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RESEARCH

Beta-glucan production of *Phaeodactylum tricornutum*, *Monodopsis subterranea* and *Cylindrotheca fusiformis* during nitrogen depletion

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Abstract

Beta-glucans are polysaccharides that can be used for different applications, for example as an immunomodulator in food or feed or for managing high cholesterol levels. Certain microalgae species use beta-glucans as energy storage, accumulating them during nutrient depletion. In this study, we examined and compared beta-glucan production during nitrogen depletion in three different algae species, *Phaeodactylum tricornutum*, *Monodopsis subterranea* and *Cylindrotheca fusiformis,* grown in artificially illuminated flat panel airlift reactors, in order to determine the most promising microalgae species for beta-glucan production. Co-products such as fatty acids (especially eicosapentaenoic acid) and the carotenoid fucoxanthin (not produced by *M. subterranea*) were also considered. Biomass analysis showed that *P. tricornutum* cultures reached a maximal beta-glucan content of 317 ± 9 mg g_{DW}^{-1}, *M. subterranea* cultures reached 188 ± 6 mg g_{DW}^{-1} and *C. fusiformis* cultures reached 129 ± 13 mg g_{DW}^{-1}. Furthermore, beta-glucan production was faster in *P. tricornutum* cultures. However, the maximum volumetric beta-glucan concentration reached was higher in *M. subterranea* cultures compared to *P. tricornutum* cultures as *M. subterranea* cultures produced more biomass during nitrogen depletion. In terms of possible co-products, *P. tricornutum* produced fucoxanthin and EPA, whereas *M. subterranea* did not produce fucoxanthin. However, *M. subterranea* exhibited a higher EPA content, which remained above 45 mg g^{-1} even after several days of nitrogen depletion. Overall, our results suggest that *P. tricornutum* and *M. subterranea* are both suitable species for beta-glucan production in flat panel airlift reactors.

Keywords *Phaeodactylum tricornutum* · *Cylindrotheca fusiformis* · *Monodopsis subterranea* · Beta-glucan · Microalgae · Nitrogen depletion

Introduction

Beta-glucans (ß-glucans) can be found in human diets in the form of yeast or cereals (Ciecierska et al. 2019). Additionally, various products containing ß-glucans are available on the market. For instance, ß-glucans from yeast are sold as animal feed (MacroGard™, Offra). Macro- and microalgae also contain ß-glucans and are therefore possible alternative sources (Myklestad 1989; Aziz et al. 2003).

Microalgae produce ß-glucans in different forms, for example as unbranched 1,3-ß-glucan (paramylon) or as branched 1,3–1,6-ß-glucan (chrysolaminarin). These structural differences affect solubility. Chrysolaminarin is found dissolved in the cytosol, while paramylon is found undissolved as granules (Kiss et al. 1988; Espinoza-Gallardo et al. 2017; Gruber and Kroth 2017). All three microalgae species examined here, *Phaeodactylum tricornutum*, *Monodopsis subterranea* and *Cylindrotheca fusiformis,* are known to accumulate ß-glucans as carbon and energy storage during nutrient depletion. However, *P. tricornutum* and *C. fusiformis* produce water-soluble branched chrysolaminarin, while *M. subterranea* produces unbranched paramylon (Vieler et al. 2012; Eliáš et al. 2017; Gao et al. 2017). Besides carbohydrates, microalgae also accumulate triglycerides as carbon and energy storage during nutrient depletion. These triglycerides contain saturated and unsaturated fatty acids with different chain lengths, such as C16:0, C16:1 and C18:1 (Yodsuwan et al. 2017; Adamakis et al. 2018).

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Therefore, the accumulation of these fatty acids, which are used for energy storage, must be taken into account when examining the production of ß-glucans.

Various possible applications have been described for the use of algae-derived ß-glucans, for example in food, feed or agriculture. It has been reported that ß-glucans show anti-oxidative, immunomodulatory, anti-inflammatory and antitumor activity in animals and humans (Neyrinck et al. 2007; Sugiyama et al. 2010; Ji et al. 2012; Xia et al. 2014). Several recent studies addressed the application of ß-glucan from the microalga *P. tricornutum*. This ß-glucan showed promising cholesterol-lowering properties in zebrafish. The effect of the ß-glucan was similar to Simvastin, a drug used to treat high cholesterol levels (Gora et al. 2022). Reis et al. (2021) showed that ß-glucan from *P. tricornutum* promotes the health in juvenile fish, allowing for possible application in aqua-feed. Furthermore, feeding trials with ß-glucan-rich *P. tricornutum* biomass in mice showed gut-related benefits. For example, an increase in short-chain fatty acids was observed in this study (Stiefvatter et al. 2022b). Another recent study with humans described further potentially beneficial effects for healthy aging (Stiefvatter et al. 2022a). These results make algae-derived ß-glucans interesting for human nutrition as well.

Algae-derived ß-glucans can also be used in agriculture. They can act as elicitors to trigger defence mechanisms in vascular plants when they come into contact with their leaves (Kobayashi et al. 1993; Inui et al. 1997; Klarzynski et al. 2000; Aziz et al. 2003; Neyrinck et al. 2007; Sugiyama et al. 2010; Ji et al. 2012; Xia et al. 2014; Wanke et al. 2020). A product with ß-glucan derived from macroalgae is already on the market (Vacciplant™, Stähler). Vascular plants react to a contact with ß-glucans by activating various defence systems against pathogenic fungi such as *Plasmopara viticola*. Application of ß-glucans may lead to fewer infection events with pathogenic fungi. In grapevine plants treated with ß-glucans, up to 75% fewer infection events with *P. viticola* were reported compared to the untreated control (Aziz et al. 2003). Other publications report a similar effect for various vascular plants and pathogenic fungi (Kobayashi et al. 1993; Inui et al. 1997; Klarzynski et al. 2000; Wanke et al. 2020).

