analysis

The relationship between the growth parameters and meteorological data were analyzed by canonical correlation and regression analysis for each microalga strain and the mixed Chlorellaceae culture. Outliers were detected using Tukey's fences and the canonical correlation was computed using standardized data. Growth parameters considered were daily dry matter production (mg L-1 day 1), daily change in cell number (cell number mL-1 day-1) and daily change in cell size (µm2 day-1). Meteorological variables considered were average air temperature (°C), minimum air temperature (°C), maximum air temperature (°C), sum of daily mean of sunshine duration (h), sum of PAR (µmol photons m-2 day-1) and maximum PAR (µmol photons m-2 day-1). Three canonical roots were extracted but only the first and the most significant root was interpreted. The canonical correlation coefficient (R) was determined for each strain to the first canonical root. The Chi-square test was applied to R for each strain to determine the significance of R. Linear regression (Pearson) was applied to confirm the relationship between the average air temperature and sum of PAR on the daily change in cell number and dry matter production for each strain.

Multifactor ANOVA was applied to analyze the effect of day of harvest (day 5 and day 10) and the strain on the protein and lipid content of the three monocultures and the mixed Chlorellaceae culture. In order to find significant differences between the pairs of the samples, post hoc Tukey test was carried out. The pairwise t-test was used to analyze the DPPH antioxidant activity between the positive controls (AA and BHT) and the three monocultures and the mixed culture harvested on day 5 and day 10. Multifactor ANOVA was performed to explore the effect of day of harvest (day 5 and day 10) and the strain on the plant growth-promoting activity considering the three strains and the mixed culture. ANOVA was followed by post hoc Tukey test to find significant differences between each pair of samples. In order to explore significant differences between the samples and IBA standards, pairwise t-test was applied.

All computations were carried out using Statistica 13.0

Results

Growth of microalgae strains in relation to environmental variables

Environmental parameters in the greenhouse varied over the experimental period with the average air temperature ranging from 23.2 to 29.8 °C; minimum and maximum air temperature ranging from 13.6 to 21.0 °C and 42.7 to 51.2 °C, respectively; daily hours of sunshine between 4.0

