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Growth strategies of *Chlorella vulgaris* in seawater for a high production of biomass and lipids suitable for biodiesel

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ABSTRACT

Chlorella vulgaris is a freshwater microalga that synthesises large amounts of saturated lipids, which makes it suitable for production of bioenergy and biofuels. Since its cultivation usually requires freshwater, it competes with agriculture, economic development and ecological conservation for this limited natural resource. This study investigated the possibility of the partial replacement of freshwater by seawater (50 %) in the growth medium for a more sustainable biomass and lipid production. *Chlorella vulgaris* 211-11b was cultivated as shake-flask cultures in Bold's Basal Medium (BBM) formulated with 50 % freshwater and 50 % seawater under photoautotrophic, mixotrophic and heterotrophic conditions for eight days with glucose as organic carbon source in the latter two cases. The alga's best growth performance and highest lipid contents (49 % DW^{-1}), dominated by palmitoleic and oleic acid, occurred under mixotrophic rather than photoautotrophic and heterotrophic conditions. This study demonstrates a more economic and ecologically sustainable biomass and lipid production of *C. vulgaris* by saving 50 % freshwater, which is available for other purposes.

1. Introduction

For decades microalgae have been used for the biotechnological production of a great variety of natural products [1–5]. Amongst these algae, species of the genus *Chlorella* turned out to be promising resources for the production of biodiesel, food, feed and wastewater treatment due to their high growth rates and physiological plasticity [3,6,7]. For large-scale and industrial applications, *Chlorella vulgaris* is grown under photoautotrophic conditions in photobioreactors and raceway ponds at which light and CO_2 serve as energy and carbon sources for photosynthesis, respectively. However, *C. vulgaris* can also be grown both mixo- and heterotrophically on acetate, glycerol and glucose as organic carbon sources. Glucose seems to be the most suitable carbon source because of its highest conversion rate into microalgal biomass [8]. The mixotrophic biomass production of *C. vulgaris* on glucose is often more effective than photoautotrophy and heterotrophy because of shorter cultivation periods and higher cell densities [8,9]. Moreover, the alga's growth performance and lipid production depend on the organic carbon source supplied with the medium. A comparison between substrates revealed that glucose promoted the biomass productivity, while glycerol and acetate resulted in a higher productivity and accumulation of lipids [3,8]. Since the lipid composition of *C. vulgaris* and its closely-related species is dominated by saturated and monounsaturated fatty acids, they are desirable candidates for the production of biodiesel [3,10,11].

Chlorella vulgaris occurs in limnic ecosystem and, therefore, freshwater-formulated growth media are expected to be required for an optimal cultivation. Nevertheless, studies demonstrated that strains of *C. vulgaris* are also capable of growing in media with different salinities

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[12,13]. For example, the growth rate of a *C. vulgaris* strain isolated from a freshwater source in New Zealand were identical in medium formulated with both freshwater and 50 % seawater, while it grew slower in 100 % seawater [13]. Moreover, *C. vulgaris* UTEX-265 of freshwater origin was shown to grow rapidly until 1 % (w/v) NaCl, which is equivalent to 30 % seawater or brackish conditions, it was not able to grow above 2 % (w/v) NaCl or 57 % seawater. In contrast, *Chlorella* sp. HS2 isolated from a marine tide pool demonstrated a relatively high halotolerance by its ability to grow between 1 and 7 % NaCl (30–200 % seawater) due to its physiological adaptation to the fluctuating salinities in its habitat [14]. The physiological traits of halotolerance are related to the algae's capability of adjusting metabolically to hyperosmotic stress by accumulating compatible solutes, ions and relevant proteins through stress-induced gene expression [15]. Halotolerant strains of *Chlorella autotrophica* and *Chlorella emersonii* regulate their turgor under hypersaline conditions by the accumulation of proline as compatible solute and fluxes of Na^+, K^+, Ca^{2+} and Cl^- ions [16,17]. The oleaginous *Chlorella vulgaris* strain SAG 211-11b was also shown to be halotolerant because it was able to grow up to 0.75 M NaCl in the medium (125 % seawater). Under this osmotic stress, there was a 4.2-fold increase in intracellular sodium-ions measured, while potassium and calcium-ions decreased by 92 % and 85 %, respectively [18].

Tapping the physiological traits that allow the alga to grow under saline conditions could be an important pre-requisite for an economic and ecologically more sustainable biomass production in coastal areas where seawater is available as potential alternative to freshwater. The use of freshwater for microalgal biomass and bioenergy production may compete with its need for agricultural activities, the economic and population growth as well as the conservation of ecosystems. The industrial bioenergy production by microalgae in open ponds and photobioreactors may have a considerable impact on freshwater resources, resulting in an enormous blue water footprint (i.e. the volumes of surface and groundwaters used to produce a product, measured over the entire supply chain) of 8 to 193 m^3 GJ^{-1} [19]. However, the replacement of freshwater by seawater for the cultivation process could reduce a production's blue water footprint substantially [19]. Thus, the saved freshwater could be available as resource for other purposes such as the production of food and feed as well as for ecosystems [20]. Moreover, since climate change, growing populations and a competitive water use may cause shortages of freshwater supplies, the use of seawater for algal cultivation in coastal areas could reduce the pressure on the available freshwater resources. Since freshwater strains of *C. vulgaris* can tolerate a wide range of salinities, this study was conducted to understand the effects of a growth medium formulated with 50 % seawater on the biomass production, the photophysiology and the biochemical composition, with emphasis on the lipid production under photoautotrophic, mixotrophic and heterotrophic conditions. The results of the present study could be useful for a more sustainable biomass production of *C. vulgaris* with bioenergy-suitable lipid contents.