Besides ß-glucans, microalgae contain further valuable bioactive compounds, such as eicosapentaenoic acid (EPA) and fucoxanthin (FX). All microalgae species tested here contained EPA, while FX is only produced by the two diatoms tested (*P. tricornutum* and *C. fusiformis*). EPA is an important omega-3 fatty acid for human nutrition and is already used as a dietary supplement (Ritter et al. 2013). It shows positive effects in the prevention of cardiovascular disease and hypertension (Kang and Leaf 1996; Prisco et al. 1998; Connor 2000; Frenoux et al. 2001; Narayan et al. 2006). In addition, EPA shows antioxidant and anti-inflammatory effects in humans and animals (Kim and Chung 2007; Calder 2010). FX has been reported to exhibit anti-oxidative, anti-inflammatory and weight-reducing properties, as well as activity against non-alcoholic fatty liver disease (NAFLD) (Kotake-Nara et al. 2001; Hosokawa et al. 2004; Maeda et al. 2005, 2006; Sachindra et al. 2007; Heo et al. 2012; Fung et al. 2013; Neumann et al. 2018; Gille et al. 2019). A FX-based product against NAFLD is already available in the United States (Fucovital™, Algatech). Co-products such as FX and EPA, are also interesting for a process focusing on ß-glucan production, as it is possible to extract different compounds from the same biomass through cascaded extraction. This can improve the economical prospect of a production process (Derwenskus et al. 2020b). Therefore, EPA and FX should also be considered to complete the picture for future valorisation in a bio-refinery process.

Although ß-glucans are microalgae-based products with interesting possible applications, there is little information about their production in photobioreactors. Previously published publications on the production of compounds with diatoms tend to focus on pigments (like FX) or fatty acids (like EPA) rather than ß-glucans (Yang et al. 2020). Furthermore, publications that include ß-glucans focus on only one algae species and mostly on better-known algae species such as *P. tricornutum* (Gao et al. 2017; Frick et al. 2023). Consequently, there is a lack of data regarding the suitability of different microalgae species for the ß-glucan production in scalable commercial photobioreactors. Therefore, we examined and compared the ß-glucan production of the three different algae species *P. tricornutum*, *M. subterranea* and *C. fusiformis,* grown in flat panel airlift reactors (FPA) during nitrogen depletion (N-depletion). Besides ß-glucan, fatty acid accumulation was also analysed. Here, we focused especially on fatty acids which are used as carbon and energy storage by microalgae (here: C16:0, C16:1 and C18:1). In addition, the production of EPA and FX was also analysed, as both are valuable bioactive compounds that can be extracted from the same biomass (Derwenskus et al. 2020b). The algae species selected were a marine species (*C. fusiformis*), a brackish species (*P. tricornutum*) and a freshwater species (*M. subterranea*). In addition to the two diatoms *(P. tricornutum* and *C. fusiformis),* the eustigmatophyte *M. subterranea* was selected for its promising potential to produce EPA and fatty acids (Lu et al. 2002). Furthermore, *M. subterranea* showed carbohydrate accumulation of up to 300 mg g^{-1} at steady state in bubble column reactors (Guil-Guerrero and Rebolloso-Fuentes 2008).

Materials and methods

Algae species

Phaeodactylum tricornutum SAG 1090-1b was acquired from the Department of Experimental Phycology and Culture Collection of Algae (EPSAG) of the

Georg-August University in Göttingen, Germany. *Monodopsis subterranea* SAG 848–1 was acquired from the Department of Experimental Phycology and Culture Collection of Algae (EPSAG) of the Georg-August University in Göttingen, Germany. *Cylindrotheca fusiformis* was acquired from the Australian National Algae Culture Collection (CSIRO).

Culture medium

As brackish cultivation medium for *P. tricornutum* modified Mann & Myers medium was used, with 10 g L^{-1} NaCl, 2.4 g L^{-1} $MgSO_4.7H_2O$, 0.6 g L^{-1} $CaCl_2.2H_2O$ and 20 ml L^{-1} trace element solution (Mann and Myers 1968). The trace element solution was prepared as described in the original recipe. *Cylindrotheca fusiformis* was cultivated under seawater conditions in modified f/2-medium, with 32 g L^{-1} seawater extract (Tropic Marin, Dr. Biener GmbH), 20 mg L^{-1} $MgSO_4.7H_2O$, 10.6 mg L^{-1} SiO_3 and 2 mL L^{-1} trace element solution (Guillard and Ryther 1962). Here, the trace element solution was also prepared as described in the original recipe. *Monodopsis subterranea* was cultivated under freshwater conditions on modified OHM medium, with 493 mg L^{-1} $MgSO_4.7H_2O$, 222 mg L^{-1} $CaCl_2.2H_2O$, 5.2 mg L^{-1} Fe(III)Citrate.$5H_2O$, 0.02 mg L^{-1} $CoCl_2.6H_2O$, 0.024 mg L^{-1} $CuSO_4.5\ H_2O$, 1.98 mg L^{-1} $MnCl_2.4H_2O$, 0.24 mg L^{-1} $Na_2MoO_3.2H_2O$ (Fábregas et al. 2000). In all media, nitrogen and phosphorous were added separately. A phosphate stock solution was used (50 g L^{-1}) as phosphorous source. The phosphate stock solution was prepared from 45.35 g L^{-1} K_2HPO_4 and 35.8 g L^{-1} KH_2PO_4. Phosphate concentration in the culture medium ranged from 20 to 200 mg L^{-1} (0.2—2.1 mmol L^{-1}). As a nitrogen source, ammonium was added from a stock solution (35 g L^{-1}). The ammonium stock solution was prepared from 153.4 g L^{-1} NH_4HCO_3. In the pre-cultures (see 3.3), ammonium concentration ranged from 30 to 300 mg L^{-1} (1.7—16.6 mmol L^{-1}). During the N-depletion, no ammonium was supplied and the ammonium concentration dropped to 0 mg L^{-1}. Ammonium as well as phosphate content of the culture medium was analysed daily using flow injection analysis with a photometrical detector.

