Algal Research 82 (2024) 103597

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Alternative production of fucoxanthin and PUFAs using *Chlorochromonas danica* and *Hibberdia magna*, unicellular chrysophytes with different trophic modes

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ARTICLE INFO

Keywords:
Fucoxanthin
Polyunsaturated fatty acids
Mixotrophy
Multi-target biorefinery
Hibberdia magna
Chlorochromonas danica

ABSTRACT

Ochrophyte microalgae attract attention from the applied phycology perspective, due to their ability to grow rapidly, engage variable trophic modes, and simultaneously produce high-value compounds such as xanthophyll carotenoids and polyunsaturated fatty acids. Unlike more often considered marine diatoms and haptophytes, unicellular chrysophytes may represent a reasonable freshwater alternative. In this work, we introduced two representatives: *Chlorochromonas danica* (Ochromonadales) and *Hibberdia magna* (Hibberdiales). We compared their ability to produce target compounds in mixotrophic and photoautotrophic modes, respectively. Both organisms had a similar temperature optima (18–22 °C), but light demands were much higher in the photoautotrophic *H. magna*. This work is the first report of fucoxanthin content and productivity by *C. danica*, showing that the presence of light enhanced the content of fucoxanthin 4.5-fold compared with darkness. For fucoxanthin productivity in the mixotrophic batch cultured *C. danica*, the optimal initial glucose dose was 10 g L^{-1}. Culture medium supplemented with mineral nutrients resulted in an increase in biomass and fucoxanthin productivity of *C. danica* achieving the highest biomass productivity of 0.81 ± 0.06 g L^{-1} d^{-1}. *H. magna* accumulated a maximum of 4.54 ± 0.04 mg g^{-1} DW of fucoxanthin, which was slightly more than the maximum value for *C. danica* of 3.99 ± 0.19 mg g^{-1} DW. However, due to the lower biomass productivity of *H. magna*, the maximal fucoxanthin productivities reached very similar values of 1.15 ± 0.05 and 1.16 ± 0.01 mg L d^{-1} in *C. danica* and *H. magna*, respectively. Both organisms had a relatively high fatty acid content, accounting for 17 % and 19 % of DW in *C. danica* and *H. magna*, respectively. *H. magna* had a higher level of polyunsaturated fatty acids, which were more diverse, longer, and more unsaturated. The potential for utilization of selected chrysophytes as producers in a multi-target biorefinery is discussed.

1. Introduction

Microalgae have attracted considerable interest due to their ability to grow rapidly in artificial culture systems and produce biomass photosynthetically, utilizing widely available resources. Alternatively, they can be cultivated with added organic substrates to enhance their productivity and biomass volumetric density [1,2]. The industrial acquisition of microalgal biomass is an immense topic, on which numerous research articles have been published. A large part of this research is driven by the possible utilization of microalgal-based lipids as a biofuel feedstock [3]. However, generating such bulk products is currently not feasible due to higher production costs in comparison with conventional industrial and agricultural strategies. These efforts are under development but face several limitations and future feasibility is uncertain [4]. Hence recent attention has been shifted to specific algal-derived high-value compounds such as xanthophyll carotenoids, polyunsaturated fatty acids (PUFAs), and many other bioactive compounds [5].

PUFAs are an essential part of the vertebrate diet and have a beneficial impact on human health, such as protection against cardiovascular and coronary heart diseases [6], and have special importance for the

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https://doi.org/10.1016/j.algal.2024.103597
Received 5 February 2024; Received in revised form 7 April 2024; Accepted 28 June 2024
Available online 30 June 2024

development and functioning of the nervous system [7]. Some microalgae are widely considered as primary producers of nutritionally important long-chain PUFAs (LC-PUFAs), especially omega-3 LC-PUFAs [8]. Moreover, microalgal biomass rich in LC-PUFAs could be used as a feed supplement for livestock, poultry [9], or in aquaculture [10,11].

Fucoxanthin (FX) is a xanthophyll carotenoid produced exclusively by algae and is one of the most abundant carotenoids in nature, particularly in marine environments [12]. FX plays an important role in the light-harvesting complex of the algal photosystem, enabling the harvesting of blue-green light that penetrates deeper into the water column [13]. FX exhibits multiple beneficial biological activities for human health, including strong antioxidant, anti-inflammatory, anti-obesity, anti-diabetic, anti-cancer, and anti-hypertensive activities [14]. Currently, most commercially available FX is extracted from seaweeds (Phaeophyceae), both farmed and wild, on an industrial scale [15]. However, these macroalgae contain relatively low concentrations of FX [16], which is also poorly bio-available [12]. Therefore, recent attention in both commercial and academic sectors has been directed to surveying microalgae as a promising alternative source of FX for diverse applications [17–19].

Biorefinery of multiple high-value products from non-fossil feedstocks is an interesting topic [20], where microalgae can serve as a plausible source of biomass with expected increasing demand. However, these strategies are usually focused on a few well-studied microalgal species. Research targeting the simultaneous production of PUFAs and FX is dominated by marine diatoms and haptophytes [21,22], although saltwater-based microalgal production techniques have some significant drawbacks. Bioprospecting for less-well-known algal species needs to be carried out to raise awareness of the possibilities of using various organisms.

Freshwater chrysophyte flagellates represent highly diverse trophic strategies, ranging from obligate heterotrophs, phagotrophic and osmotrophic facultative mixotrophs to obligate autotrophs [23,24]. This makes them attractive for utilization in microalgal biomass production. Moreover, these organisms produce not only PUFAs and FX, but also other valuable carotenoid pigments [25] and other important compounds such as water-soluble beta-glucans [26], and exopolysaccharides (Fig. S1). Therefore, they are promising candidates for a multi-target biorefinery. Unusual, toxic, and probably bioactive molecules produced by some of these chrysophytes are chlorosulfolipids [27,28], which also need to be given more attention in the future.

In this work, we aim to provide an overview of the cultivation conditions, FX productivity, FA profiles, and quantities of nutritionally important LC-PUFAs by two freshwater chrysophytes with different trophic modes: osmo-mixotrophically cultivated *Chlorochromonas danica* (formerly = *Ochromonas danica*) [29] and photo-autotrophically cultivated *Hibberdia magna*. Moreover, we compare both organisms and evaluate their prospects for the multi-target biorefining of high-value compounds.

2. Materials and methods

2.1. Algal strains and seed culture preparation

The axenic culture of the microalga *Chlorochromonas danica* (formerly = *Ochromonas danica*), strain SAG 933–7, was obtained from the Culture Collection of Algae at Goettingen University (SAG, Germany). The strain was maintained in MOM 10 medium (Table 1), at a light intensity of 5 μmol m^{-2} s^{-1}, 12/12 (light/dark) photoperiod, and a temperature of 8 °C. Seed cultures were prepared in 250 mL Erlenmeyer flasks filled with 150 mL of the MOM 10 medium incubated at room temperature, under continuous white light of intensity 20 μmol m^{-2} s^{-1} provided by a panel of fluorescent light tubes. The cultures were mixed manually several times a day and as soon as they started to grow visibly (usually in 5 days), they were used as an inoculum for the experiments.

The microalga *Hibberdia magna*, strain K-1175, was obtained from the Norwegian Culture Collection of Algae (NORCCA, Norway) as a non-axenic culture. In our laboratory, the strain was maintained in 100 mL Erlenmeyer flasks with liquid WC medium [30] (Table 2) without mixing, at a temperature of 19 ± 2 °C, and continuous white light provided by fluorescent tubes at an intensity of about 10 μmol m^{-2} s^{-1}. These conditions secured the survival of the culture, however regular (6–8 weeks) re-inoculation into fresh medium was needed to keep the culture alive. Seed cultures for experiments were prepared in 1000 mL Erlenmeyer flasks filled with 500 mL of medium WC+ 115Tris (Table 2). Cultures were kept for 3 weeks under the conditions described above, mixed manually several times a week, and then used as an inoculum for the experiments.

Table 1
Compositional variants of organic growth media (mg L^{-1}). All media were adjusted to pH 7.5 using 1 % $NaHCO_3$ after autoclaving. All media contained the same amount of micro-nutrient solution (Fe, B, Mn, Mo, Zn, Co, Cu; for detailed composition see supplementary information Table 1S).