2. Material and methods

2.1. Algal strain and stock culture

Chlorella vulgaris strain SAG 211-11b (Chlorophyta, Trebouxiophyceae) was purchased from the Culture Collection of Algae at the University of Göttingen (SAG), Germany. This temperate strain was isolated from a eutrophic freshwater pond near Delft, The Netherlands, in 1889 and kept under axenic conditions at SAG. In the laboratory, the algae were maintained as batch cultures in 100 mL of Bold's Basal medium (BBM; [21]) in 250 mL Erlenmeyer flasks on an orbital shaker (120 rpm) at 22 ± 1 °C and 125 ± 5 μmol photons m^{-2} s^{-1} (continuous light, Cool daylight 36 W, Philips, The Netherlands) for 6 weeks prior to the experiments. During this period, the algae were weekly transferred to new BBM to establish a stock culture.

2.2. Growth, physiology and biochemical composition at different growth strategies with 50 % seawater in the medium

For the experiment, BBM was formulated with natural seawater (salinity: 35) that was previously adjusted to 50 % with equal parts of distilled water. This resulted in a final salinity of 17.5. The seawater was pumped up from 250 m water depth at Saltenfjord at Mørkvedbukta in Bodø, Norway, to the laboratory. It was filtered through a 0.2 μm cellulose-nitrate membrane filter (Sartorius Stedim Biotech GmbH, Göttingen, Germany) and autoclaved prior to the preparation of the BBM. In 250 mL Erlenmeyer flasks, 90 mL of the modified BBM formulated with 50 % seawater were inoculated with 10 mL of *C. vulgaris* taken from the stock culture, reaching an OD_{540} of 0.08. These cultures were grown at 22 ± 1 °C in culture cabinet (KB8400 FL, Termaks, Norway) under photoautotrophic, mixotrophic and heterotrophic conditions for 8 days. While the photoautotrophically and mixotrophically-grown *C. vulgaris* were exposed to 125 ± 5 μmol photons m^{-2} s^{-1} (continuous light, Cool daylight 36 W, Philips, The Netherlands), the heterotrophic cultures were kept dark by covering the flasks with two layers of aluminium foil. BBM used for the mixotrophic and heterotrophic cultures was supplemented with 5 g D-glucose L^{-1} (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), which is below the strain's substrate inhibition at 27–40 g L^{-1} [11]. Filtered air (0.2 μm, Acrodisc® PTFE filters, Pall Corporation, USA) was supplied to each flask at a flow of 1 vvm (100 mL min^{-1}) using a rotameter (Omega, Manchester, UK) for oxygen/CO_2 supply and mixing purposes. OD_{540} were measured at the beginning of the experiment and after 1, 2, 3, 4 and 8 days to determine growth parameters. The photosynthetic performance of *C. vulgaris* was measured at the end of the experiment, i.e. day 8. For the biochemical analysis of the algal biomass, samples were taken at day 8 and subsequently centrifuged (5000 ×*g*, 5 min, 20 °C), frozen and freeze-dried (−55 °C; Labconco, Kansas City, USA) in the dark for three days. The freeze-dried biomass was stored at room temperature in the dark before it was used for the analysis of pigments, total carbohydrates and fatty acids.

2.3. Parameters of biomass production

Algal growth in each flask was determined by measuring the absorbance of 200 μL samples in 96-well microplates photometrically at 540 nm (Sunrise A-5082, Tecan, Männedorf, Switzerland): OD_{540} values were converted into algal dry weight (DW) using a previously determined standard curve in which OD_{540} were plotted against the corresponding dry weight of *C. vulgaris* (g L^{-1}). The specific growth rates, μ (day^{-1}), were calculated as follows:

$$\mu = (lnX_t - ln\, X_0)\, t^{-1} \quad (1)$$

where X_0 and X_t are the algal dry weights (g L^{-1}) at the beginning and at a specific point in time, t (days), of the experiment, respectively.

The doubling time, t_d (h), which is the time required for a population to double, was determined from μ taken from the previous equation:

$$t_d = ln2\, \mu^{-1} \quad (2)$$

The biomass productivity, P_i (g L^{-1} day^{-1}), was calculated as follows:

$$P_i = (X_i - X_0)(t_i - t_0)^{-1} \quad (3)$$

where X_i and X_{i-1} are the algal dry weights (g L^{-1}) at two points in time, t_i and t_{i-1} (days).

2.4. Photosynthetic performance

The photosynthetic activity was measured by chlorophyll *a* fluorescence using a pulse-amplitude modulated (PAM) fluorometer (Diving-PAM, Walz GmbH, Effeltrich Germany) at 22 ± 1 °C. Algal samples (4