Precultures

The precultures used for inoculation of the experimental cultures were already cultivated for 14 days under the same cultivation conditions as in the experiments (FPA reactor, pH value, temperature, specific light availability, phosphate concentration). This was done to avoid adaption processes during the experiment. The precultures were regularly diluted so that the biomass concentration (c_{DW}) ranged between 1 and 3 g L^{-1}.

Light regime

Biomass specific light availability (I_{spec}) was used to describe the light regime. Here, the photon flux density (PFD) on the reactor surface is correlated to the culture volume and the biomass concentration of the culture. Figure S4 shows the PFD applied to the reactor surface. Only light in the PAR region was considered for the calculation. I_{spec} was described previously by Holdmann et al. (2018). I_{spec} was calculated according to Eq. 1, using illuminated reactor surface A (0.21 m^2), photon flux density PFD on the surface of the reactor, culture volume V and biomass concentration c_{DW}.

$$I_{spec} = \frac{A * PFD}{V * c_x}\left[\frac{mol_{Photons}}{g_{DW} * s}\right] \quad (1)$$

Experimental setup and cultivation conditions

For each of the three examined microalgae species, three separate flat panel airlift photobioreactors (culture volume 6 L) were inoculated from the same preculture. These experimental cultures were grown for ten days as batch cultivations. The nitrogen depletion experiments were therefore carried out in biological triplicate for each microalgae species. The duration of N-depletion was chosen based on previous results (Frick et al. 2023). During N-depletion, phosphorous was added throughout the experiment, while no further ammonium was added to the cultures. Subsequently, the ammonium content of the medium dropped to 0 mg L^{-1}. The first day without ammonium in the medium was considered as the start of the N-depletion (Day 0). In all experiments, this was the first day after inoculation, which is why the data is presented up to day 9 of N-depletion.

In all experiments, commercially available flat panel airlift photobioreactors (FPA) with a culture volume of 6 L were used (Subitec GmbH, Germany). The FPA reactor is a variation of a flat plate reactor. An air/CO_2 mixture is injected through a silicone membrane at the bottom of the reactor to pneumatically mix the microalgae culture in the reactor. The shape of the reactor has been modified to improve the intermixing of the culture inside. Furthermore, the shape increases the time that the gas bubbles are in the culture medium, which has a positive effect on the gas transfer between gas bubbles and culture medium. The FPA reactors used were equipped with LED panels for artificial illumination (Nichia, NSSL157AT-H3). The LED panels were placed on one side of the reactor at a distance of 2 cm. The LEDs used emitted a light spectrum comparable to sunlight (3000 K, CRI > 90). The FPA reactor was continuously

illuminated and the illuminated reactor surface was 0.21 m^2. The photon flux density (PFD) on the reactor surface was correlated to the biomass concentration of the culture inside (I_{spec}). Pre-cultures as well as experimental cultures were cultivated at an I_{spec} of 5 $\mu mol_{photons}\ g_{DW}^{-1}\ s^{-1}$. I_{spec} was reset daily to this value by adjusting the impinging PFD on the reactor surface. To control the cultivation conditions, each reactor was equipped with a reactor control unit (Siemens SPS, Germany) that automatically regulated pH value, CO_2 content of the gas flow and temperature. PFD on the reactor surface and the addition of phosphate and ammonium had to be adjusted manually. During the experiments, the temperature ranged between 20.0 and 20.5 °C and pH between 7.1 and 7.5 for each species tested. In order to keep the temperature stable, the lower part of the reactors (10 cm) was immersed in a water bath whose temperature was controlled by the reactor control unit. Pressurised air was used for aeration (180 L h^{-1}). To keep the pH value constant, pure CO_2 (1—20 L h^{-1}) was automatically added to the airflow.

Analytical procedures

During the experiments, biomass samples were taken daily from each culture using a syringe. The determination of biomass dry weight was carried out with a fresh culture sample. For the analysis of the compounds, the biomass samples were concentrated by centrifugation, washed twice to remove excess medium, frozen and freeze-dried. In preparation for the determination of the beta-glucan content, the fatty acid content, and the fucoxanthin content, cell disruption was carried out using a homogenizer (Precellys24, Bertin technologies, France).

Determination of biomass dry weight

The biomass concentration c_{DW} was determined according to Frick et al. (2023). A sample of 5 mL was put on a pre-dried and pre-weight glass-fibre filter (pore size: 0.2 µm; MN 85/70, Macherey–Nagel GmbH, Germany), placed on a Büchner funnel. After filtering the sample 5 mL of ddH_2O was used to remove residual medium from the sample. This step was carried out twice. The filter with the sample then was dried and weighed on an analytical balance. Finally, the biomass concentration c_{DW} was calculated as the difference between the filter loaded with biomass and the empty filter.

Determination of beta-glucan content

The ß-glucan content $\omega_{ß\text{-glucan}}$ (in mg g_{DW}^{-1}) was analysed with an enzymatic test kit (K-EBHLG 08/18, Megazyme, Ireland), which had been used previously for the quantification of ß-glucan from micro- and macroalgae (Danielson et al. 2010; Frick et al. 2023). The test was performed according to the manufacturer's instructions but scaled down by a factor of 5. In this test, the ß-glucan is enzymatically digested into glucose molecules using a β-1,3-glucanase. Afterwards a glucose oxidase/peroxidase reagent was added, which reacted with the resulting glucose molecules. The resulting colour change was measured photometrically and converted to the initial amount of ß-glucan in the sample (McCleary and Draga 2016). The ß-glucan content of the experimental cultures $\omega_{ß\text{-glucan}}$ was analysed daily in biological triplicates.

Determination of fatty acid content

The total fatty acid content ω_{TFA}, and the content of the specific fatty acids ω_{EPA}, $\omega_{C16:0}$, $\omega_{C16:1}$ and $\omega_{C18:1}$ (all in mg g_{DW}^{-1}) were analysed by gas chromatography (7890A, Agilent, USA) according to the transesterification method described by Lepage and Roy (1984). The content of the different fatty acids of the experimental cultures was analysed daily in biological triplicates.