Component	Growth medium variant					
	ABR	MOM 2	MOM 5	MOM 10	MOM 20	MOM 40
Glucose[a]	10,000	2000	5000	10,000	20,000	40,000
Beef extract[b]	2000	2000	2000	2000	2000	2000
Yeast extract[c]	2000	2000	2000	2000	2000	2000
NH_4NO_3	800	0	0	0	0	0
K_2HPO_4	195	0	0	0	0	0
KH_2PO_4	101	0	0	0	0	0
$MgSO_4.7H_2O$	75	0	0	0	0	0
$CaCl_2$	20	0	0	0	0	0

[a] Lach:Ner, Czechia.
[b] Thermo Fisher Scientific, USA.
[c] Glentham Life Sciences, United Kingdom.

Table 2
Compositional variants of WC growth media (mg L^{-1}). All media were adjusted to pH 6 using 2 % HCl before autoclaving. All media contained the same amount of vitamin solution (B_1, B_{12}, H; for detailed composition see supplementary information Table 2S).

Component	Growth medium variant				
	WC original	WC+ 115 Tris	2WC+ 1000 Tris	2WC+ No Buff	2WC+ 1000MES
Buffers					
Tris	500	115	1000	0	0
MES	0	0	0	0	1000
Macro-nutrients					
$CaCl_2 \cdot 2H_2O$	36.8	36.8	73.6	73.6	73.6
$MgSO_4 \cdot 7H_2O$	37	37	74	74	74
$NaHCO_3$	12.6	12.6	25.2	25.2	25.2
$NaNO_3$	8.7	87	87	87	87
K_2HPO_4	85	850	850	850	850
$Na_2SiO_3 \cdot 9H_2O$	28.4	28.4	56.8	56.8	56.8
Micro-nutrients					
Na_2EDTA	4.36	4.36	8.72	8.72	8.72
$FeCl_3 \cdot 6H_2O$	3.15	3.15	6.3	6.3	6.3
H_3BO_3	1	1	2	2	2
$MnCl_2 \cdot 4H_2O$	0.18	0.18	0.36	0.36	0.36
$ZnSO_4 \cdot 7H_2O$	0.022	0.022	0.044	0.044	0.044
$CoCl_2 \cdot 6H_2O$	0.01	0.01	0.02	0.02	0.02
$CuSO_4 \cdot 5H_2O$	0.01	0.01	0.02	0.02	0.02
$Na_2MoO_4 \cdot 2H_2O$	0.006	0.006	0.012	0.012	0.012

2.2. Experimental design

For light and temperature preferences of *C. danica*, a custom-made cross-gradient cultivation device (Labio, Czechia) was used. For a detailed description of this device, see Kvíderová et al. [31]. Cultures were grown in Petri-dishes sealed with parafilm using ABR medium (Table 1). Cultures were exposed to cross-gradient temperatures of 15, 19, 24, 27.5, 30.5, and 32.5 °C, and light intensities of 0, 20, 50, and 120 μmol m^{-2} s^{-1}. The temperature was measured using a resistance thermometer equipped with a 310 (GRYF HB, Czechia) bead thermistor sensor, and light intensity was measured using a PU550 light meter equipped with a custom-made photosynthetic active radiation light sensor (Metra Blansko, Czechia). The cultures were collected after 4 days of cultivation for estimation of biomass growth.

To examine biomass growth and FX productivity of *C. danica*, several organic growth media supplied with microelements (Fe, B, Mn, Mo, Zn, Co, Cu; Table S1) varying in content and ratio of glucose, beef extract, and yeast extract were tested. Seed cultures were inoculated by 10 times dilution using the appropriate medium into 250 mL Erlenmeyer flasks with a working volume of 70–100 mL, and cultivated in the same way as during pre-cultivation. An experiment comparing cultivation under dark and light conditions was performed in MOM 10 medium (Table 1) and the dark-grown variants were shaded with aluminum foil. For growth estimation, cultures were sampled at regular intervals, and at the end of the experiments, biomass was collected by centrifugation (800 ×*g*, 8 min, room temperature), washed with *Z*-medium [32] to remove traces of organic compounds, centrifuged again and immediately frozen at −20 °C for further analysis. All variants were cultivated in biological triplicates.

Two separate cultivation experiments with *H. magna* were performed using a similar design. Briefly, 40 mm in diameter round-bottom glass cultivation tubes filled with 170 mL of medium were mixed by bubbling with air enriched with 1 % CO_2 (v/v). Cultivation tubes were placed into the temperature-controlled water baths and each culture tube was provided with a dimmable LED strip of full spectra white light. In advance of the experiments, light intensity was controlled by a LI-250 (LI-COR Environmental, USA) photometer equipped with a spherical probe placed in the middle of the cultivation tube. At the beginning of the experiments, the seed culture of *H. magna* was inoculated at an initial concentration of 1 × 10^6 cells mL^{-1}. The cell count was determined using a Multisizer4 Coulter Counter (Beckman Coulter, USA). For the light-temperature cross-gradient experiment, the temperature-controlled water baths were set at 10, 15, 20, 25, and 30 °C (for all temperatures ±1 °C). In each bath, four culture tubes were placed and illuminated with 60, 120, 240, and 480 μmol m^{-2} s^{-1}. The culture medium used for this experiment was WC+ 115Tris (Table 2). The cultures were monitored daily for biomass density. The experiment was terminated after reaching stationary growth phase and was not replicated.

For subsequent examination of FX productivity of *H. magna*, four different mineral culture media (Table 2) supplied with vitamins (B_1, B_{12}, H; Table S2) were tested under identical culture conditions (22 °C and 200 μmol m^{-2} s^{-1}). The cultures were sampled daily for biomass density and pH determination. After 8 and 10 days, 20 mL of the cultures were harvested by centrifugation (1670 ×*g*, 12 min, room temperature), and kept at −70 °C for further analysis. Cultures grown in WC Original cultivation medium were collected only after 8 days because this variant did not show relevant growth. All variants were cultivated in biological triplicates.

2.3. Growth and pH

For dry biomass volumetric density (DW = dry weight) analysis, aliquots of *C. danica* cultures were centrifuged (800 ×*g*, 8 min, room temperature) in pre-weighed micro-tubes and the pellet was resuspended in *Z*-medium [32] and recentrifuged. The pellet was dried at 105 °C and weighed on an XS 205 Dual Range (Mettler Toledo, Germany) analytical balance until a constant weight was achieved. The pH of the samples was measured using Machery-Nagel Tritest L indicator strips. For *H. magna* DW evaluation, a known volume (2–5 mL) of fresh algal culture suspension was vacuum filtered through a pre-dried and pre-weighed 55 mm diameter, 1.2 μm glass microfibers filter (VWR International, USA), dried at 90 °C overnight in an oven, cooled in a desiccator and weighed on an R160P (Sartorius, Germany) analytical balance. pH of the sample was measured using the Dosatest pH color-fixed indicator strips.

2.4. Fucoxanthin and fatty acids analysis

The FX and FA contents were determined as described in our previous study [33]. Briefly, lyophilized biomass (*C. danica* using a Heto PowerDry PL3000 freeze dryer; *H. magna* using a Scanvac, CoolSafe freeze dryer) of known weight (~4 mg) was used for both analyses. For FX quantification, the sample was extracted in 100 % ethanol using a Mini-Beadbeater-16, a high-energy cell disruptor (BioSpec Products, USA), and immediately analyzed using a Dionex UltiMate 3000 HPLC (Thermo Scientific, USA) high-performance liquid chromatography (HPLC) system equipped with a diode array detector set to 450 nm. A commercial FX standard (Sigma Aldrich, Germany) was used for quantification. Quantitative and qualitative analyses of the trans-esterified FAs were performed using a Trace 1300 (Thermo Scientific, USA) gas chromatograph equipped with a flame ionization detector. A TR-FAME column (60 m × 0.32 mm, df 0.25 μm) was used for separation with hydrogen as the carrier gas. The retention times of FA methyl esters were compared to known standards (Supelco® 37 Component FAME Mix), supplemented with analytical standards of stearidonic acid (C18:4n3) and osbond acid (C22:5n6) methyl esters (both supplied from Cayman Chemical, USA). The quantity of individual FAs was calculated using glycerol-tripentadecanoate as an internal standard.

2.5. Calculations

The specific growth rate (μ) was calculated from DW data using the equation: $\mu = \ln(DW_{t1} / DW_{t0}) / (t_1 - t_0)$, where DW_{ti} are values of DW at the sampling time (t_1) and at the start time of the experiment (t_0). DW volumetric productivity was calculated using the equation: $Prod_{DW} = (DW_{t1} - DW_{t0}) / (t_1 - t_0)$, where DW_{ti} represents values of DW at given times (t_1, t_0). The FX volumetric productivity was calculated using the equation: $Prod_{FX} = (DW_{t1} * FX_{t1} - DW_{t0} * FX_{t0}) / (t_1 - t_0)$, where DW_{ti} represents values of DW at given times (t_1, t_0) and FX_{ti} are values of FX content per DW at given times (t_1, t_0). Graphs and regression analyses were carried out using the Sigma Plot 11.0 or 14.0 (Systat Software, USA) and Prism 9 (GraphPad Software, USA) software.