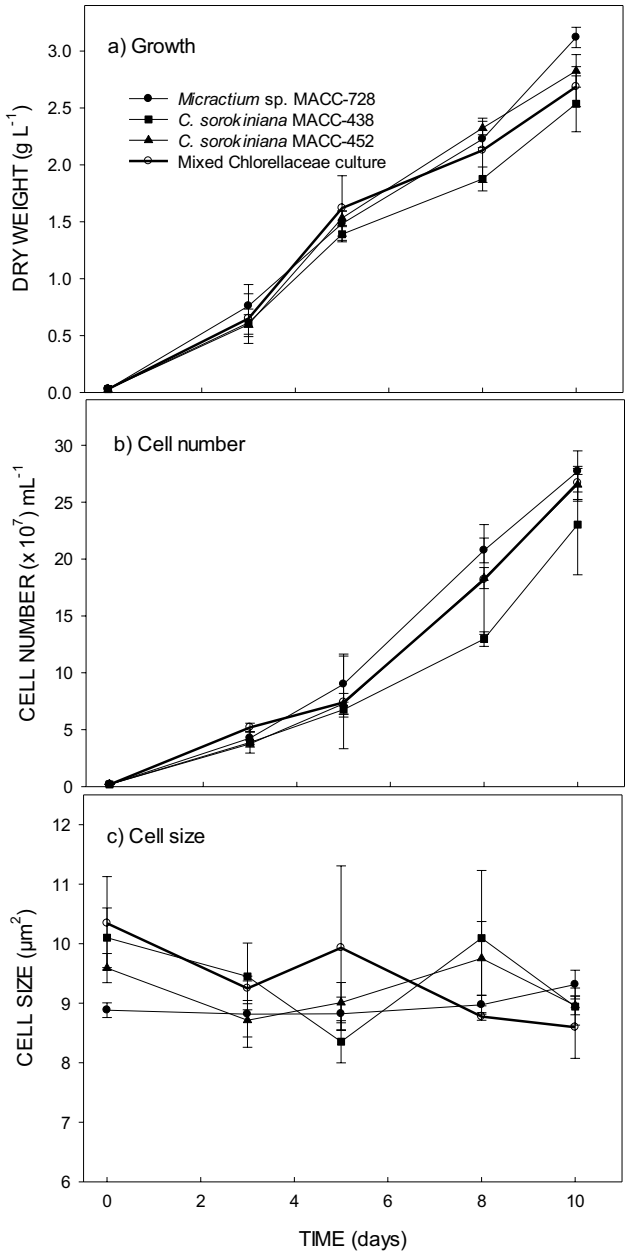

Fig. 1 Growth of three microalgae strains and the mixed Chlorellaceae culture grown in a greenhouse over a 3-month period (April–July 2018) showing **a** biomass (dry weight), **b** cell number, and **c** cell size. Results are the average of three experiments and are presented as mean ± SE ($p < 0.05$)

and 6.1 h; and sum of PAR and maximum PAR ranging from 7.23 to 12.37 mol photons m^{-2} day^{-1} and 1100 to 1582 µmol photons m^{-2} day^{-1} respectively over all the experiments (Table 1).

On average, *Micractinium* sp. produced the most biomass (DW) and had the highest cell number. *Chlorella sorokiniana* MACC-438 produced the lowest biomass and cell number. Growth of the mixed Chlorellaceae culture was less than

Table 2 Average daily biomass productivity in the three microalgae strains and a mixed Chlorellaceae culture grown for 10 days in a greenhouse during spring and summer 2018 at the Széchenyi István University, Mosonmagyaróvár, Hungary

Experiment	Daily biomass production (mg L^{-1} day^{-1})			
	Day 1–3	Day 4–5	Day 6–8	Day 9–10
Micractinium sp. MACC-728				
1	172.8 ± 5.5	590.8 ± 14.1	201.2 ± 22.1	491.8 ± 46.8
2	388.6 ± 20.7	132.7 ± 21.2	226.9 ± 10.8	456.2 ± 14.3
3	212.4 ± 13.0	361.2 ± 42.6	315.1 ± 41.2	391.2 ± 36.3
C. sorokiniana MACC-438				
1	130.4 ± 5.9	468.3 ± 33.3	213.1 ± 40.8	422.3 ± 71.5
2	208.9 ± 6.5	346.0 ± 26.1	117.7 ± 38.4	538.7 ± 15.0
3	278.4 ± 9.9	352.7 ± 3.8	154.8 ± 14.2	29.0 ± 3.1
C. sorokiniana MACC-452				
1	139.4 ± 5.6	507.8 ± 21.8	329.6 ± 42.1	337.2 ± 64.6
2	232.9 ± 6.4	483.2 ± 27.9	189.4 ± 25.6	261.0 ± 27.3
3	228.8 ± 9.2	411.3 ± 13.0	271.9 ± 37.6	155.0 ± 36.2
Mixed Chlorellaceae culture				
1	65.5 ± 4.1	420.3 ± 26.5	172.7 ± 1.8	382.5 ± 22.2
2	303.7 ± 3.9	527.8 ± 37.8	122.6 ± 10.6	296.2 ± 25.5
3	287.5 ± 10.6	508.3 ± 23.7	212.0 ± 14.3	159.7 ± 27.2

Micractinium sp. monoculture and *C. sorokiniana* MACC-452 monoculture but greater than *C. sorokiniana* MACC-438 monoculture (Fig. 1a and b). Cell size was very variable over the three experiments for each strain conducted over the 3 months (Fig. 1c). Average daily biomass production (mg L^{-1} day^{-1}) peaked between day 4 and 5 for all strains except for *Micractinium* sp. grown over 10 days (Table 2).