Determination of fucoxanthin content

The FX content ω_{FX} (in mg g_{DW}^{-1}) was analysed by HPLC (1200 Infinity, Agilent, USA). The method applied was described by Derwenskus et al. (2020a) and is based on the method described by Gille et al. (2015). The FX content ω_{FX} of the experimental cultures was analysed daily in biological triplicates.

Calculations

Volumetric concentration of a compound

The volumetric concentration of a compound X c_x (in mg L^{-1}) describes the amount of a compound (here: ß-glucan, FX or fatty acids) per litre culture medium. It was calculated using Eq. 2 with biomass concentration c_{DW} (in g L^{-1}) and the content of the compound X ω_X (in mg g_{DW}^{-1}).

$$c_X = c_{DW} * \omega_X[\frac{mg_X}{L}] \quad (2)$$

Biomass specific beta-glucan productivity

The biomass specific ß-glucan productivity $q_{ß\text{-glucan}}$ (in mg g_{DW}^{-1} day^{-1}) describes the amount of ß-glucan which was produced per gram biomass on the previous day. It was calculated with Eq. 3 using the ß-glucan concentration (in mg L^{-1}) at day *n* ($c_{ß\text{-glucan}}$ (n)) and at the previous day ($c_{ß\text{-glucan}}$ (n-1)), as well as the biomass concentration (in g L^{-1}) at the previous day (c_{DW} (n-1)). Furthermore, the observed time window has to be taken into account (here: 1 day).

$$q_{\beta-glucan} = \frac{c_{\beta-glucan}(n) - c_{\beta-glucan}(n-1)}{c_{DW}(n-1) * 1d}[\frac{mg_{\beta-glucan}}{g_{DW} * d}] \quad (3)$$

Statistical analysis

Statistical analysis was performed similar to our latest publication (Frick et al. 2023) using Matlab R2022b (MathWorks, USA). To test for the statistical significance of our results, we employed analysis of variance (ANOVA). The assumptions for ANOVA were checked using the Jarque–Bera test for normality (Matlab function: "jbtest") and the Bartlett's test for equal variances (Matlab function: "vartestn"). If these assumptions were not met, Kruskal–Wallis test was used as an alternative to ANOVA to test the statistical significance of our results (Sullivan et al. 2016). ANOVA results are reported using F(df1,df2) and p, where „F" is the F-value, „df1″ and „df2″ are the degrees of freedom and „p" is the p-value. Kruskal–Wallis test results are reported using χ^2 (df1, df2) and p. Here, "χ^2" is the chi square and "df1" and "df2" are also the degrees of freedom and "p" stands for the p-value. If a significant difference is shown by ANOVA (or Kruskal–Wallis), we used the Tukey post hoc test to determine the significance between the groups. For fucoxanthin analysis, where only two species were compared, we used t-test (Matlab function "ttest"). Results of the t-test are reported using t(df) and p, where „t" is the t-value, „df" is the degree of freedom and „p" is the p-value.

Significant differences ($p \leq 0.05$), analysed with ANOVA (or Kruskal–Wallis) and the Tukey post hoc test, are represented with different lowercase letters above the values in tables. If two values are marked with the same letter, this means that there is no significant difference between these two values. The detailed results of all statistical tests performed are presented in Tables S4, S5 and S6.

Results

As described above (see 3.5), the cultures of the three algae species tested were cultivated for nine days under nitrogen depleted conditions. During this period, the increase of the biomass concentration in *P. tricornutum* and *C. fusiformis* cultures stopped, whereas biomass concentration in *M. subterranea* cultures continued to increase thereafter (see Fig. 1). Therefore, the experiments for *M. subterranea* were continued until biomass increase eventually stopped (day 16). However, to ensure comparability of the results between species, only the first 9 days of N-limitation of all tested algal species were considered in this paper. Additional data on *M. subterranea*, including protein content, can be found in the appendix (see S3).

Biomass increase during nitrogen depletion

Starting with a c_{DW} of 1 g L^{-1}, the biomass concentration c_{DW} increased in all three tested algae species during N-depletion. The maximal biomass concentration differed significantly between *C. fusiformis*, *M. subterranea* and *P. tricornutum* and was highest in *M. subterranea* cultures (see Table S1). In *M. subterranea* cultures, the biomass concentration c_{DW} increased up to the last day of the observation period and reached a maximum of 5.18 ± 0.15 g L^{-1}. In *P. tricornutum* cultures, the highest biomass concentration of 2.70 ± 0.13 g L^{-1} was measured on day 8 of N-depletion. In *C. fusiformis* cultures, the biomass concentration did not increase after day 4. The highest biomass concentration for *C. fusiformis* was measured on day 8 of N-depletion with 1.49 ± 0.31 g L^{-1}.

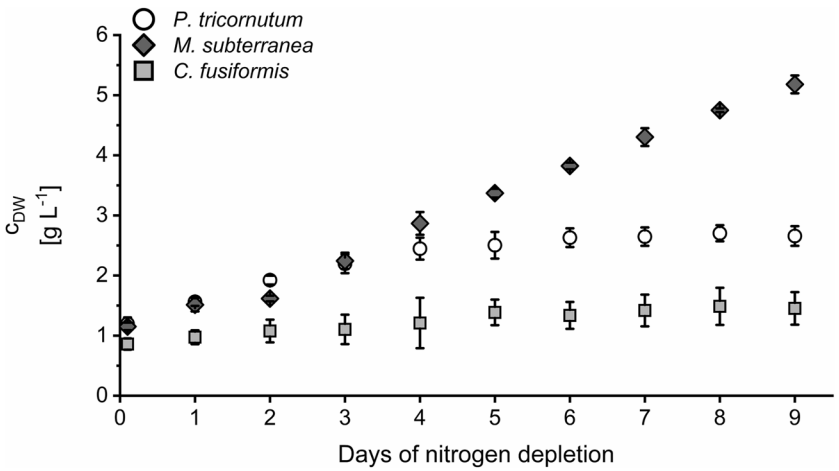

Fig. 1 Biomass concentration c_{DW} of *P. tricornutum*, *M. subterranea* and *C. fusiformis* during nitrogen depletion. $I_{spec.}$ was reset daily to 5 $\mu mol_{photons}$ g_{DW}^{-1} $s.^{-1}$ via adjusting the PFD on the reactor surface (see 3.4). (±SD, n = 3 analysed as biological triplicate). Data for *P. tricornutum* previously published in Frick et al. (2023)