mL) taken from the experimental flasks were adjusted to 1.0 g DW L^{-1} using BBM formulated with 50 % seawater before they were transferred into a 3 mL-cuvette with a 1 cm optical light path. The suspensions were homogenized throughout the measurements by stirring them with a Teflon-coated stirring bar. After 60 min of dark-adaptation to fully oxidise the photosystem II (PSII), the minimum (F_0) and maximum (Fm) fluorescence yields were measured using a weak red measuring light (λ = 680 nm, <1 µmol m^{-2} s^{-1}) and applying a saturation pulse (>9000 µmol photons m^{-2} s^{-1}, 0.6 s), respectively. The maximum PSII-quantum yields were calculated: Fv/Fm = (Fm − F_0)/Fm [22]. Subsequently, the effective PSII-quantum yields [Fv′/Fm′= (Fm′ − F_0′)/Fm′] were measured from minimal (F_0′) and maximum fluorescence (Fm′) yields of the same algal samples. They were exposed to incrementally increasing actinic light intensities (E_{AL} = 41–1452 µmol photons m^{-2} s^{-1}) after the application of a saturation pulse (>9000 µmol photons m^{-2} s^{-1}, 0.6 s) every 10 s. The relative photosynthetic electron transport rates (ETRs) were determined by multiplying Fv′/Fm′ with the corresponding E_{AL} (ETR = Fv′ / Fm′ × E_{AL}) and plotted against E_{AL}. ETR-*E* curves were fitted using *R* version 3.53 to estimate the photosynthetic characteristics: the maximum ETRs (ETR_{max}), the light saturation points (E_k), and initial slopes of the ETR-*E* curves (α_{ETR}) [23,24].

2.5. Pigment analysis

For the pigment analysis, 100 % methanol (1.5 mL) and a mixture of glass (0.1 mm) and ceramic beads (1.4 mm) were added to algal samples (2.0 mg DW). The samples were ground to a fine powder by three 20 s-cycles of beat-milling (6000 rpm) with a 120 s-break on ice using a Precellys Evolution tissue homogenizer (Bertin Technologies SAS, Montigny-le-Bretonneux, France). Afterwards, the samples were stored on ice in the dark for 2 h before they were vortex-mixed and centrifuged (7000 ×*g*, 20 °C, 10 min). The absorbance of the supernatant (1 mL) was recorded between 400 and 700 nm using a spectrophotometer (Uviline 9400, SCHOTT Instrument GmbH, Germany). All steps were performed in the dark. The contents of the chlorophylls *a* (Chl *a*) and *b* (Chl *b*) as well as of the total carotenoids (Car) were determined and expressed as µg mL^{-1} [25]. The total chlorophyll content (µg mL^{-1}) is the sum of Chl *a* and *b*.

$$Chl\ a = 15.65 \times A_{666} - 7.34 \times A_{653} \quad (4)$$

$$Chl\ b = 27.05 \times A_{653} - 11.21 \times A_{666} \quad (5)$$

$$Car = (1000 \times A_{470} - 2.68 \times Chl\ a - 129.2 \times Chl\ b)\ 221^{-1} \quad (6)$$

2.6. Analysis of total carbohydrates, proteins and lipids

For the analysis of the biochemical composition, 80 mL of each algal suspension were centrifuged (1500 ×*g*, 20 °C, 5 min) and the pellet was resuspended in 10 mL of 0.5 M ammonium formate (NH_4HCO_2). After centrifugation (1500 ×*g*, 20 °C, 5 min) the pellets were freeze-dried at −55 °C and stored at −80 °C until analysis.

The content of total carbohydrates was quantified by the Anthrone method [26]. The samples were hydrolysed with 3 mL of 3 M HCl at 100 °C for 80 min and cooled-down to room temperature. Then, 4 mL of the Anthrone reagent (500 mg anthrone dissolved in 250 mL of 96 % H_2SO_4) was added to 1 mL of each sample, vortex-mixed and incubated for 10 min at 70 °C. After the samples were cooled-down to room temperature, their absorbance was measured photometrically at 620 nm. The content of total carbohydrates was quantified by comparison with a standard curve of a known D-glucose concentration and expressed as % DW^{-1}.

Total lipids were extracted using organic solvents and gravimetrically quantified [27]. The fatty acid methyl esters (FAMES) from triacylglycerols (TAGs) and polar lipids were obtained from total lipids by solid-phase extraction using 6 mL volume, 1 g silica cartridges (Supelco, USA) [28]. TAGs and polar lipids were derivatised to FAMES according to Breuer et al., (2013) and analysed in a GC fitted with a Flame Ionization Detector (Scion 436, Bruker, USA) and an Agilent CP-Wax 52 CB column (Agilent Technologies, USA) using a splitless injector. To identify and quantify the most common FAMEs, external Supelco® 37-component standards (Sigma-Aldrich, USA) were used. Blanks were included in the extraction process to eliminate background trace peaks.

The protein content was determined from hydrolysed algal samples (1 N NaOH) in comparison to a standard curve of BSA with known concentrations and expressed as % DW^{-1} [29].

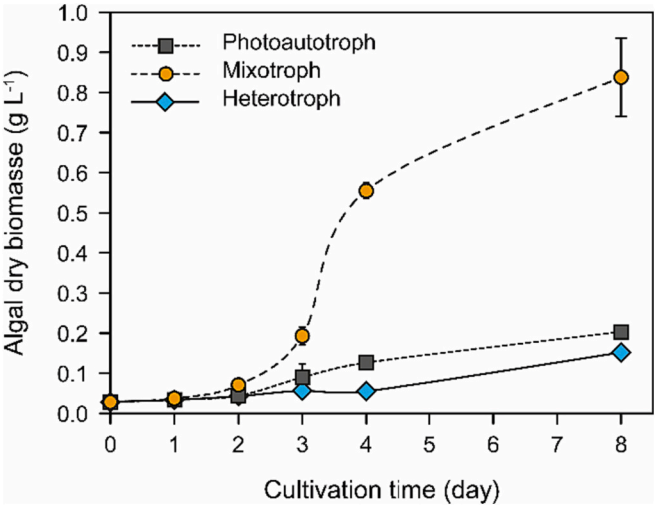

Fig. 1. Growth curve of *Chlorella vulgaris* over 8 days under photoautotrophic (grey squares, straight line), mixotrophic (orange circles, dashed line) and heterotrophic (blue diamonds, dotted line) conditions in Bold's Basal Medium (BBM) formulated with 50 % freshwater and 50 % seawater. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.7. Statistical analysis