The canonical correlation coefficient (R) showed that the environmental variables had the strongest influence on the growth parameters of *C. sorokiniana* MACC-438 and had less effect on *C. sorokiniana* MACC-452 and the mixed Chlorellaceae culture. R was not significant for *Micractinium* sp., showing that the set of measured environmental parameters had no significant effect on the measured growth parameters of this strain (Table 3). For *C. sorokiniana* MACC-438, average and maximum air temperature and the sum of PAR had the most influence on cell number with

Table 3 Canonical correlation coefficient (R) for each microalgae strain and the mixed Chlorellaceae culture showing the relationship between growth parameters and meteorological variables. The significance of R was determined by the chi-square test with the first root removed ($p = 0.05$)

Strain	Canonical R	p
Micractinium sp. MACC-728	0.438	0.490
C. sorokiniana MACC-438	0.767	0.000
C. sorokiniana MACC-452	0.615	0.014
Mixed Chlorellaceae culture	0.636	0.039

Table 4 Canonical coefficients of the factor structure of the most significant root (root 1) for each microalgae strain and the mixed Chlorellaceae culture for the meteorological variables and the growth parameters

Variable	*Micractinium* sp. MACC-728	*C. sorokiniana* MACC-438	*C. sorokiniana* MACC-452	Mixed culture
Meteorological variables				
Average air temperature (°C)	0.964	0.580	0.832	−0.936
Minimum air temperature (°C)	0.709	0.238	0.402	−0.952
Maximum air temperature (°C)	0.354	0.452	0.549	−0.437
Daily mean of sunshine duration (h)	0.091	0.054	0.169	0.416
Sum of PAR (µmol photons m^{-2} day^{-1})	0.614	0.438	0.694	−0.498
Maximum PAR (µmol photons m^{-2} s^{-1})	−0.170	−0.037	0.294	−0.022
Growth parameters				
Daily dry matter production (mg L^{-1} day^{-1})	0.984	0.589	0.016	0.692
Daily change in cell number (mL day^{-1})	0.261	0.980	0.961	− 0.764
Daily mean change in cell size (µm^2 day^{-1})	0.216	0.416	0.553	0.463

less effect on dry matter production and cell size. For *C. sorokiniana* MACC-452, average, minimum, and maximum air temperature and the sum of PAR had the most influence on cell number with less effect on cell size. In the mixed culture, average, minimum, and maximum air temperature, daily mean of sunshine duration, and the sum of PAR influenced dry matter production, cell number, and cell size (Table 4). These results were confirmed by the correlation coefficients calculated using linear regression (Table 5). Thus, the most influential parameters were average air temperature and sum of PAR with the most effected growth

Table 5 Correlation coefficients (Pearson) calculated by linear regression showing the correlation (significant at p = 0.05) between selected meteorological variables and growth parameters in three microalgae strains and the mixed Chlorellaceae culture

Strain	Correlation coefficients	
	R	p
Daily change in cell number vs average air temperature		
Micractinium sp. MACC-728	0.098	0.517
C. sorokiniana MACC-438	0.436	0.004
C. sorokiniana MACC-452	0.488	0.000
Mixed Chlorellaceae culture	0.531	0.000
Daily dry matter production vs average air temperature		
Micractinium sp. MACC-728	0.461	0.001
C. sorokiniana MACC-438	0.260	0.097
C. sorokiniana MACC-452	0.040	0.789
Mixed Chlorellaceae culture	0.311	0.048
Daily change in cell number vs sum of radiation		
Micractinium sp. MACC-728	0.179	0.235
C. sorokiniana MACC-438	0.337	0.029
C. sorokiniana MACC-452	0.409	0.004
Mixed Chlorellaceae culture	0.354	0.023
Daily dry matter production vs sum of radiation		
Micractinium sp. MACC-728	0.305	0.031
C. sorokiniana MACC-438	0.201	0.201
C. sorokiniana MACC-452	0.128	0.387
Mixed Chlorellaceae culture	0.095	0.557