Accumulation of beta-glucan during nitrogen depletion

The ß-glucan content $\omega_{ß\text{-glucan}}$ increased in all three species during N-depletion. In *P. tricornutum* and *C. fusiformis* cultures the ß-glucan content increased fastest in the first few days of N-depletion and did not increase further towards the end of the experiments. The maximal $\omega_{ß\text{-glucan}}$ differed significantly between the species tested (see Table S1). Even though $\omega_{ß\text{-glucan}}$ did not increase in *P. tricornutum* cultures after day 4, *P. tricornutum* cultures reached the highest $\omega_{ß\text{-glucan}}$ of all three species tested with 317 ± 9 mg g_{DW}^{-1} (see Fig. 2 and Table S1). *Cylindrotheca fusiformis* cultures started with the highest $\omega_{ß\text{-glucan}}$, but showed the lowest maximal $\omega_{ß\text{-glucan}}$ (129 ± 13 mg g_{DW}^{-1}). After day 4, there was no more increase of $\omega_{ß\text{-glucan}}$ in *C. fusiformis* cultures. However, in *M. subterranea* cultures, $\omega_{ß\text{-glucan}}$ increased until the end of the experiment and reached a maximal value of 188 ± 9 mg g_{DW}^{-1}.

With 195 ± 55 mg L^{-1} *C. fusiformis* cultures reached a significantly lower maximal volumetric ß-glucan concentration $c_{ß\text{-glucan}}$ compared to *P. tricornutum* and *M. subterranea* cultures. *Phaeodactylum tricornutum* cultures reached their maximum $c_{ß\text{-glucan}}$ of 840 ± 66 mg L^{-1} on day 8 of N-depletion. Whereas in *M. subterranea* cultures, increasing ß-glucan content and especially increasing biomass concentration led to an increase of $c_{ß\text{-glucan}}$ until the last day of the experiment (day 9). Subsequently, *M. subterranea* cultures showed the highest $c_{ß\text{-glucan}}$ of the three species tested with 975 ± 76 mg L^{-1}.

Accumulation of fatty acids during nitrogen depletion

The accumulation of fatty acids during N-depletion differed between the three species tested with regard to the maximal value and the time course. Total fatty acid (TFA) content ω_{TFA} increased in all three species during N-depletion. Compared to the other two species, *M. subterranea* cultures showed a significantly higher ω_{TFA} with 299 ± 9 mg g_{DW}^{-1} on day 9 on N-depletion (see Table 1 and Table S1). In all three species, mostly C16:0 and C16:1 fatty acids were accumulated, as shown by the increasing content of C16:0 $\omega_{C16:0}$ and of C16:1 $\omega_{C16:1}$ (see Table 1). In addition, M. *subterranea* accumulated C18:1. The EPA content ω_{EPA} remained almost unchanged during N-depletion in *C. fusiformis* and *P. tricornutum* but increased in *M. subterranea*. Overall, *M. subterranea* exhibited a significantly higher ω_{EPA} with up to 57 ± 2 mg g_{DW}^{-1} compared to the other two species (see Table 1 and Table S1).

Fucoxanthin under nitrogen depletion

The fucoxanthin content ω_{FX} decreased during N-depletion in *P. tricornutum* and *C. fusiformis* cultures. The volumetric fucoxanthin concentration c_{FX} decreased as well in *P. tricornutum* cultures. Whereas in *C. fusiformis* cultures, c_{FX} did not change in the progress of N-depletion (see Fig. 3). As a eustigmatophyte, *M. subterranea* did not produce FX.

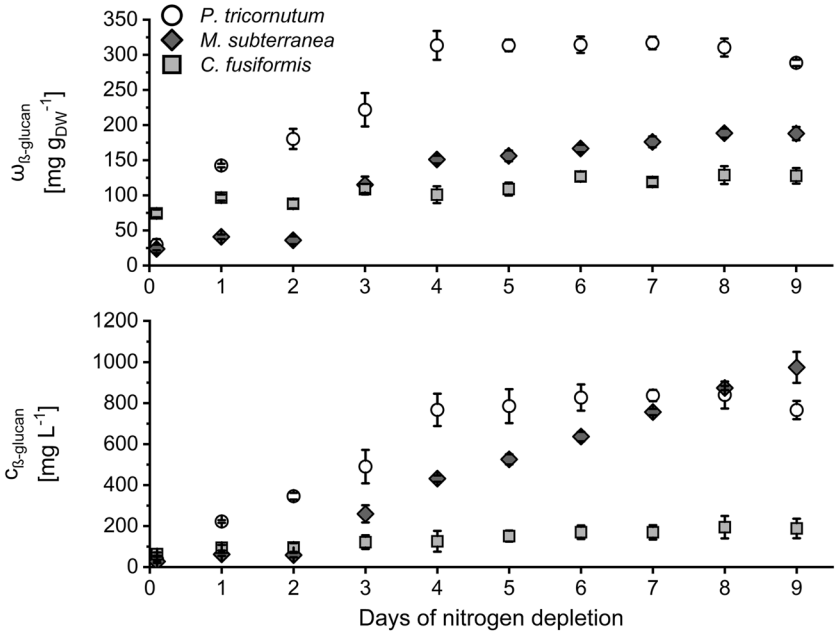

Fig. 2 ß-glucan content $\omega_{ß\text{-glucan}}$ and volumetric ß-glucan concentration $c_{ß\text{-glucan}}$ of *P. tricornutum*, *M. subterranea* and *C. fusiformis* during nitrogen depletion. $I_{spec.}$ was reset daily to 5 $\mu mol_{photons}$ g_{DW}^{-1} $s.^{-1}$ via adjusting the PFD on the reactor surface (see 3.4). (± SD, n = 3 analysed as biological triplicate see 3.5). Data for $\omega_{ß\text{-glucan}}$ of *P. tricornutum* previously published in Frick et al. (2023)