Means and standard deviations were calculated from three independent replicates per treatment (n = 3) in the experiment. Normal distribution and homoscedasticity as assumptions of the analysis of variance (ANOVA) were tested using the Shapiro Wilk-test and the Levene test, respectively. When these assumptions were met, a 1-way ANOVA with Tukey's honestly significant difference (HSD) post-hoc test were performed. At heteroscedasticity, a 1-way Welch-ANOVA with the Games-Howel post-hoc test was conducted. A 5 %-significance level (P = 0.05) was applied in all statistical tests, which were performed using the software packages JMP version 14.0, JMP Pro 15.2.0 (SAS Institute Inc., Cary, North Carolina, USA) and *R* version 4.1.2 with the package 'rstatix' version 0.7.0 [24].

2.8. Results and discussion

The oleaginous green microalga *Chlorella vulgaris* SAG 211-11b can tolerate a wide range of salinities despite its freshwater origin due to its capability of adapting to osmotic stress [18]. The preliminary investigation to the present study confirmed this adaptability by showing no apparent difference of algal growth between BBM formulated with 100 % freshwater and 50 % seawater/50 % freshwater over five days (Fig. S1). A similar halotolerance was also demonstrated for closely-related strains of *C. vulgaris*, e.g. from Hwajinpo Lake in South Korea [30]. Based on this broad halotolerance, it is crucial to understand the physiological and biochemical responses to high salinities in the medium for a high biomass and lipid production under different growth strategies for industrial applications. Using BBM formulated with 50 % seawater and 50 % freshwater, the mixotrophic cultivation of *C. vulgaris* after

Table 1
Growth characteristics of *Chlorella vulgaris* over eight days with different growth strategies and 50 % seawater. For mixotrophic and heterotrophic growth, 5 g glucose L^{-1} were added to the medium. Data are means and standard deviations. Different superscript small case letters behind the results indicate statistically significant differences between the growth strategies for each parameter individually (specific growth rate and doubling time: Welch-ANOVA, Games-Howel post-hoc test, $P < 0.05$; biomass productivity and algal dry biomass: 1-way ANOVA, Tukey HSD post-hoc test, $P < 0.05$).

Growth strategy	Specific growth rate (day^{-1})	Doubling time (h)	Biomass productivity (g L^{-1} day^{-1})	Algal dry biomass after 8 days (g L^{-1})
Photoautotrophy	0.193 ± 0.002^{b}	86.2 ± 0.9^{a}	0.135 ± 0.002^{b}	0.152 ± 0.010^{b}
Mixotrophy	0.289 ± 0.002^{a}	57.6 ± 0.4^{b}	0.451 ± 0.011^{a}	0.838 ± 0.097^{a}
Heterotrophy	0.181 ± 0.011^{b}	92.0 ± 5.6^{a}	0.033 ± 0.002^{c}	0.124 ± 0.004^{b}

eight days on glucose resulted in a significant higher growth rate (1.5–1.6×), biomass yield (5.5–6-8×) and productivity (1.4–3.3×) as well as a substantially lower doubling time (63–67 %) than under photoautotrophic and heterotrophic conditions after the same period of time (Fig. 1 and Table 1). This result demonstrates that mixotrophy is the preferred of the three tested growth strategies for a fast and high biomass production in 50 % seawater, which is in accordance with other studies on the same strain in solely freshwater-formulated BBM in another independent study [31].

Under the mixotrophic conditions, the highest growth rates and biomass yields revealed the synergistic effects of light and glucose [31–34]. Photosynthesis and respiration are closely coupled processes in mixotrophic *Chlorella* because the oxidation of glucose leads to an increase in photosynthesis [33,34]. In the present study, however, both the maximum PSII-quantum yields (Fv/Fm: 0.587–0.662 r.u.) and the photosynthetic parameters [ETR_{max}: 49.4–49.5 r.u.; E_k: 197–281 µmol m^{-2} s^{-1}; α_{ETR}: 0.183–0.251 (µmol m^{-2} s^{-1})$^{-1}$] of *C. vulgaris* were statistically similar between the mixotrophic and photoautotrophic conditions after eight days of cultivation (Fig. 2). By contrast, the chlorophyll and carotenoid contents were generally 1.8–2.2× higher in the mixotrophically than in photoautotrophically grown algae. Since mixotrophy led to higher Chl *a* (2.13×) than Chl *b* (1.81×) content compared to photoautotrophy, the mixotrophic algae had a significantly higher Chl *a*/*b* ratio of 2.73 (Table 2). These effects can be most likely ascribed to the different physiological stages of *C. vulgaris* after eight days of cultivation: while the mixotrophically grown algae reached the

Table 2
Photosynthetic and accessory pigments of *Chlorella vulgaris* after eight days grown under different growth strategies and 50 % seawater. Under mixotrophic and heterotrophic conditions, 5 g glucose L^{-1} was added to medium. Chl: chlorophyll, Car: carotenoids, DW: dry weight. Data are means and standard deviations. Different superscript small-case letters indicate statistically significant difference between the different growth strategies (1-way ANOVA, Tukey HSD post-hoc test, $P < 0.05$).