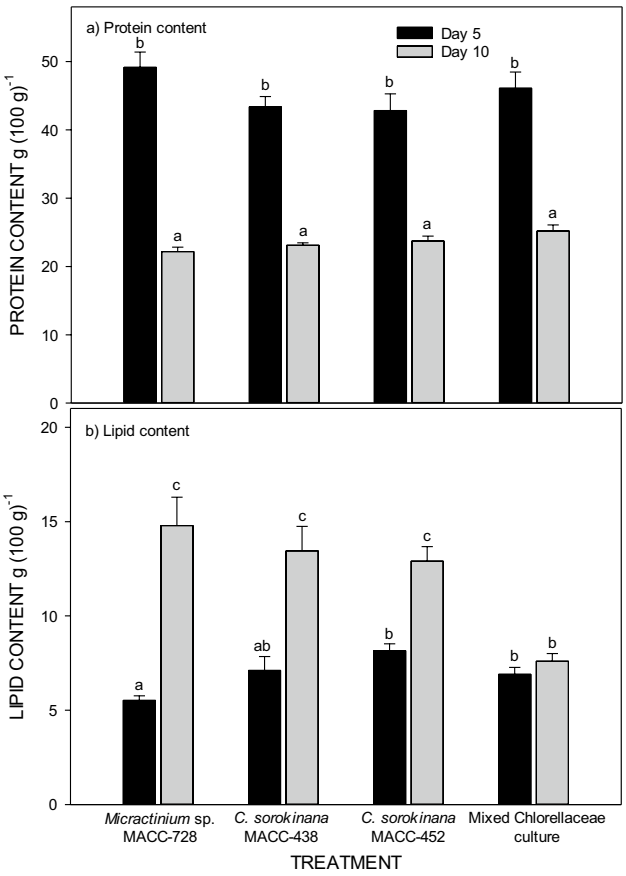

Fig. 2 **a** Protein and **b** lipid content of the three microalgae strains and the mixed Chlorellaceae culture grown in a greenhouse over a 3-month period (April–July 2018). Results are the average of three experiments and are presented as mean ± SE ($p < 0.05$)

parameters being daily change in cell number and daily dry matter production.

Macromolecule content

Protein content was significantly higher on day 5 and decreased by day 10 in all the cultures. *Micractinium* sp. had the highest protein content, and the two *C. sorokiniana* strains had the lowest protein content although these differences were not significant. The protein content was higher in the mixed Chlorellaceae culture compared to the *C. sorokiniana* monocultures but lower than the *Micractinium* sp. culture on day 5. On day 10, the mixed culture had the highest protein content compared to the three monocultures (Fig. 2a). *Micractinium* sp. had a significantly lower lipid content compared to *C. sorokiniana* MACC-452 and the mixed culture on day 5. The lipid content increased significantly in the monocultures from day 5 to day 10 with the largest increase in the *Micractinium* sp. However, the lipid content did not increase in the mixed *Micractinium* sp. culture and was significantly lower compared to the monocultures on day 10 (Fig. 2b).

Antioxidant activity

Antioxidant activity measured using the DPPH assay was significantly higher in samples harvested on day 5 compared to the samples harvested on day 10 for each microalgae strain (Fig. 3). On day 5, *Micractinium* sp. and the mixed Chlorellaceae culture had slightly higher activity compared to the activity of the two *C. sorokiniana* strains. By day 10, *Micractinium* sp. had significantly lower activity than *C. sorokiniana* MACC-438 and the mixed culture (Fig. 3).

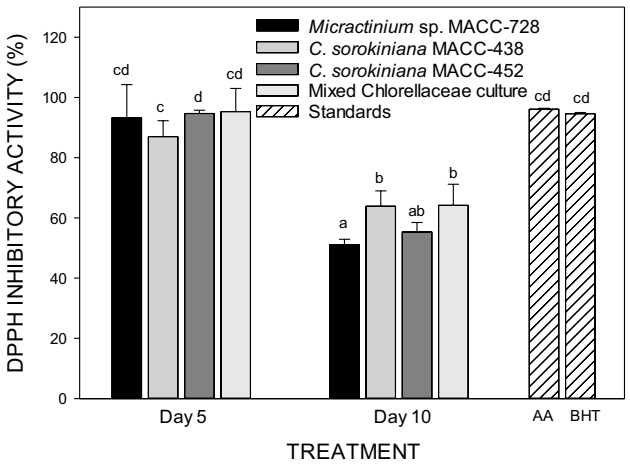

Fig. 3 Antioxidant activity measured in the DPPH assay for dichloromethane extracts of three microalgae strains and the mixed Chlorellaceae culture harvested on day 5 and day 10. Ascorbic acid (AA) and butylated hydroxytoluene (BHT) were included as positive controls. Results are the average of three experiments and are presented as mean ± SE ($p < 0.05$)