Table 1 Total fatty content ω_{TFA}, the content of C16:0 fatty acids $\omega_{C16:0}$, C16:1 fatty acids $\omega_{C16:1}$, C18:1 fatty acids $\omega_{C18:1}$ and EPA content ω_{EPA} of *P. tricornutum*, *M. subterranea* and *C. fusiformis* during nitrogen depletion. $I_{spec.}$ was reset daily to 5 $\mu mol_{photons}\ g_{DW}^{-1}\ s^{-1}$ via adjusting the PFD on the reactor surface. (±SD, n = 3 analysed as biological triplicate). Data for ω_{EPA} of *P. tricornutum* previously published in Frick et al. (2023)

	Day of nitrogen depletion									
		0	1	2	3	4	5	6	7	8	9
P. tricornutum	ω_{TFA} [mg g_{DW}^{-1}]	54 ± 1	62 ± 3	86 ± 4	117 ± 10	184 ± 11	194 ± 7	223 ± 13	232 ± 8	237 ± 5	253 ± 2
	$\omega_{C16:0}$ [mg g_{DW}^{-1}]	10 ± 0	13 ± 1	22 ± 1	35 ± 3	57 ± 4	63 ± 2	74 ± 4	77 ± 3	81 ± 2	86 ± 1
	$\omega_{C16:1}$ [mg g_{DW}^{-1}]	17 ± 1	20 ± 0	29 ± 2	42 ± 3	69 ± 4	75 ± 1	88 ± 4	92 ± 1	95 ± 1	101 ± 1
	$\omega_{C18:1}$ [mg g_{DW}^{-1}]	2 ± 0	3 ± 0	5 ± 0	7 ± 1	12 ± 3	12 ± 1	13 ± 1	14 ± 1	14 ± 0	15 ± 0
	ω_{EPA} [mg g_{DW}^{-1}]	20 ± 1	20 ± 1	20 ± 1	19 ± 2	24 ± 1	23 ± 1	24 ± 3	24 ± 1	23 ± 1	24 ± 1
M. subterranea	ω_{TFA} [mg g_{DW}^{-1}]	61 ± 19	95 ± 11	104 ± 14	162 ± 3	202 ± 3	234 ± 19	269 ± 13	286 ± 5	280 ± 24	299 ± 9
	$\omega_{C16:0}$ [mg g_{DW}^{-1}]	14 ± 4	21 ± 1	22 ± 3	37 ± 1	45 ± 1	53 ± 4	63 ± 3	65 ± 2	64 ± 6	68 ± 2
	$\omega_{C16:1}$ [mg g_{DW}^{-1}]	14 ± 3	20 ± 4	24 ± 3	39 ± 1	52 ± 2	64 ± 7	76 ± 5	83 ± 2	86 ± 8	93 ± 2
	$\omega_{C18:1}$ [mg g_{DW}^{-1}]	2 ± 1	3 ± 0	4 ± 1	11 ± 1	19 ± 2	27 ± 3	37 ± 2	44 ± 1	44 ± 4	49 ± 2
	ω_{EPA} [mg g_{DW}^{-1}]	22 ± 9	37 ± 7	40 ± 5	53 ± 2	57 ± 2	55 ± 3	53 ± 2	52 ± 3	47 ± 3	48 ± 2
C. fusiformis	ω_{TFA} [mg g_{DW}^{-1}]	150 ± 11	148 ± 9	178 ± 1	213 ± 13	213 ± 9	243 ± 8	236 ± 20	245 ± 9	263 ± 11	257 ± 13
	$\omega_{C16:0}$ [mg g_{DW}^{-1}]	38 ± 1	38 ± 3	50 ± 1	59 ± 2	58 ± 3	67 ± 0	65 ± 4	68 ± 3	72 ± 4	71 ± 4
	$\omega_{C16:1}$ [mg g_{DW}^{-1}]	50 ± 4	51 ± 2	63 ± 1	79 ± 5	82 ± 4	95 ± 3	93 ± 9	97 ± 5	106 ± 6	105 ± 7
	$\omega_{C18:1}$ [mg g_{DW}^{-1}]	1 ± 0	1 ± 0	2 ± 0	2 ± 0	2 ± 0	3 ± 0	3 ± 0	3 ± 0	3 ± 0	3 ± 0
	ω_{EPA} [mg g_{DW}^{-1}]	30 ± 3	28 ± 1	28 ± 1	31 ± 2	29 ± 0	30 ± 1	29 ± 2	29 ± 0	29 ± 1	28 ± 1

Discussion

The production of any compound always depends on the biomass increase (biomass productivity). Biomass productivity is lower during nutrient depleted cultivation conditions compared to nutrient repleted conditions (Frick et al. 2023). Therefore, N-depletion is mainly favourable for products that accumulate during N-depletion, such as storage compounds like ß-glucans. In our experiments, biomass increased in all three species tested during N-depletion. However, biomass increase in *M. subterranea* was higher and lasted for a longer period of time compared to the other two species. This had a positive effect on the compounds produced during the process (ß-glucan and fatty acids).