3. Results

3.1. Chlorochromonas danica growth and FX productivity

In the cross-gradient of light and temperature, the specific growth rates of *C. danica* showed two maxima, one at 18 °C in darkness, the second at 22 °C and a light intensity of 20 μmol m^{-2} s^{-1}. Data suggest that *C. danica* can grow in the dark solely on organic substrates as an obligate heterotroph. This ability was limited or even suppressed at temperatures above 26 °C. Light availability enhanced its tolerance to higher temperatures and improved its growth rate at higher temperatures (Fig. 1a). As there were two maxima of growth rate, we further evaluated the role of light for *C. danica* growth and FX content. Although both dark and light variants grew well within the first four days, there were some differences in growth curves (Fig. 1b). Cultures in darkness had a prominent lag phase within the first day, probably arising from the use of a light-adapted inoculum. These cultures reached the highest DW density of 1.98 ± 0.25 g L^{-1} after four days of growth. Cultures at a light

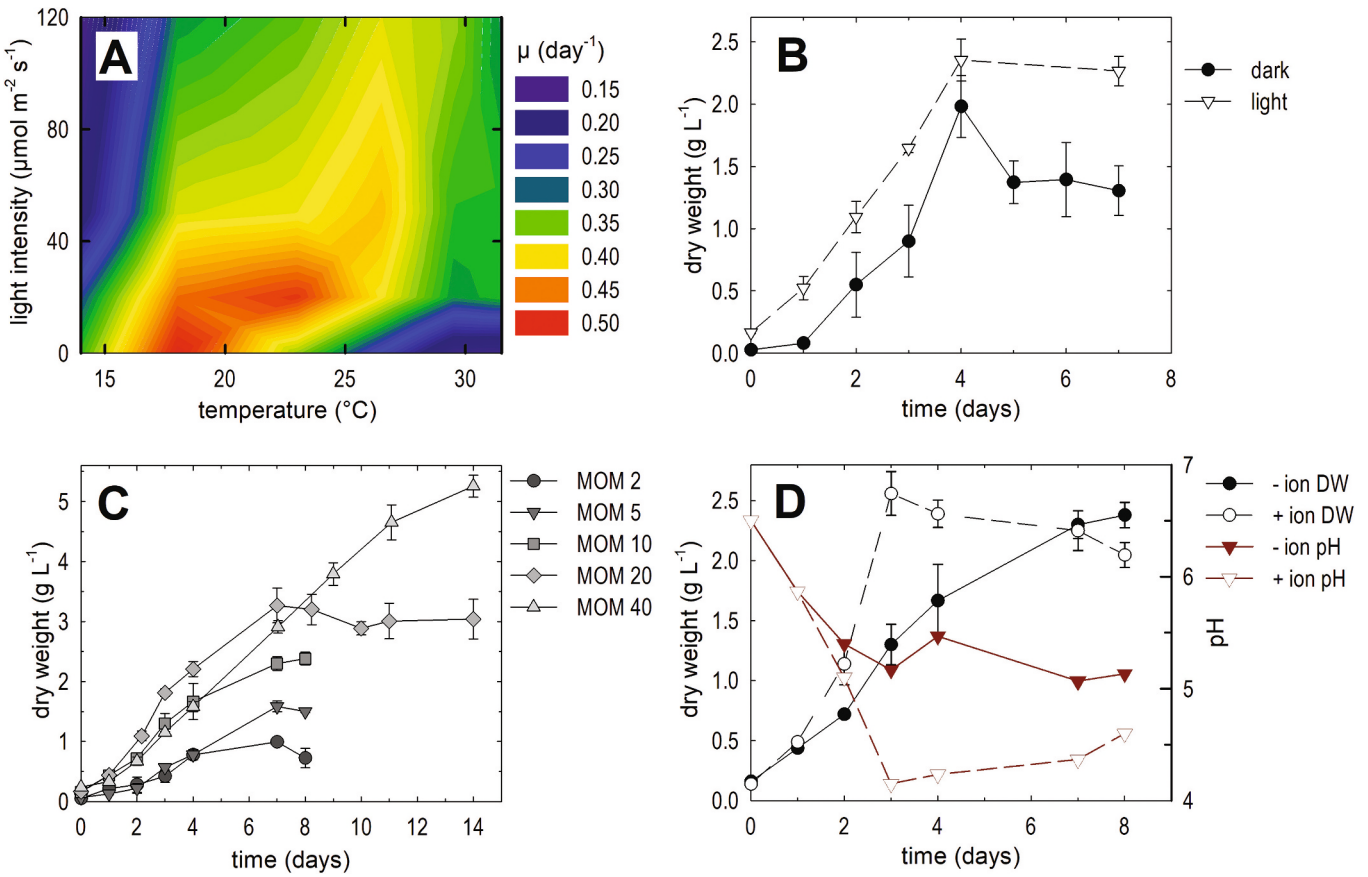

Fig. 1. Growth of *Chlorochromonas danica*: A. Contour plot of specific growth rates in crossed gradients of light and temperature based on DW values; B. Growth curves of cultures in organic medium (MOM10) – darkness (circles); light (20 µE m^{-2} s^{-1}) (triangles); C. Growth curves of cultures in organic media of different initial glucose doses: MOM2 (2 g L^{-1}, circles), MOM5 (5 g L^{-1}, triangles down), MOM10 (10 g L^{-1}, squares), MOM20 (20 g L^{-1}, diamonds), and MOM40 (40 g L^{-1}, triangles up) D. The time courses of mixotrophic growth (black circles) and pH (colored triangles) in different media – without (filled; medium MOM 10) and with (empty; medium ABR) added inorganic macronutrient ions (see Table 1 for detail); Symbols represent means (n = 3), and error bars represent standard deviation.

intensity of 20 µmol m^{-2} s^{-1} grew almost linearly without lag phase and reached a DW density of 2.35 ± 0.17 g L^{-1} after four days of growth. Despite differences in growth dynamics, there was no statistical difference ($p = 0.054$) in the maximal DW densities achieved. After reaching the highest DW densities, the light-grown cultures entered stationary phase. In contrast, DW density in darkness decreased to 1.31 ± 0.20 g L^{-1} on day 7 (Fig. 1b). FX content was evaluated at the end of the experiment, after seven days of growth, and showed a substantial difference. Cultures grown in darkness had an FX content of 0.37 ± 0.08 mg g^{-1} DW whereas in light, the FX content was 4.5-fold higher (1.69 ± 0.16 mg g^{-1} DW).

In the next experiment, the effect of different glucose concentrations on *C. danica* osmo-mixotrophic growth and FX production was tested. During the first 7–8 days of the experiment, growth was fully dose-dependent up to 20 g L^{-1} and stationary phase was reached within 8 days under all experimental conditions (Fig. 1c). A further increase in the glucose concentration to 40 g L^{-1} did not lead to an increase in biomass productivity rate. However, growth proceeded for a longer period and stationary phase was not reached, even within 14 days. The maximal DW density of over 5 g L^{-1} DW was achieved at this highest glucose concentration (Fig. 1c). Thus, the final biomass densities fully reflected the initial glucose doses. The optimal glucose concentration for overall specific growth rate and biomass productivity in the 7–8 days batch mode experiment was 20 g L^{-1}, with values of 0.43 ± 0.02 d^{-1} and 0.38 ± 0.13 g L^{-1} d^{-1}, respectively. However, the variant with a glucose concentration of 40 g L^{-1} did not show any different biomass productivity (0.38 ± 0.02 g L^{-1} d^{-1}). FX content was affected by the glucose concentration and differed according to the growth phase. When the cultures were in linear or exponential growth phases, the FX content was lower (1.35–1.87 mg g^{-1} DW), but after the cultures reached stationary phase and organic substrates were probably no longer sufficient (not quantified), the FX content usually increased to over 3 mg g^{-1} DW

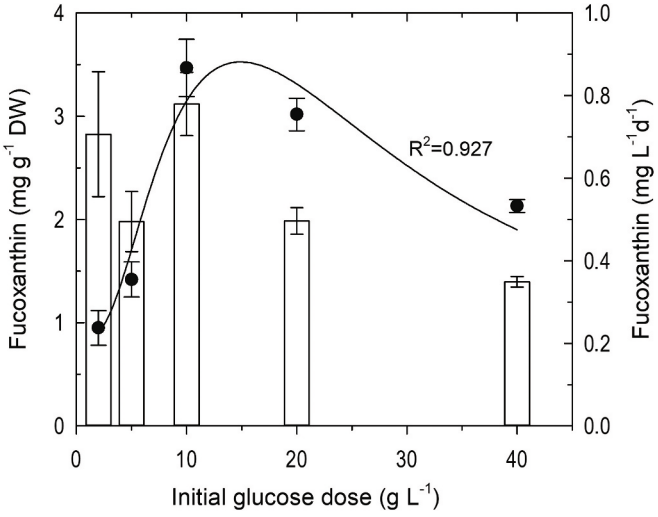

Fig. 2. Fucoxanthin content per biomass (bars) and fucoxanthin volumetric productivity (filled circles) after 7–8 days of mixotrophic growth of *C. danica* in organic medium of various initial glucose concentrations (see Table 1 for detail). Data represent means and standard deviations ($n = 3$).