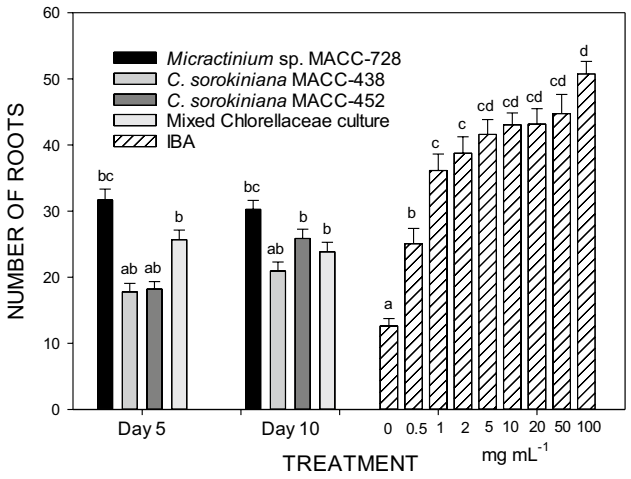

Fig. 4 Rooting activity of water extracts of three microalgae strains and the mixed Chlorellaceae culture harvested on day 5 and day 10 and tested in the mung bean rooting assay. IBA standards were included as a positive control. Results are the average of three experiments and are presented as mean ± SE ($p < 0.05$)

Plant growth-stimulating activity

The water extract of *Micractinium* sp. produced significantly higher rooting activity compared to the other strains. This activity was similar whether the biomass was harvested on day 5 or day 10. The rooting activity was equivalent to that elicited by 0.5–2 mg L^{-1} IBA (Fig. 4). The water extracts of *C. sorokiniana* MACC-438 and *C. sorokiniana* MACC-452 produced significantly lower rooting activity, being equivalent to 0–0.5 mg L^{-1} IBA. The rooting activity of *C. sorokiniana* MACC-452 increased from day 5 to day 10. The rooting activity of the mixed Chlorellaceae culture was moderate, being equivalent to 0.5 mg L^{-1} IBA. Activity was similar regardless of day of harvest (Fig. 4).

Discussion

The present experimental setup used 2-L closed bioreactors situated in a non-climate-controlled greenhouse to test the performance of the microalgae strains in spring and early summer. Temperatures fluctuated over the 3-month experimental period with a 6.6 °C difference in average air temperature between the various experiments and a 7.4 °C minimum and 8.5 °C maximum air temperature difference between experiments. Light parameters were also variable over the experimental period with a 2.1 h difference in the

average mean sunshine duration for the experiments and 5.14 mol photons m^{-2} day^{-1} and 482 µmol photons m^{-2} s^{-1} difference in the sum of PAR and maximum PAR, respectively (Table 1). These sunshine values characterized the immediate environment of the microalgae cultures and were influenced by, for example, the design, location, and environmental conditions (e.g., shading effect) of the greenhouse. The environmental parameters that were the most influential on the growth rates of the three microalgae strains used in the present experiment were the average and maximum air temperatures and the total amount of radiation received (sum of PAR), followed by daily mean of sunshine duration. The minimum air temperature and maximum PAR had less effect on biomass production (Tables 4 and 5).