Growth strategy	Chl *a* (mg g^{-1} DW)	Chl *b* (mg g^{-1} DW)	Chl *a*/*b*	Chl_{total} (mg g^{-1} DW)	Car (mg g^{-1} DW)
Photoautotrophy	13.36 ± 1.09^{b}	5.77 ± 0.69^{b}	2.32^{b}	19.13 ± 1.52^{a}	2.72 ± 0.24^{a}
Mixotrophy	28.48 ± 2.14^{a}	10.48 ± 0.54^{a}	2.72^{a}	38.97 ± 2.68^{b}	6.07 ± 0.36^{b}
Heterotrophy	7.15 ± 2.80^{c}	2.62 ± 0.93^{c}	2.73^{a}	9.78 ± 3.74^{c}	1.50 ± 0.65^{c}

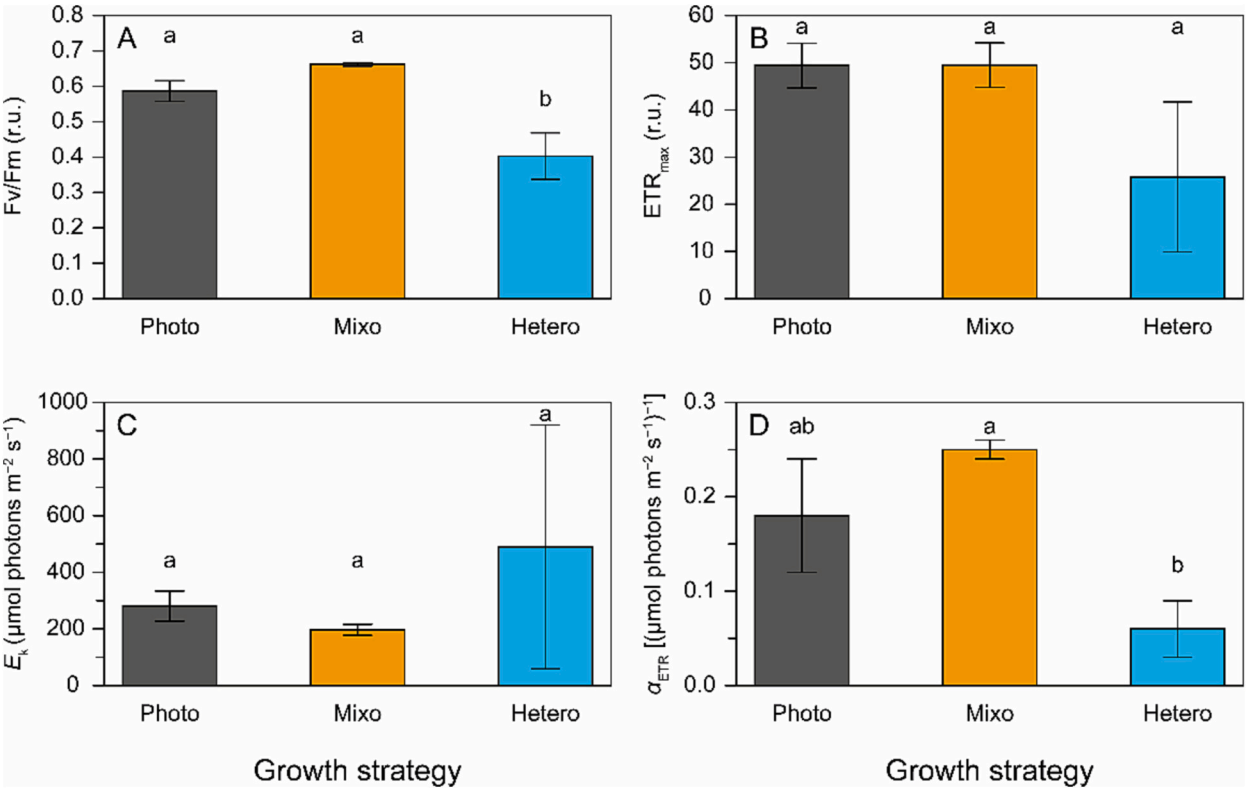

Fig. 2. Photosynthetic performance of *Chlorella vulgaris* after eight days grown under photoautotrophic (grey), mixotrophic (orange) and heterotrophic conditions (blue) with 50 % seawater. (A) Maximum quantum yields of photosystem II (Fv/Fm), (B) relative photosynthetic electron transport capacities (ETR_{max}), (C) light saturation points of photosynthesis (E_k) and (D) the initial slopes of the ETR-*E* curves (α_{ETR}). Under mixotrophic and heterotrophic conditions, 5 g glucose L^{-1} were added to the BBM. Data are means with standard deviations as error bars. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