(Fig. 2, Table 3). Only the MOM 5 variant with a glucose concentration of 5 g L^{-1} deviated from this and reached a slightly lower FX content (1.98 ± 0.29 mg g^{-1} DW) in stationary phase. This is difficult to explain, but possibly organic substrates remained for a longer time and the transition to phototrophy was not complete in these cultures. The highest FX volumetric productivity (0.91 ± 0.11 mg L^{-1} d^{-1}) was observed for the medium variant with 10 g of glucose per L (MOM 10, Fig. 2), which corresponds to the optimal combination of glucose concentration and harvest time in this particular design.

Finally, growth and FX productivity of *C. danica* were tested with the addition of elementary inorganic macronutrients (N, P, Mg, S, Ca, Cl, K, medium ABR, Table 1) for the medium variant, that reached the highest FX productivity in the previous experiments (medium MOM 10, Table 1). Growth curves and time courses of pH for this experiment are presented (Fig. 1d). The time course of culture pH negatively correlated with the biomass increase in both media variants. The ABR medium results showed that biomass productivity was substantially enhanced within the first three days of growth and achieved the highest DW productivity value for *C. danica* among all experiments (0.81 ± 0.06 g L^{-1} d^{-1}). For comparison, DW productivity in MOM 10 medium was only 0.38 ± 0.06 g L^{-1} d^{-1} over the same period. After three days, cultures in ABR medium entered stationary growth phase and their DW declined slightly. The MOM 10 variant grew more steadily following a sigmoidal growth curve of lower slope and eventually attained a similar DW density of about 2.4 g L^{-1} (Fig. 1d). These results demonstrate that *C. danica* sustained osmo-mixotrophic growth without inorganic macronutrient supplementation and achieved similar densities at stationary phase. On the other hand, the presence of inorganic macronutrients enhanced its biomass productivity 2.1-fold within the first three days of growth. FX productivity was also positively impacted by ABR medium. The FX content was very similar in both variants (ABR: 1.96 ± 0.06 mg g^{-1} DW; MOM 10: 1.87 ± 0.10 mg g^{-1} DW) on day 4 of growth. However, due to faster growth in ABR medium, FX productivity was almost 1.6-fold higher than for MOM 10 medium and achieved a value of 1.15 ± 0.05 mg L^{-1} d^{-1}, which was the highest FX productivity observed among all experiments with *C. danica*. On day 8, cultures grown in ABR medium were already in late stationary phase and the FX content increased considerably to a value of 3.99 ± 0.19 mg g^{-1} DW, which was the highest observed FX concentration for *C. danica* among all experiments. However, FX productivity decreased (1.01 ± 0.03 mg L^{-1} d^{-1}) due to the decrease in DW density between day 3 and day 8 in ABR medium (Fig. 1d).

3.2. *Hibberdia magna* growth and FX productivity

The temperature and light preferences of *H. magna* were examined in the cross-gradient experiment, showing broader temperature tolerance under dim light conditions, whereas at the higher light intensity, the optimum was restricted to a moderate temperature of 20 °C (Fig. 3a). Temperatures over 25 °C were lethal, regardless of light intensity. The specific growth rate increased with increasing light intensity, indicating dominance of the photoautotrophic metabolism of *H. magna*. Specific growth rate reached the highest values at a temperature of 20 °C and a light intensity of 480 μmol m^{-2} s^{-1} (0.50 d^{-1}), however, the highest final DW density of 1.38 g L^{-1} was achieved at a lower light intensity of 240 μmol m^{-2} s^{-1}. *H. magna* grew faster at higher light intensity but achieved a lower final biomass densitiy compared to that at a lower light intensity [33].

H. magna biomass and FX productivity were evaluated in four different modifications of WC medium (Table 2). The variant with the lowest nutrition loads of the original WC medium resulted in the lowest biomass productivities, indicating conspicuous nutrient limitation after only two days of the experiment. The remaining variants grew similarly up to day 7, after that the WC medium variant without buffer reached a slightly higher final DW density (2.48 ± 0.12 g L^{-1}) than the others (Fig. 3c). The specific growth rates and DW productivity values at the end of growth (day 8) and at stationary phase (day 10) were calculated (Table 3). The initial pH of the cultures was influenced by the presence and type of buffer used. It ranged from a pH 5.5 for the medium supplemented with 1000 mg of MES buffer per L to 7.6 for the medium with 1000 mg of Tris buffer per L, however, the buffers were not able to maintain the pH at initial levels during the experiment (Fig. 3b). Nevertheless, apparently the influence of pH on culture growth was negligible within this pH range.

The highest FX content achieved for *H. magna* biomass (13.04 mg g^{-1} DW, $n = 1$), was recorded in the seed culture used as an experimental inoculum. All experimental cultures showed a decrease in FX content compared to the inoculum, indicating that the experimental conditions were not optimal regarding FX accumulation. The lowest FX content was recorded in the nutrition-limited variant of the original WC medium. Conversely, the highest FX content was observed in cultures grown in modified WC medium without buffer, reaching a value of 4.54 ± 0.04 mg g^{-1} DW and 4.10 ± 0.20 mg g^{-1} DW, on day 8 and 10, respectively. This also led to the highest overall FX productivities in this culture medium variant, 1.16 ± 0.01 mg L^{-1} d^{-1} and 0.89 ± 0.05 mg L^{-1} d^{-1} after 8 and 10 days of growth, respectively (Table 3).

Table 3
Growth and FX productivity parameters of mixotrophically cultivated *C. danica* and autotrophically cultivated *H. magna* in different culture media. Values represent the means and standard deviations of three replicates. The highest values for each organism are highlighted (**bold**).

Culture medium	Growth time (d)	DW spec. growth rate (d^{-1})	DW vol. productivity (g L^{-1} d^{-1})	FX absolute concentration (mg g^{-1} DW)	FX vol. productivity (mg L^{-1} d^{-1})
Chloroochormonas danica					
MOM 2	7	0.332 ± 0.036	0.084 ± 0.020	2.826 ± 0.605	0.237 ± 0.042
MOM 5	7	0.391 ± 0.015	0.179 ± 0.003	1.980 ± 0.290	0.354 ± 0.042
MOM 10	8	0.338 ± 0.007	0.278 ± 0.013	**3.118 ± 0.305**	**0.912 ± 0.113**
MOM 20	7	**0.426 ± 0.015**	**0.380 ± 0.129**	1.986 ± 0.127	0.754 ± 0.040
	14	0.208 ± 0.008	0.205 ± 0.024	**3.051 ± 0.303**	0.626 ± 0.051
MOM 40	7	0.354 ± 0.007	**0.381 ± 0.015**	1.397 ± 0.051	0.533 ± 0.016
	14	0.218 ± 0.003	0.348 ± 0.007	1.354 ± 0.051	0.472 ± 0.015
Hibberdia magna					
WC	8	0.199 ± 0.004	0.047 ± 0.002	1.532 ± 0.098	−0.065 ± 0.006
2WC+ 1000	8	0.375 ± 0.003	0.226 ± 0.006	3.200 ± 0.146	0.607 ± 0.035
Tris	10	0.296 ± 0.006	0.175 ± 0.011	2.691 ± 0.247	0.372 ± 0.046
2WC+ No Buff	8	**0.399 ± 0.005**	**0.278 ± 0.010**	**4.543 ± 0.040**	**1.161 ± 0.012**
	10	0.326 ± 0.005	0.238 ± 0.012	4.095 ± 0.200	0.891 ± 0.049
2WC+ 1000	8	0.388 ± 0.006	0.253 ± 0.012	3.475 ± 0.312	0.764 ± 0.082
MES	10	0.305 ± 0.009	0.192 ± 0.019	3.387 ± 0.634	0.558 ± 0.127