The three strains were selected for the current experiment based on their slightly different temperature optimum determined in preliminary laboratory experiments where the strains were grown in N-limiting conditions (3% N). In controlled laboratory conditions, *C. sorokiniana* MACC-452 and *Micractinium* sp. had the most rapid growth at 30 °C. *C. sorokiniana* MACC-438 had a broader temperature optimum with the most rapid growth at 25–30 °C (Online resource 2a). The difference in temperature optimum of the two *C. sorokiniana* strains may be attributed to slow generational adaptation which occurs progressively over many generations (Ras et al. 2013). In the present study carried out in a greenhouse with non-controlled environmental conditions, *Micractinium* sp. had higher biomass accumulation compared to the two *C. sorokiniana* strains. It was less sensitive to heat with its growth not being affected by the high temperature and light (Fig. 1 and Table 3). In contrast, temperature and light had the strongest influence on *C. sorokiniana* MACC-438 and this strain produced the lowest biomass (Fig. 1 and Table 3). The air temperatures in the greenhouse were higher (up to 51 °C) than in laboratory experiments, indicating that *Micractinium* sp. had a wider thermal tolerance and was better able to acclimate to the higher temperatures than the *C. sorokiniana* strains. Temperature optimum determined under laboratory conditions often do not coincide with temperature optimum of cultures grown outdoors (Butterwick et al. 2005; Ras et al. 2013) as microalgae are able to acclimatize to outdoor conditions, thus altering their performance. For example, the optimum temperature for growth of *Chrysotila carterae* (previously *Pleurochrysis carterae*) was 25 °C when grown in the laboratory and increased to 32 °C when grown in an outdoor raceway pond (Moheimani and Borowitzka 2007).

Light is indispensable for photosynthesis, promoting cell growth. The average irradiance and its seasonal and daily variations have a large impact on productivity in outdoor microalgae cultures (Borowitzka and Vonshak 2017). These changes range from being light limiting up to light saturation, maybe even causing photoinhibition and photodamage, leading to a decrease in productivity. Outdoor cultures are often exposed to higher irradiances than those encountered under laboratory conditions (Borowitzka 2016a). In the present study, the sum of PAR and daily mean of sunshine were the most influential light parameters on growth rates of the three microalgae strains (Tables 4 and 5).

Temperature and light are closely linked factors influencing microalgae growth. For example, temperature influences enzymatic activity in the photosynthetic pathway and high temperatures disrupt the electron transport chain and affect the stability of the photosynthetic structures (Grimaud et al. 2017). Microalgae cells are more susceptibility to photoinhibition when grown at lower temperatures as the rate of CO_2 fixation is slower (Butterwick et al. 2005; Ras et al. 2013; Borowitzka 2016a). Microalgae have different strategies to acclimate to temperature changes such as adjusting the biosynthesis of key components, e.g., increasing chlorophyll content (Moheimani and Borowitzka 2007; Grimaud et al. 2017) or decreasing cell size to reduce metabolic costs (Ras et al. 2013). In the present study, both temperature and light parameters significantly influenced the growth in the *C. sorokiniana* strains. The growth parameter most affected by the environmental conditions in the greenhouse was the daily change in cell number (Tables 4 and 5), indicating that the rate of cell division was affected in the microalgae strains used in the present study. Cell size was variable with little correlation to the environmental parameters measured (Fig. 1c and Table 4). Cell division in many microalgae is triggered once cells reached a critical threshold size and have sufficient energy stored in the form of starch, lipids, and polyphosphates. For example, in *Chlamydomonas reinhardtii*, the length of the cell cycle is determined by the growth rate which is modulated by a combination of light intensity and temperature (Vítová et al. 2011a, b). Cultures grown in low light conditions had a slower growth rate and longer cell cycle (up to 72 h) compared to cultures grown in higher light intensities which had a faster growth rate and shorter cell cycle (10 h). Increasing temperature also increased the growth rate and shortened the cell cycle in *Chlamydomonas reinhardtii* with the length of the cell cycle decreasing from 34 h at 15 °C to 15 h at 28 °C (optimum temperature) and was blocked at 39 °C (Vítová et al. 2011b).

Polycultures ensure resilience to fluctuating abiotic and biotic conditions (Newby et al. 2016). Species with different temperature optimum should be selected when assembling a polyculture consortium so that at least one species will dominate within the temperature range (Newby et al. 2016). Generally, higher growth rates and yields are obtained in polycultures made up of divergent species compared to the monocultures as this species diversity ensures more efficient utilization of resources between species with different temperature-growth profiles (Schabhüttl et al. 2013; Shurin