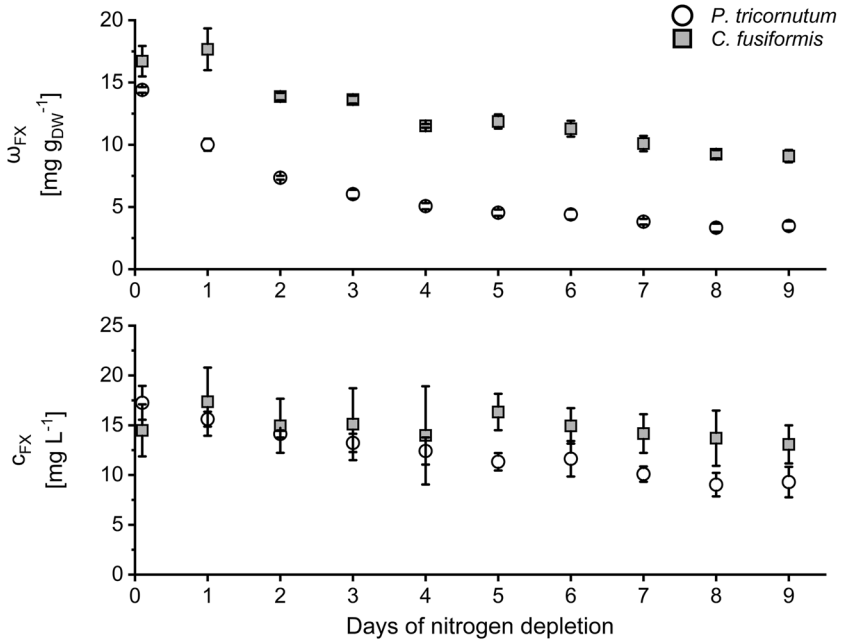

Fig. 3 Fucoxanthin content ω_{FX} and volumetric fucoxanthin concentration c_{FX} of *P. tricornutum* and *C. fusiformis* during nitrogen depletion. As a eustigmatophyte, *M. subterranea* did not contain any FX. $I_{spec.}$ was reset daily to 5 $\mu mol_{photons}\ g_{DW}^{-1}\ s.^{-1}$ via adjusting the PFD on the reactor surface (see 3.4). (±SD, n = 3 analysed as biological triplicate). Data for ω_{FX} of *P. tricornutum* previously published in Frick et al. (2023)

Production of ß-glucan and fatty acids

The accumulation of carbohydrates and fatty acids used as energy storage are closely connected to each other (Siaut et al. 2011; Recht et al. 2012). Microalgae use both carbohydrates and fatty acids as energy storage. It is also reported that carbohydrates such as ß-glucans are metabolised in favour of fatty acids after a longer period of nutrient depletion (Li et al. 2011; Gao et al. 2017). Therefore, the accumulation of ß-glucans and fatty acids during N-depletion should be regarded in context.

For a ß-glucan production process it would be beneficial if the production strain would start accumulating ß-glucan before accumulating fatty acids. In the best case, there would be a considerable amount of time between the starting points. That way, more energy is stored in the form of ß-glucan instead of fatty acids. *P. tricornutum* cultures started to accumulate ß-glucan prior to fatty acids used as energy storage (C16:0, C16:1). It has been previously described that *P. tricornutum* accumulates ß-glucan as energy storage prior to the accumulation of fatty acids (Gao et al. 2017). In contrast to *P. tricornutum*, *M. subterranea* started the accumulation of ß-glucan and fatty acids simultaneously. Unfortunately, there is no publication to date describing the response of *M. subterranea* to N-depletion in detail. However, Recht et al. (2012) examined the response of the related eustigmatophyte *Nannochloropsis* sp. to N-depletion. They reported that *Nannochloropsis* sp. showed a stable total carbohydrate content during nitrogen depletion. While they did not observe an accumulation of carbohydrates during nitrogen depletion, *Nannochloropsis* sp. accumulated fatty acids up to 500 mg g^{-1} (Recht et al. 2012). *Cylindrotheca fusiformis* accumulated fewer energy storage molecules in response to N-depletion compared to the other two species. Nevertheless, our results for *C. fusiformis* suggest that the ß-glucan accumulation started before the accumulation of fatty acids. For *C. fusiformis* there are also no published data describing its response in the progress of nutrient depletion in detail.

The three species showed differences regarding the ratio of ß-glucan and fatty acids used for energy storage (C16:0, C16:1, C18:1). There are microalgae species that prefer fatty acid as energy storage, such as *Chlorella vulgaris,* and other species favour glucans (starch or ß-glucans) like *Chlorella sorokiniana* (Mujtaba et al. 2012; Gifuni et al. 2018). For a ß-glucan production process, it would be beneficial if the production strain would favour ß-glucan over fatty acids as energy storage. In this way, more resources (energy and carbon) would be directed to the accumulation of ß-glucan. However, in our experiments, *C. fusiformis* accumulated more fatty acids than ß-glucan, which is in line with data from a previous publication which suggests that *C. fusiformis* favours the accumulation of fatty acids over ß-glucan (Schulze et al. 2016). In *P. tricornutum* cultures, the ß-glucan content was higher than the total fatty acid content in our experiments throughout the N-depletion period. This is in contrast to previous publications (Gao et al. 2017). However, the comparison with other publications is difficult due to differences in the culture conditions, for example light intensity, culture vessel and temperature. In addition, we reported in a previous publication, that the ratio between energy storage molecules differs between different strains of *P. tricornutum* (Frick et al. 2023). *Monodopsis subterranea* showed an equal distribution of energy storage molecules in our experiments, which shifted in favour of fatty acids over time. Although there are no published data on this ratio for *M. subterranea* so far, Recht et al. (2012) reported that the related eustigmatophyte *Nannochloropsis* sp. accumulated fatty acids rather than carbohydrates during N-depletion. They observed a carbohydrate content of around 200 mg g^{-1} and a fatty acids content of up to 500 mg g^{-1} after five days of N-depletion (Recht et al. 2012). This indicates to a species-specific adaption to unfavourable environmental conditions such as N-depletion and emphasises the careful selection of a production strain. However, compared to other publications, the total fatty acid content we found in *M. subterranea* was rather low (Khozin-Goldberg and Cohen 2006; Hu et al. 2019). This would influence the ratio of ß-glucan to fatty acids in favour of ß-glucan, which would be beneficial for a ß-glucan production process.