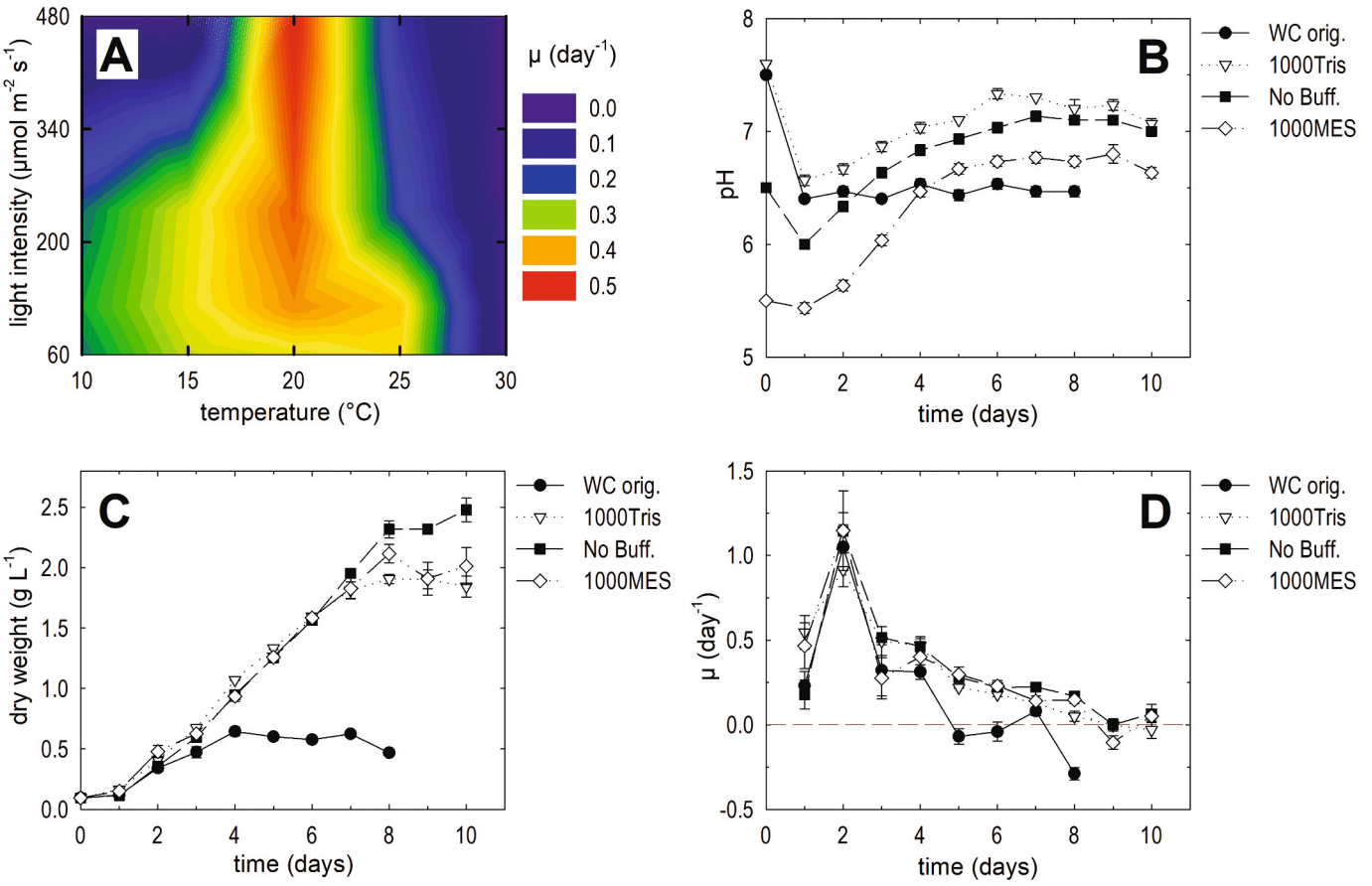

Fig. 3. Growth of *Hibberdia magna*: A. Contour plot of specific growth rates in crossed gradients of light and temperature based on DW values; B. The time courses of pH values for four variants of WC cultivation media; C. Growth curves of cultures grown in four variants of WC cultivation media; D. The time courses of specific growth rates of cultures grown in four variants of WC cultivation media; Media used (see Table 2 for detail) were: WC original (filled circles), 2WC+ 1000Tris (empty triangles), 2WC+ No Buff (filled squares), and 2WC+ 1000MES (open diamonds); Symbols represent means (n = 3), and error bars represent standard deviation.

3.3. Fatty acid content

Analysis of the FA profile was performed for both organisms in selected samples. To present the most important outcomes, only FA profiles of the stationary phase cultures of the most productive condition (medium MOM 20, day 14 for *C. danica*; medium 2WC+ No Buff, day 10 for *H. magna*) are shown (Fig. 4). The other FA profiles of the organisms were very similar to those presented here (Tables S3, S4, S5, S6). Both organisms had a relatively high level of total FA per biomass of 169.0 ± 13.2 mg g^{-1} DW, and 188.7 ± 5.3 mg g^{-1} DW for *C. danica* and *H. magna*, respectively. *H. magna* had a substantially higher proportion of PUFAs (62.3 ± 0.2 %) than *C. danica* (41.3 ± 1.6 %), while the proportion of monounsaturated fatty acids (MUFAs) was less than 2 % in *H. magna*, compared to more than 14 % in *C. danica*. The saturated fatty acid (SFA) proportion was closest in these two organisms, i.e. about 36 % in *H. magna* and 44 % in *C. danica*.

The major FA was myristic acid (C14:0) for both organisms, but otherwise their FA profiles were quite different (Fig. 4). Generally, *C. danica* had a higher proportion of shorter and less unsaturated FA: mainly palmitic acid (C16:0, 14.3 ± 1.4 %), octadecenoic acid (C18:1 more isomers, 13.0 ± 0.6 %) and its most abundant PUFA, linoleic acid (LA, C18:2n6, 18.3 ± 0.7 %). Other PUFAs were much less abundant as only two of them exceeded the value of 5 % (alfa-linolenic acid, ALA, C18:3n3, 5.9 ± 0.1 %, and arachidonic acid, ARA, C20:4n6, 5.5 ± 0.4 %). This means that *C. danica* contained quite a low level of omega 3 PUFAs. Consequently, the omega 6:3 PUFA ratio was 3.09 ± 0.07 in the selected samples. The proportion of high-value LC-PUFAs (PUFAs with 20 or more carbons) in *C. danica* biomass was 10.1 ± 0.3 %, mainly on account of omega 6 PUFAs. Apart from the already mentioned ARA, other omega 6 LC-PUFAs were osbond acid (n-6 DPA, C22:5n6, 3.0 ± 0.1 %), and dihomo-gamma-linolenic acid (DGLA, C20:3n6, 1.1 ± 0.0 %). The most valuable omega 3 LC-PUFAs for human nutrition, eicosapentaenoic acid (EPA, 20:5n3) and docosapentaenoic acid (DHA, 22:6n3), were present in very limited amounts. On the other hand, *H. magna* had a higher proportion of longer-chain and more unsaturated FAs in comparison with *C. danica*, and it also had a more diverse profile of these PUFAs. While *C. danica* predominantly contained standard PUFAs, like LA, ALA, and ARA, the *H. magna* PUFA profile was shifted to higher unsaturation. The predominant omega 6 PUFA in *C. danica* was gamma-linolenic acid (GLA, C18:3n6, 17.1 ± 0.1 %) instead of the more usual LA (9.6 ± 0.1 %) which was the second most abundant omega 6 PUFA in *H. magna*. Similarly, the third most abundant omega 6 PUFA was n-6 DPA (7.8 ± 0.0 %) instead of the more usual ARA (2.8 ± 0.0 %), which was up to fifth-ranked in *H. magna* because DGLA (3.1 ± 0.1 %) was more abundant. A similar situation was observed for omega 3 PUFAs. The most abundant omega 3 PUFA in *H. magna* was stearidonic acid (SDA, C18:4n3, 16.1 ± 0.2 %), which greatly exceeded the level of ALA (1.6 ± 0.0 %). The content of the most desirable omega 3 PUFAs in *H. magna* was not prominent (EPA 1.7 ± 0.0 %, DHA 2.6 ± 0.0 %), but still reasonably higher than in *C. danica* (Fig. 4). The proportion of LC-PUFA was 18.0 ± 0.1 % and the omega 6:3 PUFA ratio was 1.83 ± 0.03 in the selected samples of *H. magna*.