et al. 2014). However, low yields (underyielding) may occur when two highly productive strains are paired, highlighting the care that must be taken to select compatible strains for co-culturing (Shurin et al. 2014). The three strains used in the present experiment are single-celled, freshwater species belonging to the Class Trebouxiophyceae, Order Chlorellales, Family Chlorellaceae (Guiry and Guiry 2016) with each strain having similar traits and good productivity. The mixed Chlorellaceae culture performed well with biomass production exceeding that of the *C. sorokiniana* monocultures, suggesting that these strains were compatible (Table 2). Growth performance in cooler months (late summer to autumn) needs to be investigated to determine if this consortium of microalgae can perform satisfactorily at suboptimal lower temperatures and light levels. From a screening study of 300 strains, *Micractinium reisseri*, *Chlorella vulgaris*, and *Scenedesmus rubescens* were selected based on good growth traits and a high lipid and carbohydrate content for an outdoor winter trial. These strains were successfully cultivated at cool temperatures (10–20 °C with 0 °C minimum temperature) and low light intensities and duration, albeit with a slower growth rate and a change in lipid and carbohydrate profiles (Dahlin et al. 2018).

Proteins are required in actively growing cultures. Under stress conditions where the growth rate decreases, there is a shift in biosynthetic pathways so that photoassimilates are stored in lipid bodies in the cytoplasm. Thus, there is generally an inverse correlation between protein and lipid content in microalgae (Rodolfi et al. 2009; Ördög et al. 2013). In the present study, there was a decrease in protein content and an increase in lipid content from day 5 to day 10 in the monocultures. *Micractinium* sp. had a significantly lower lipid content of day 5 compared to the *C. sorokiniana* strains. However, it had the highest biomass accumulation, the highest protein content on day 5, and the highest lipid content on day 10 (Figs. 1 and 2).

While nitrogen depletion is most commonly used to induce lipid biosynthesis in microalgae, light and temperature also influence the macromolecule content. For example, higher irradiance levels increase chloroplast activity, leading to a decrease in lipid content as lipids are a major component of chloroplasts (de Morais et al. 2015), and decreasing temperatures below the growth optimum favors lipid production (Ras et al. 2013). When the strains used in the present study were grown at three temperatures under laboratory conditions, *C. sorokiniana* MACC-452 had the largest changes in protein and lipid content with lipid content increasing at higher temperatures and protein content decreasing with increasing temperature. *Chlorella sorokiniana* MACC-438 accumulated more lipids and fewer proteins at 20 °C compared to higher temperatures. Lipid content in *Micractinium* sp. increased with higher temperatures while the protein content was not affected and was the lowest of the three strains (Online resource 2b and c). In the present study, *Micractinium* sp. had the highest protein content (and highest biomass accumulation), providing evidence that was the strain best able to acclimate to the environmental conditions in the greenhouse.

There is a positive link between species richness and lipid productivity (Stockenreiter et al. 2012) especially when divergent microalgae that utilize different wavelengths make up the consortium as this allows for more efficient light use (Stockenreiter et al. 2012, 2013). For example, there was a non-significant increase in lipid content with increasing species number when all the species were from the same class and a significant increase in lipid content when species from different classes were combined (Stockenreiter et al. 2013). In contrast, in the present study, the mixed Chlorellaceae culture was underyielding with the lipid content not increasing with culture age and was significantly lower on day 10 compared to the monocultures. This low lipid content may be linked to the mixed culture having a higher protein content on day 10 compared to the monocultures (Fig. 2). The reason for lipid underyielding in the mixed Chlorellaceae culture needs further investigation.

Environmental conditions also affect the synthesis of secondary metabolites in microalgae with many metabolites produced in response to stress conditions (Borowitzka 2016a; Rossi and De Philippis 2016). As reactive oxygen species (ROS) are a by-product of photosynthesis, their generation is affected by light and temperature conditions (Borowitzka 2016a). Microalgae synthesize secondary metabolites with antioxidant activity which scavenge free radicals and thus provide protection against oxidative stress which is increased at high temperatures due to the inactivation of the oxygen evolving capability of PSII (Ras et al. 2013). In the present study, *Micracintium* sp. had slightly higher antioxidant activity in the DPPH assay compared to the two *C. sorokiniana* strains on day 5. Antioxidant activity decreased significantly over time with *Micractinium* sp. having the largest decrease so that its activity was significantly lower on day 10 compared to the *C. sorokiniana* strains (Fig. 3). Similarly, antioxidant activity (measured in the DPPH and β-carotene-linoleic acid assays) was highest in three *Chlorella* strains grown in nitrogen-sufficient conditions compared to nitrogen-limiting conditions with activity decreasing over time as cultures became more stressed (Aremu et al. 2016). The species composition in the polyculture may also influence the quality of the biomass produced. Species richness alone does not ensure higher biomass quality but rather selection of strains with specific quality characteristics should be included in the microalgae consortium (Newby et al. 2016). In the present study, antioxidant activity of the mixed Chlorellaceae culture was higher on both day 5 and day 10 compared to the monocultures (Fig. 3). This suggests that mixed cultures comprising suitable fast-growing microalgae strains may improve the quality of the