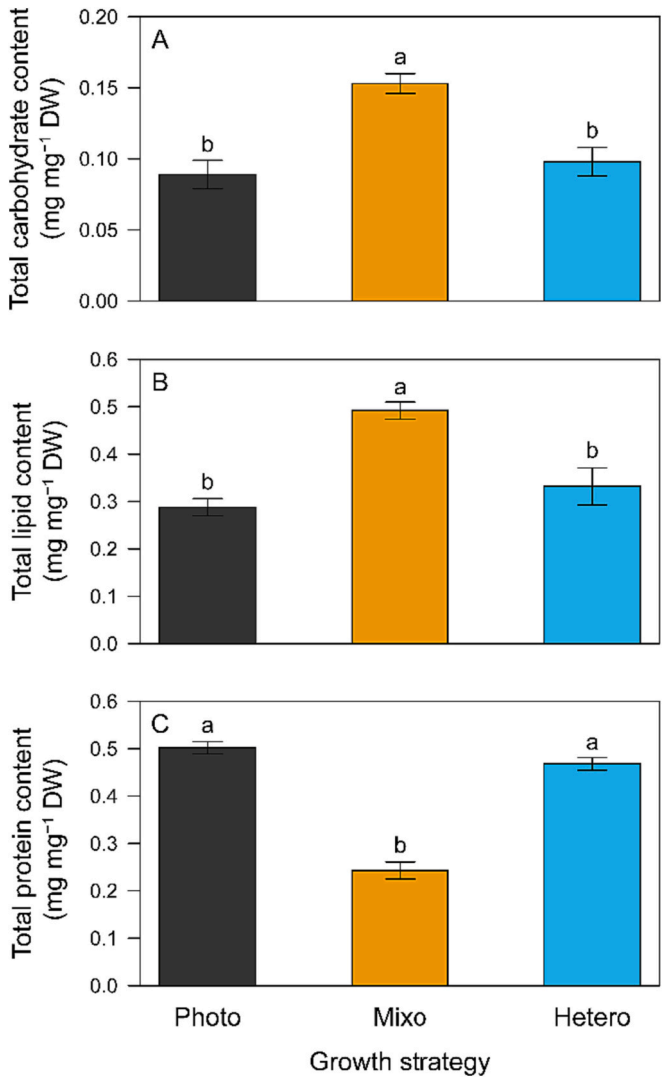

Fig. 3. Biochemical composition of *Chlorella vulgaris* after 8 days grown under photoautotrophic (grey), mixotrophic and heterotrophic conditions with 50 % seawater. Contents of (A) total carbohydrates, (B) total lipids and (C) total soluble proteins. Under mixotrophic and heterotrophic conditions, 5 g glucose L^{-1} were added to the BBM. Data are means with standard deviations as error bars.

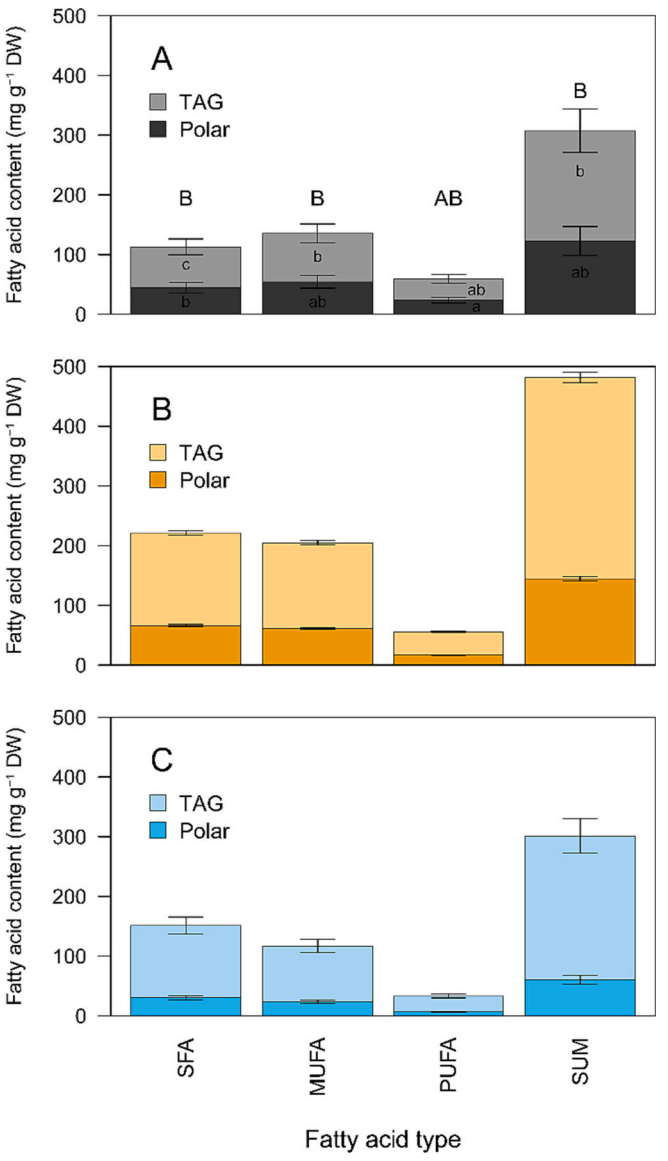

Fig. 4. Fatty acid methyl esters in *Chlorella vulgaris* after 8 days grown under (A) photoautotrophic (grey), (B) mixotrophic (orange) and (C) heterotrophic conditions (blue) and 50 % seawater. Under mixotrophic and heterotrophic conditions, 5 g glucose L^{-1} was added to the BBM. Upper light column part: polar fractions; lower darker column part: triacylglycerol (TAG) fractions. Data are means with standard deviations as error bars of triplicates per growth strategy (n = 3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