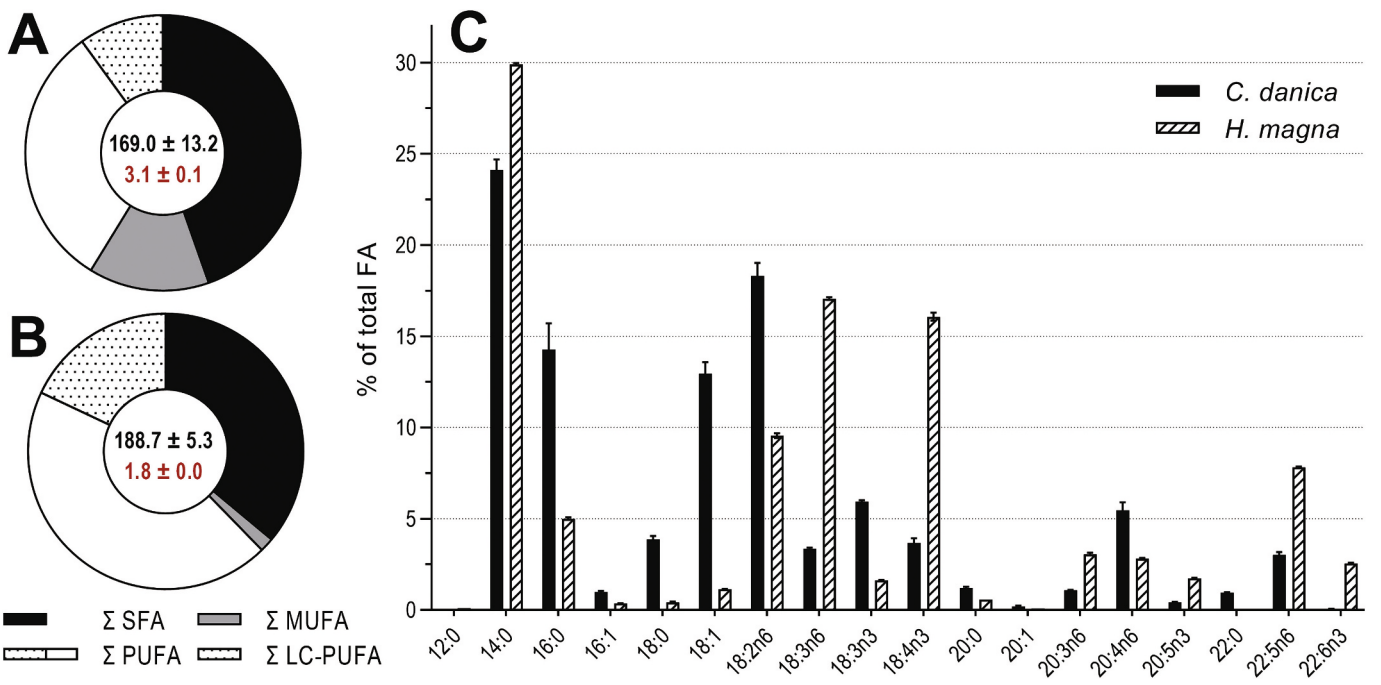

Fig. 4. Fatty acid (FA) composition of *Chlorochromonas danica* (MOM20 medium, day 14) and *Hibberdia magna* (2WC+ No Buff medium, day 10) samples: A. (*C. danica*), B. (*H. magna*) Pie plots of the content of major FA groups: saturated FAs (SFA, black fill); monounsaturated FAs (MUFA, grey fill); polyunsaturated FAs (PUFA, white fill together with dotted pattern fill); and long-chain PUFAs (LC-PUFA) as a subgroup of PUFAs (dotted pattern fill); the numbers in the middle of pie plots represent total FA quantity per biomass (black) and the Omega 6:3 PUFA ratio (red); C. Bar chart of FA profile (% proportion of total FA) of *C. danica* (black bars) and *H. magna* (hatched bars) major FAs; Data represent means and standard deviations (n = 3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

4.1. Chrysophyceae in applied phycology context

Freshwater chrysophyte flagellates are indeed an important protist group from many perspectives. Namely, some mixotrophic members of the order Ochromonadales evolved quite a successful trophic strategy. Ochromonadales are the most diverse taxon within Chrysophyceae [24]; they are widespread in freshwater habitats and also occur in some marine environments. They can play a significant role in the microbial loop as so-called mixoplankton. There is an almost complete spectrum of nutritional strategies within this order, from the obligate heterotrophs (e.g. genus *Spumella*) to the obligate phototrophic mixotrophs (e.g. genus *Dinobryon*), but phagocytosis is common to all members of the order [23]. A member of this large and heterogeneous group is the polyphyletic genus *Ochromonas*, which represents organisms of similar morphologies and trophic modes but falling into distinct molecular phylogenetic lineages [29], so we will further refer to them as "*Ochromonas*-like organisms" rather than *Ochromonas* sp. In vitro, comparative experiments with some *Ochromonas*-like organisms have already proved to be not only phylogenetic but also to express biological intragenus diversity in important traits such as salinity tolerance, trophic mode, light dependence [34], toxicity [35], and transcriptomic profiles [36]. This implies that biotechnological potential would not be the same for all *Ochromonas*-like organisms and a thorough comparison of these species is important. This confusion also complicates a proper literature review among others, hence further revision of this polyphyletic taxon is needed.

Some of these *Ochromonas*-like organisms, mostly from the genera *Poterioochromonas* and *Chlorochromonas*, recently attracted attention from an applied phycology perspective. Doubtless, they are interesting from the multi-target biorefinery point of view due to their content of high-value compounds such as PUFAs, β-glucans, and carotenoids, including their significant plasticity, versatility of trophic modes and the ability to transform waste substrates to algal biomass. They also have overall robustness and capacity for culture under artificial conditions [37]. Multiple works by a renowned research group from the Institute of Hydrobiology, Wuhan, China, focused on the experimental cultivation of a strain of *Poterioochromonas malhamensis* isolated from a massive outdoor *Chlorella* culture [38]. They investigated the ability of a high-cell-density culture to produce FX [19], water-soluble β-1,3-glucans with significant antioxidative and regenerative activities [26], and to control cyanobacterial blooms [39]. The research group from the University of Akron, Akron, USA, works with the same strain of *C. danica* as was used in this study. Among others, they focused on its ability to transform different organic waste substrates like ketchup [40], waste grease [41], and wastewater bacteria [42] into biomass with a high lipid content for utilization as a biofuel precursor. They also tested its application in wastewater sludge treatment [43]. Further, extracts of *C. danica* were tested for antibiotic activity with positive results [44]. Lastly, research on a strain of *Poterioochromonas* sp. isolated from a contaminated cyanobacterial culture is worth mentioning because of its focus on FX production [45], but other records about the biotechnological potential of *Ochromonas*-like organisms are scarce. Evaluation of the combined productivity of FX and PUFA by the strain *C. danica* has not been reported.

A different situation can be seen in the order Hibberdiales, which is two orders of magnitude less diverse than Ochromonadales, and is also less well studied and abundant in nature. The potential of Hibberdiales from the applied phycology perspective was only evaluated in our previous research [33] and photoautotrophic chrysophytes in general are mostly neglected from the biotechnology point of view. The few recent references about its applied potential are the research of Petrushkina et al. [46], where one strain of freshwater *Mallomonas* sp. was evaluated for FX production at different light intensities; Ruffell et al. [47], where the marine chrysophyte *Boekelovia hooglandii* was evaluated for potential utilization as aquaculture feed; and Klaveness [48], who reported the filamentous colonial alga *Hydrurus foetidus* as a potential producer of

PUFAs. Application-oriented algal research for FX and PUFA production is mostly focused on a few species of marine diatoms and haptophytes, while others have been overlooked [14]. Here, we introduce two freshwater Chrysophyceae flagellates with different trophic modes as candidates for a multi-target biorefinery approach.