biomass being harvested regardless of the environmental conditions and harvest time and thus could be a viable strategy in a biorefinery approach.

The concentration of plant-promoting compounds in the microalgae feedstock will vary with microalgae species and culture conditions. The mung bean bioassay used in the present study measured the root-promoting activity of the extract. Positive results have previously been achieved in this bioassay using microalgae extracts where two *Chlorella* strains had higher rooting activity than *Scenedesmus acutus* (Stirk et al. 2020). In the present study, *Micractinium* sp. was the most biologically active with similar rooting activity in extracts harvested on day 5 and day 10. The two *C. sorokiniana* strains had significantly lower activity with activity increasing in extracts harvested on day 10. The rooting activity of the mixed Chlorellaceae culture was greater than the *C. sorokiniana* extracts but less than the *Micractinium* sp. extract. This activity was consistent in the extracts made from biomass harvested on day 5 and day 10 (Fig. 4). There is a dose-dependent response in plants treated with microalgae extracts (Garcia-Gonzalez and Sommerfeld 2016), and thus, achieving a consistent level of biological activity in mass-cultured microalgae is desirable if it is to be used as a plant biostimulant. This suggests that in a biorefinery approach, inclusion of *Micractinium* sp. in *Chlorella* cultures grown for other purposes, e.g., lipid production, could improve the plant biostimulant activity of the extract.

In conclusion, it is important to evaluate the physiological responses and metabolite accumulation in microalgae strains grown in outdoor conditions to establish if they can acclimate to the prevailing environmental conditions. The 2-L experimental setup in the greenhouse used in the current study allowed for relatively easy and quick evaluation of the microalgae strains when exposed to a range of high temperature and light conditions and thus could guide the selection of suitable strains for outdoor cultivation. The most influential environmental parameters on microalgae growth were average air temperature and the sum of PAR, followed by maximum air temperature. The growth parameter most affected was daily change in cell number, suggesting that the length of the cell cycle was temperature and light-dependent. *Chlorella sorokiniana* MACC-438 produced the lowest biomass and was most affected by high temperature and light. *Micractinium* sp. produced the highest biomass and was least affected, suggesting it has a wider thermal tolerance, making it the most suitable for outdoor cultivation of the three strains tested. However, the lipid content was significantly lower compared to the *C. sorokiniana* strains on day 5 but was the highest by day 10. The mixed Chlorellaceae culture performed well with biomass production exceeding that of the *C. sorokiniana* monocultures. The mixed culture also had a higher protein content on day 10 compared to the monocultures but was significantly underyielding in lipid content. This highlights the importance of selecting compatible strains when assembling a polyculture, even if using taxonomically close species. Antioxidant activity and the root-stimulating activity of the extracts varied with strain and culture age with *Micractinium* sp. having the highest but most variable activity. Bioactivity in the mixed culture was more consistent, remaining high regardless of culture age and environmental conditions. Thus, mixed cultures made up of productive strains could be a useful strategy to ensure stable and high-quality biomass production in outdoor cultivation with fluctuating environmental conditions.

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Author contribution WAS wrote the manuscript and carried out the antioxidant and mung bean assays; PB conducted the growth experiments; GM conducted the molecular identification; ZV participated in the collection and processing of the meteorological data and the assessment of the meteorological impacts; ZL carried out the statistical analysis; JvS edited the manuscript; and VÖ conceptualized and designed the experiment and edited the manuscript.

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Data availability The datasets generated during this study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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