stationary phase, the slower growing photoautotrophic algae were apparently still in the physiologically more active exponential phase (Fig. 1). In *Chlorella sorokiniana*, the photosynthetic activity of mixotrophically grown algae was highest in the exponential phase but shortly decreased in the stationary phase to activities similar to those of the photoautotrophic algae in the exponential phase [34]. Accordingly, the photosynthetic capacity (ETR_{max}) of the mixotrophic *C. vulgaris* could also have been higher in the exponential growth phase than in the stationary phase. Then photosynthesis could have decreased to a level that was similar to that in the exponentially growing photoautotrophic algae while remaining the PSII activity. The high Fv/Fm of the mixotrophic *C. vulgaris* indicates to undamaged, fully functional PSII reaction centres, which is in contrast to *C. sorokiniana* [34]. Hence, the down-regulation of photosynthesis of the mixotrophically grown *C. vulgaris* could be associated with changes in the light capture strategy, which resemble to high light acclimation. The high Chl *a/b* ratio suggests that the presence of smaller PSII-associated light harvesting antennae (i.e. LHCII) that restrict the transfer of the absorbed light energy to PSII. The decline in the photosynthetic capacity (ETR_{max}) in the stationary phase of the mixotrophic algae and the restricted light capture could also have resulted in a decrease in the photon use efficiency (α_{ETR}) and the light saturation point of photosynthesis (E_k) to the level of the photoautotrophic algae. The down-regulation of photosynthesis of the mixotrophically grown algae in the stationary phase may reflect the transition from the physiologically active growth phase into phase in which energy reserves are reduced.

The heterotrophically grown *C. vulgaris* showed significantly lower Fv/Fm (0.403 r.u.) and α_{ETR} [0.062 (μmol m^{-2} s^{-1})$^{-1}$] than the photoautotrophic and mixotrophic algae, while the photosynthetic capacity (ETR_{max}: 25.8 r.u.) and light saturation point (E_k: 489 μmol m^{-2} s^{-1}) were similar amongst all growth strategies (Fig. 2). Although the chlorophyll and carotenoid contents were 4× lower than under mixotrophic conditions, the Chl *a/b* ratio of 2.72 was statistically similar (Table 2). This indicates to an equal degradation of the photosynthetic and accessory pigments in the dark because they are unused by

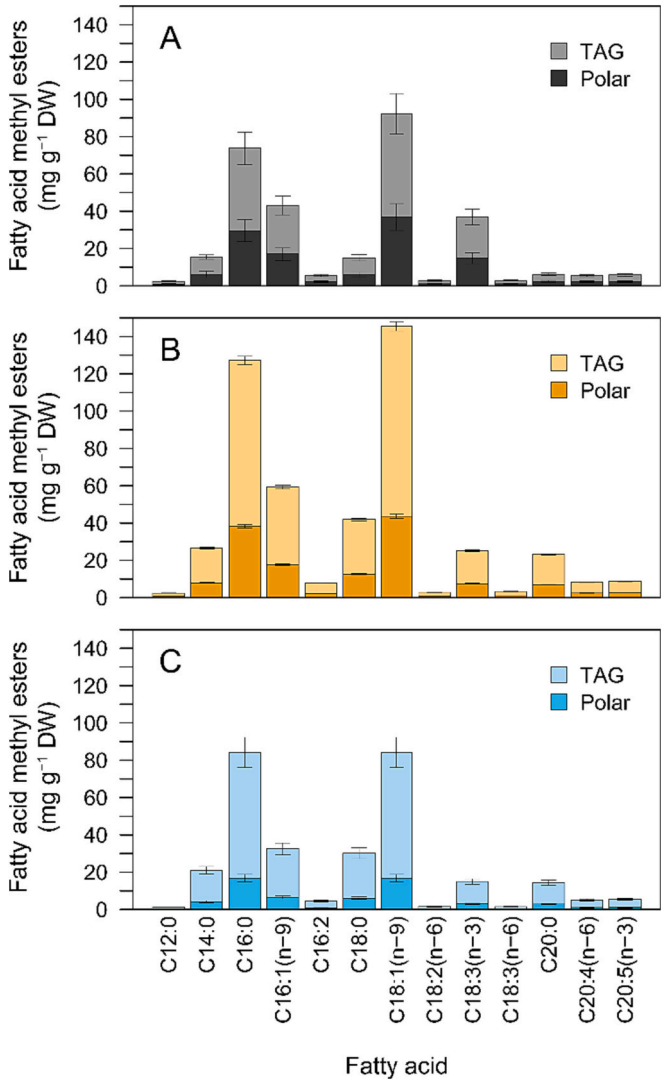

Fig. 5. Fatty acid methyl esters in *Chlorella vulgaris* after 8 days grown under (A) photoautotrophic, (B) mixotrophic and (C) heterotrophic conditions and 50 % (half-strength) seawater. Under mixotrophic and heterotrophic conditions, 5 g glucose L^{-1} was added to the BBM. White columns: polar fractions; grey columns: triacylglycerol (TAG) fractions. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, SUM: sum of SFA, MUFA and PUFA. Data are means with standard deviations as error bars of triplicates per growth strategy (n = 3).

photosynthesis. Heterotrophically grown *C. vulgaris* is known to remain pigments such as chlorophylls, while other green microalgae (e.g. *Chlamydomonas acidophila*) bleach and cannot grow on glucose in the dark [31]. Heterotrophically grown *C. vulgaris* seem to maintain the ability to photosynthesise but they apparently reduce the functionality of PSII and photon use efficiency due to the lower chlorophyll contents.