4.2. Light and temperature optima

Our results showed that both organisms studied preferred moderate temperatures between 16 °C and 23 °C. Interestingly we found two discrete optima for *C. danica*, one at 18 °C without light ($\mu = 0.50 \pm 0.03$ d^{-1}), the other at 23 °C and dim light of 20 μmol m^{-2} s^{-1} ($\mu = 0.48 \pm 0.03$ d^{-1}, Fig. 1a). This observation could reflect an adaptation of *C. danica* to its natural habitat of small water bodies from whence this strain was isolated [49]. The impact of light on the growth rate of these *Ochromonas*-like mixotrophs has been frequently investigated but with contradictory observations. Some authors observed a positive impact of light on mixotrophically grown cultures [50–52]. In other cases, no significant increase in growth rates in light was observed [53,54], while cell size and bio-volume rather than growth rates were impacted by light [55,56]. This heterogeneity of results can be caused by the previously mentioned polyphyly of this taxon, but our results showed a different reaction to the light in a single species at different temperatures. Previous research showed a certain relationship between temperature decrease and an increased rate of heterotrophic carbon acquisition in *Dinobryon sociale*. Lower temperatures generally caused a decrease in growth rate of *D. sociale* [57]. Our result showing a temperature-dependent reaction to darkness in *C. danica* is not fully congruent with comparable research and may deserve deeper investigation. *H. magna*, on the other hand, performed more like standard photoautotrophic algae. Its growth rate optimum was at 20 °C and a higher light intensity of 480 μmol m^{-2} s^{-1} ($\mu = 0.50$ d^{-1}) (Fig. 3a) but the highest final DW density was achieved at 20 °C and lower light of 240 μmol m^{-2} s^{-1} (1.38 g L^{-1}). Previously, another photoautotrophic chrysophyte, *Mallomonas* sp., was found to be similarly tolerant to higher light intensities over 200 μmol m^{-2} s^{-1}, however, its temperature optimum was 28.5 °C, probably due to its tropical origin [46].

4.3. Trophic strategy of C. danica

Although *C. danica* did not show an obligatory requirement for light, and growth rates were not decreased in the dark, its FX content was strongly affected. Cultures grown in the dark had a very low FX content (0.37 ± 0.08 mg g^{-1}DW), whereas FX content in the presence of light exceeded values of 3 mg g^{-1} DW (Table 3). These observations correspond with the suggested trophic strategy for *C. danica*, which is a type of mixotrophy called photoheterotrophy [25]. The metabolic investment of nutrients and energy in the fully operational photosynthetic apparatus is very high [50]; hence, the mixotrophs usually reduce this machinery if organic carbon sources are available and build it up only when the organic sources are unavailable [53,56]. *Ochromonas*-like photoheterotrophs are known to acquire only a negligible quantity of carbon via photosynthesis when they are grown mixotrophically [52,58]. Therefore, it is not surprising that the difference in growth curves we observed was not significant when we compared mixotrophic and heterotrophic growth (Fig. 1b). When organic carbon sources are scarce, these *Ochromonas*-like photoheterotrophs do not express a fully-fledged photosynthetic apparatus and do not switch to the complete photoautotrophy [25]; they express it only at such a level as to provide sufficient energy to survive [36]. Our results showed that *C. danica* cultured in the dark depleted previously gained biomass after reaching stationary phase. This was likely accounted for by catabolism after the organic sources were depleted. Cultures grown in light increased their FX content and maintained biomass quantity, which signals that they were able to utilize light energy to compensate for respiration (Fig. 1b). Using photosynthesis only as a survival strategy and not for proliferation is quite a well-documented phenomenon for *Ochromonas*-like organisms [55,58,59], and it can be regarded as an advantage in the optimization of FX production. FX is an integral part of this occasionally expressed photosynthetic apparatus, and its quantities correlate with those of chlorophyll *a* [19]. FX increase after probable organic substrate depletion is well documented by our results in ABR medium-grown cultures where ~103 % increase in FX content was observed between days 4 and 8 and in MOM 20 medium where ~53 % increase in FX content was observed between days 7 and 14 (Table 3). This approach, when biomass was grown heterotrophically and FX production was induced in the second step by light addition was already tested in the marine diatom *Nitzschia laevis*. It produced the highest ever reported value of FX volumetric productivity by any microalgae (16.5 mg L^{-1} d^{-1}) [17]. *Ochromonas*-like photoheterotrophs could be even more suitable with this technique because the enhancement of FX after organic substrate depletion is their natural strategy. Furthermore, unlike diatoms, they have phagocytic ability, which significantly expands the range of potential substrates for feeding.

4.4. Effect of culture medium on C. danica

Different glucose concentrations tested in the batch mode culture experiment showed clear dose-dependency for *C. danica* growth. Growth was suppressed, probably as a result of organic substrate depletion, however, their concentration in the medium was not monitored. It was demonstrated previously that *Ochromonas*-like organisms were very efficient in sugar consumption [60], with a quite high ratio (40 %) of substrate transfer into biomass (substrate yield) [40]. Our calculations showed a mean substrate yield of ~16 % (yield = final DW density (g L^{-1}) / initial glucose level in the medium (g L^{-1}) + initial organic extracts in the medium (g L^{-1})). The glucose concentration of 40 g L^{-1} showed a considerable suppression of growth rate at the beginning of cultivation, but the subsequent growth was the longest and yielded the highest DW densities. This slower growth was most probably caused by high glucose concentration stress. According to Ma et al. [39], glucose concentrations of 20 g L^{-1} and higher were found to inhibit growth of *P. malhamensis*, while its growth was fastest at a glucose concentration of 5 g L^{-1}.

To evaluate the dependence of *C. danica* on inorganic nutrients, we tested variants of culture medium with or without mineral salts of the macronutrients (N, P, Mg, S, Ca, Cl, K). On one hand, *C. danica* achieved similar DW density at stationary phase in both media (~2.5 g L^{-1}). On the other hand, the medium variant with supplementation of inorganic ions achieved maximal DW density in 3 days, which was more than twice as fast as without supplementation. For large-scale microalgal production, there are obvious expenses associated with growth time (energy, maintenance, labor, facility space and capacity, failure risk, etc.), and these are probably much higher than expenses for widely available mineral fertilizers. Similarly, the best growth performance with culture media combining organic and inorganic N supplies was shown for *P. malhamensis* [19]. Furthermore, the FX concentration achieved in the medium variant supplemented with mineral ions at late stationary phase was the highest observed for *C. danica* among all experiments (3.99 ± 0.19 mg g^{-1} DW); this was probably caused by the increase in FX concentration in substrate-limited cultures, but also to higher N concentrations in the culture medium [61]. Further optimization of medium should be carried out for biotechnological applications of *C. danica*.

4.5. FX productivity

FX concentration in *C. danica* biomass is mostly impacted by the presence of light, organic substrates, and mineral nutrients. According to our results, the FX volumetric productivities in *C. danica* were the highest (1.15 ± 0.05 mg L^{-1} d^{-1}) for the medium variant containing 10 g L^{-1} of glucose supplemented with inorganic mineral salts (ABR

medium). FX productivity could be further enhanced by employing an optimized chemostat [18] or fed-batch two-stage cultivation mode [17]. The above research described biomass densities of over 30 g L^{-1} for *C. danica* if it was cultivated in the optimized fed-batch mode in 3 L fermenters [40,62]. For *P. malhamensis*, two comparable works focused on FX production employing different cultivation techniques. Jin et al. [19] cultivated this organism in osmo-mixotrophic high-cell-density fed-batch mode and achieved a remarkably high biomass concentration reaching 32.9 g L^{-1} but a relatively low FX content of 1.56 mg g^{-1} DW. However, in combination with a high growth rate they achieved FX volumetric productivity of 6.31 mg L^{-1} d^{-1}, which was more than 5.5 times higher than in our study. Secondly, Gao et al. [45], applied a different approach to the different strain of *Poterioochromonas* sp. They utilized phago-mixotrophical batch-culture mode as they fed it with live cells of the cyanobacterium *Microcystis aeruginosa*. Unfortunately, these authors do not provide data about DW density, only cell count, which was 4.6 × 10^6 cells mL^{-1} at the end of the experiment. These authors observed a remarkably high FX content, 11.58 ± 0.37 mg g^{-1} DW, more than 7 times higher than in Jin et al. [19] and 2.9 times higher than then in our study, but the FX volumetric productivity was 1.32 ± 0.02 mg L^{-1} d^{-1}, therefore similar to our results [45].