Mixotrophic cultivation of *C. vulgaris* also had a great impact on the alga's biochemical composition. After eight days of cultivation, the total carbohydrate (8.9–9.9 % DW^{-1}), protein (46.8–50.2 % DW^{-1}), pigment (1.1–2.2 % DW^{-1}) and lipid (28.8–33.2 % DW^{-1}) contents were relatively similar under photoautotrophic and heterotrophic conditions. However, the carbohydrate (15.4 % DW^{-1}) and lipid (49.2 % DW^{-1}) contents increased significantly by 48–72 % after eight days of mixotrophic growth, while the protein content was halved ($P < 0.01$; Fig. 3).

This difference can probably be attributed to the metabolic shift from the utilisation of either photosynthetically produced or added sugars for the production of biomass during exponential growth to the accumulation of energy reserves at the stationary phase. As mentioned above, photoautotrophically and heterotrophically grown *C. vulgaris* were physiologically at the exponential growth phase on day 8 of the experiment. In contrast, mixotrophically grown algae seemed to enter the stationary growth phase one or two days prior (on days 6–7), which could be possibly ascribed to nutrient limitation. Although the nutrient concentrations in the medium were not analysed in this study, the significant increase in both carbohydrate and lipid contents implies nutrient limitation [35,36]. The simultaneous increase in the carbohydrate and lipid contents suggests that *C. vulgaris* can accumulate both classes, which can occur sequentially. Starch accumulates during the early stationary growth and, during prolonged nutrient limitation it is converted into lipids as shown for several species, including *C. vulgaris* [37–40]. The total lipid content of 49.2 % DW^{-1} in *C. vulgaris* 211-11b was close to the strain's 53.4 % of total lipids detected under S limitation, while those under P and N limitation were 17.4 and 21.4 %, respectively [11]. This suggests that *C. vulgaris* has already converted the majority of carbohydrates into lipids due to prolong S limitation in the stationary growth phase. The high lipid content under mixotrophic growth can be ascribed to an overall increase in SFAs and MUFAs, while PUFAs remained relatively low (Fig. 4).

In either case, these lipids were mainly present as neutral lipids or triacylglycerols (TAGs: 60–80 %) with a smaller fraction of polar lipids (20–40 %). Since polar lipids are essential elements of biological membranes and cellular processes, they could be associated with growth and metabolic maintenance in *C. vulgaris* [41–43]. The specific increase in TAGs in *C. vulgaris* could be a response to the assumed prolonged nutrient depletion under mixotrophic growth. Oleic (C18:1, n−9) and palmitic (C16:0) acid were the predominant fatty acids in the FAME profile with 89.0 ± 2.2 mg g^{-1} DW (total: 127.2 ± 2.2 mg g^{-1} DW) and 101.8 ± 2.6 mg g^{-1} DW (total: 145.5 ± 3.7 mg g^{-1} DW) of TAGs, respectively (Fig. 5). Although the TAGs of palmitioleic (C16:1, n−9), stearic (C18:0) and other fatty acids also increased under mixotrophic condition, their share remained below 40–50 % of the contents of palmitic and oleic acid, respectively. The predominance of oleic and palmitic acid with lower contents of palmitioleic and stearic acid in the FAME profile were also detected under photoautotrophic and heterotrophic growth.

Although this pattern is similar to the FAME profile of *C. vulgaris* 211-11b under S, N and P limitation as well as a wide pH range [11,44], it can also be dominated by the α-linolenic acid (C18:3 n−3) at the three growth strategies [31]. Nevertheless, the dominance of SFAs and MUFAs make *C. vulgaris* to a promising resource for the production of biodiesel in accordance with European (EN14214) and American (ASTM6751–02) standards [45,46]. Major problems in SFA and MUFA-rich biodiesel produced by algae, however, are their reduced cold flow and oxidative stability, which can be overcome by using feedstocks with inherently different fatty acid profiles [10].

Mixotrophic growth on glucose seems to be a favourable growth strategy for *C. vulgaris* to accumulate high contents of lipids. In addition, since these high lipid contents produced under freshwater conditions are similar to those of *C. vulgaris* 211-11b grown mixotrophically on medium with a 50 %-share of seawater, it is reasonable to argue that the alga's fast and high lipid production is more environmentally sustainable and economic than when grown under freshwater conditions.

3. Conclusions

Mixotrophic growth of *Chlorella vulgaris* 211-11b on glucose and in BBM formulated with 50 % seawater is a promising approach for a fast and high production of biomass, and lipids. *Chlorella vulgaris* could serve for tailored biotechnological applications such as biodiesel production due to the high contents of oleic and palmitic acids. Due to the alga's capability of growing well in 50 % seawater, freshwater in growth media could be partially replaced by seawater. Therefore, the cultivation of *C. vulgaris* could be performed in both a more environmentally

sustainable and economic manner, than the common freshwater cultivation of this species. So far, the results of the present study reflect the alga's physiological and biochemical responses to glucose and half-strength seawater under laboratory conditions. Future experiments, however, need to demonstrate in how far the large-scale cultivation of *C. vulgaris* is sustainable and viable under industrial conditions.

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CRediT authorship contribution statement

Ralf Rautenberger: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Alexandre Détain:** Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Kari Skjånes:** Resources, Writing – original draft, Writing – review & editing. **Peter S.C. Schulze:** Validation, Writing – original draft, Writing – review & editing. **Viswanath Kiron:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review & editing. **Daniela Morales-Sánchez:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors did not use generative AI and AI-assisted technologies during the writing process of this work.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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