For comparison, we introduced *H. magna*, a related freshwater chrysophyte, but with a photoautotrophic mode of nutrition. *H. magna* cultivated even in the best working medium had lower specific growth rates and lower biomass productivity than *C. danica*, but it had a higher FX content per biomass and very similar FX productivity (Table 3). Such FX productivities (1.16 ± 0.01 mg L^{-1} d^{-1}) may be considered relevant according to other photoautotrophic FX microalgae producers [14]. Unlike *C. danica*, the FX content in *H. magna* decreased in time under all experimental conditions and for the medium variant with low N and P concentrations, the FX content was lowest. This indicates that *H. magna* degrades its photosynthetic apparatus and restricts its activity after reaching stationary phase. This implies that a different culture strategy than for *Ochromonas*-like organisms should be utilized to optimize its FX productivity in biotechnological applications. Our previous research focused solely on *H. magna* demonstrated a significant light dependence of its FX concentration and productivity [33]. According to this research, FX content in *H. magna* biomass increased exponentially with decreasing light intensities and reached 12.74 ± 2.57 mg g^{-1} DW when the culture was light-limited. This value was similar to FX content in inoculum used in recent work (13.04 mg g^{-1} DW). FX productivity in the previous study, however, reached similar values as in the current work due to the suppression of growth rate in light-limited cultures. The phylogenetic position of *H. magna* suggests potential mixotrophy of this species [24]. However, according to our light microscopy, no phagocytosis was observed, and high numbers of bacteria were apparent in the older culture of *H. magna* (Fig. S2). The culture of *H. magna* was not axenic, which impeded us from performing experiments with added glucose and/or other organic substrates. Many authors have shown a multiplicative increase in biomass and FX productivities in glucose-supplemented algal cultures [17,63]; this would presumably enhance *H. magna* productivity and should be tested.

4.6. pH tolerance

Monitoring of pH showed that the studied organisms performed well within the pH ranges of the experimental set-up (pH 4.5–7 for *C. danica*, and pH 5.5–7.5 for *H. magna)*. Previously, phagotrophic growth and the effect of pH on *C. danica* were investigated [41] with similar findings that *C. danica* grew well between pH 5–7. *Poterioochromonas*, the related *Ochromonas*-like genus, was found to be more tolerant with a pH range from pH 3.5 [64] to pH 11 [65]. For the pH preference of *H. magna*, no references are available. However, our pilot tests showed similar growth performance in the pH range of 4–7; a pH higher than 7 was not tested (Fig. S3). We tested the application of different pH buffers to the growth medium of *H. magna*. The results showed that the buffers used were not able to maintain the pH at initial levels during culture growth, and the medium variant without buffer showed the best growth rates and FX productivities (Table 3, Fig. 3c,d). This can be considered a positive outcome from the application perspective because the consumption of very costly buffers can represent additional expenses for large-scale algae cultivation processes.

4.7. Fatty acids

The total FA content of ~17 % DW for *C. danica* and ~19 % DW for *H. magna* was reasonable in the context that no optimization to enhance FA content was applied. In our previous research, total FA content in *H. magna* was 21 % of DW at low temperatures and high light conditions [33]. Lin et al. [62] enhanced FA accumulation in *C. danica* biomass by supplementation with glycerol and acetate to already grown high cell density cultures. They achieved an overwhelming intracellular lipid content reaching 70–80 % (w/w) using sequential addition of glycerol-acetate. Whether this approach can also be applied to *H. magna* is uncertain. The FA profiles of both organisms were mostly consistent with other published FA profiles for Chrysophyceae [66,67]. Besides the other FAs, they contained a certain amount of SDA (18:4n3) and n-6 DPA (22:5n6), which are considered as biomarkers FAs for Chrysophyceae [68].

H. magna had a higher proportion of PUFAs (~62 %) and LC-PUFAs (~18 %) and a lower omega 6:3 PUFA ratio (~1.8) than *C. danica*. *H. magna* also showed quite a variety of PUFAs containing five omega 6 PUFAs (LA, GLA, DGLA, ARA, and n-6 DPA) and four omega 3 PUFAs (ALA, SDA, EPA, DHA) in a reasonably high amount (more than 1 % of total FAs). The most desirable omega 3 LC-PUFAs for the food and feed market (EPA and DHA) were in lower amounts in *H. magna* (together ~4,3 % of total FA) than is known for instance in diatoms or Eustigmatophyceae [69–71], but the diversity and high proportion of PUFAs in *H. magna* biomass could still bring benefits. The FA profile of *C. danica* contained more SFAs and MUFAs and was much poorer on highly unsaturated FAs (Fig. 4). The major PUFA was LA (18:2n6) and the content of the most desirable omega 3 LC-PUFAs was only about 0.5 % (together EPA + DHA). Two recent articles [41,72] analyzed the FA profile of *C. danica* and they showed very similar FA profiles to our results, despite minor differences in the quality and quantity of FAs that we found. Utilization of *C. danica* biomass as a precursor for the production of high-value PUFAs is therefore questionable and its high lipid production could rather be used in less quality applications. Still, the unanswered question is whether the presence of toxic chlorosulfolipids in the *C. danica* cell membranes [27] can hinder FA production from any nutrition-connected applications of *C. danica* products (this also applies to FX) or whether the chlorosulfolipids can be separated, degraded or even used in the multi-targeted biorefinery as another high-value product of this impressive organism.

4.8. Comparison of C. danica and H. magna

In conclusion, we can evaluate the pros and cons of each organism. *C. danica* and order Ochromonadales generally have been studied more and knowledge of its physiology, productivity, and cultivation techniques is more detailed. Thanks to its facultative photo-heterotrophy and phagocytotic ability, it can utilize a broad spectrum of energy resources including waste organic materials or prey microorganisms. It can grow to high biomass densities with high productivity, and is able to be upscaled using standard bioreactors. Moreover, it is a quite robust and tolerant organism, able to cope with certain microbial contaminations thanks to its phagocytosis. These all have great advantages in the context of applied phycology. *C. danica* is probably accessible to a two-stage cultivation technique when at first it accumulates biomass heterotrophically and then synthesizes target carotenoids using light. This natural trait can be utilized beneficially. *H. magna*, on the other hand, has some features, that are advantageous over *C. danica*. First, it can

grow purely autotrophically, so it does not need expensive energy resources to produce biomass; furthermore, its ability to grow mixotrophically is still unexplored. Secondly, its FX content is higher and with optimization of the culture technology, it could be significantly enhanced. Third, it has a much better PUFA profile in the sense of diversity, the quantity of high-value LC-PUFA, and the omega 6:3 PUFA ratio. So, its biomass is of higher quality from the perspective of our target products, FX and PUFAs. Therefore, both Chrysophyceae strains studied have prospects and deserve further research as well-founded alternatives for FX and PUFA production.

5. Summary

We introduced two freshwater chrysophyte flagellates as candidates for a multi-target biorefinery. We compared their growth in different cultivation media and their content of FX and PUFA in lab-scale batch mode cultivation experiments. Their distinct trophic strategies and their implications in applied phycology were evaluated. This was the first report of FX content and productivity by *Chlorochromonas danica*, which is an otherwise known and successfully cultivated mixotrophic alga with a distinctive predatory behavior and ability to grow to high densities of biomass. *Hibberdia magna* is a less studied chrysophyte autotroph that is capable of cultivation under artificial conditions. *C. danica* had a higher biomass productivity but lower content of monitored products. *H. magna* has a very diverse PUFA profile and is FX-rich. Both organisms can be recommended for further experimental work and use due to their high FX productivity accompanied by PUFA content, metabolic plasticity, and technical benefits related to their freshwater nature.

Author statement

All authors, Pavel Přibyl, Antonín Střížek, Martin Lukeš, Pavel Hrouzek, Mykola Mylenko, Jaromír Lukavský, and Linda Nedbalová, state that the manuscript entitled “Alternative production of fucoxanthin and PUFAs using *Chlorochromonas danica* and *Hibberdia magna*, unicellular chrysophytes with different trophic modes” is the result of their original work. The MS has not been submitted for publication before, and authors do not intend to submit any related manuscript elsewhere.

Funding

The work was supported by the Technology Agency of the Czech Republic (grants No. TN010000048 and TN02000044), and a long-term research development project No. RVO 67985939.

CRediT authorship contribution statement

Antonín Střížek: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Martin Lukeš:** Writing – review & editing, Methodology. **Pavel Hrouzek:** Writing – review & editing, Supervision. **Mykola Mylenko:** Methodology. **Jaromír Lukavský:** Methodology. **Linda Nedbalová:** Writing – review & editing. **Pavel Přibyl:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Conceptualization.

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

All available data for this study are included in this published article and its supplementary files.

Acknowledgements

Authors warmly thank research interns Aizhan Karabekova and Irena Knetlová for technical assistance during cultivation experiments. The collaboration with Mr. Martin Dobřichovský and the support of the company Algamo (www.algamo.cz) is greatly appreciated as well.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2024.103597.

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