The three tested algae species started the formation of energy storage molecules at different times after ammonium was depleted from the medium. It would be advantageous for a ß-glucan production process if the production strain would start the accumulation of ß-glucan shortly after the ammonium in the medium was depleted. In this case, the depletion phase would be shorter. *Monodopsis subterranea* showed a delayed response to N-depletion regarding the formation of energy storage molecules. The ß-glucan content as well as the content of fatty acids used for energy storage (C:16:0, C16:1, C18:1) started to increase on the third day of N-depletion, whereas the ß-glucan content of *P. tricornutum* started to increase on the first day. Due to the overall small increase of ß-glucan in *C. fusiformis*, the beginning of the reaction in this species could not be clearly determined. There are also no published data on this topic so far. The delayed response of *M. subterranea* to N-depletion indicates that it either has a larger nitrogen storage pool or is able to redistribute its nitrogen resources. In addition to the delayed response, *M. subterranea* was capable of maintaining biomass formation for a longer period of time (16 days) without ammonium in the culture medium (see Table S2), compared to the other two species (see Fig. 1 and Table S3). This also indicates that *M. subterranea* is capable to redistribute its nitrogen resources to compensate for the lack of

nitrogen in the medium. Furthermore, *M. subterranea* produces additional ß-glucan far longer compared to the other two species tested.

Overall, our experiments indicate that *P. tricornutum* and *M. subterranea* are suitable for ß-glucan production. As described above, *P. tricornutum* accumulated more ß-glucan than fatty acids and started the accumulation of ß-glucan prior to the accumulation of fatty acids. Accumulation of ß-glucan also began shortly after nitrogen was depleted from the medium and *P. tricornutum* reached its maximal volumetric ß-glucan concentration earlier compared to *M. subterranea*, which might be beneficial regarding a possible ß-glucan production process due to a higher space–time yield. In addition, *P. tricornutum* showed the highest ß-glucan content (see Fig. 2), which is beneficial for possible downstream processing including extraction. Although biomass specific ß-glucan productivity $q_{ß\text{-glucan}}$ was also higher in *P. tricornutum* cultures compared to *M. subterranea* cultures in the beginning of N-depletion (see Figure S3), the maximal volumetric ß-glucan concentration was higher in *M. subterranea* cultures (see Fig. 2). Taking into account the extended N-depletion time during which *M. subterranea* produced additional ß-glucan, the difference in the maximal volumetric ß-glucan concentration becomes even greater (see Tables S1 and S3). After 16 days of N-depletion, *M. subterranea* showed nearly twice the volumetric ß-glucan concentration compared to *P. tricornutum*. Thus, even considering all the advantages of *P. tricornutum* listed above, *M. subterranea* seems to be equally well suited for ß-glucan production. When choosing between these two algae species for ß-glucan production, it might ultimately come down to *P. tricornutum* producing a soluble ß-glucan (chrysolaminarin) and *M. subterranea* producing an insoluble ß-glucan (paramylon). *C. fusiformis,* on the other hand, seems to be less suitable for a ß-glucan production process in the tested setup.

However, the results regarding the ß-glucan production only apply to the tested experimental setup. Cultivation conditions have a great impact on the composition of microalgae biomass. Therefore, light regime, culture density and depleted nutrients might have an impact on the accumulation of ß-glucan as well. Furthermore, it is reported that the cultivation system also has an impact on the performance of the algae (Derwenskus 2020). Flat panel airlift reactors have a good light distribution but impose other requirements on the algae, such as high sheer forces, which can affect the performance of the cultivated algae species (Bergmann 2018; Wang and Lan 2018).

Production of other valuable compounds

The focus of this paper was on the production of ß-glucans. However, to increase the economic feasibility of a potential production process of microalgae compounds, a cascaded extraction as proposed by Derwenskus et al. (2020b) can be beneficial. Therefore, other value-adding components such as FX and fatty acids (especially EPA) have to be considered. FX has been reported to be the major contributor to the biomass value of *P. tricornutum* due to its high price (Derwenskus et al. 2020b). In a cascaded extraction, the ß-glucan can be separated from the lipophilic compounds (FX, EPA, other fatty acids) by a separation step after cell disruption. To further increase its value, FX can be purified and separated from other lipophilic compounds like EPA. However, this may require an elaborate process such as the chromatography method described by Xiao et al. (2012).

Fucoxanthin

Of the three tested species, FX is only found in the two diatoms, *P. tricornutum* and *C. fusiformis*. The FX content decreased in both diatom species during N-depletion. This is consistent with previous publications (Gao et al. 2017). Alipanah et al. (2015) examined the response of *P. tricornutum* to nutrient depletion at the genetic level and reported that genes associated with FX formation were downregulated during N-depletion. However, although N-depletion is unfavourable for FX production, there is still FX remaining in the biomass to be extracted, even if a short depletion phase would be favourable (see Fig. 3).

EPA

The EPA content of *P. tricornutum* and *M. subterranea* increased at the beginning of the N-depletion phase (see Table 1). This is unusual, as EPA is not part of the energy storage pool in the cell. Moreover, other publications reported that EPA content decreased during N-depletion (Khozin-Goldberg and Cohen 2006; Hu et al. 2019). Furthermore, the EPA content of *M. subterranea* was low at the beginning of the experiments compared to previous publications (Hu et al. 2019). This indicates that the increase in EPA content in our experiments is rather a consequence of the low content at the beginning of the experiment and not due to N-depletion. Nevertheless, our results show that a rather high EPA content (over 45 $mg_{DW}\ g^{-1}$) can be achieved during N-depletion (see Table 1). This makes EPA a promising candidate for a cascaded extraction process, especially when using *M. subterranea*, as EPA content in *M. subterranea* cultures was still above 45 $mg_{DW}\ g^{-1}$ even after 16 days of N-depletion.

During N-depletion, all three tested microalgae species accumulated fatty acids for energy storage (C16:0, C16:1 and C18:1), which could also be extracted after N-depletion (see Table 1). For a cascaded extraction process, *M. subterranea* and *P. tricornutum* could be used. The decision

depends on the aim of the process. *M. subterranea* produced more EPA, whereas *P. tricornutum* additionally produces FX. The high EPA content of *M. subterranea* makes the biomass also interesting for various applications, such as aquafeed. Here, the insolubility of its ß-glucan would also be beneficial, as it would not dissolve in the water before being eaten. However, for this application, no extraction would be necessary, only a cell disruption to ensure bioavailability would be needed.

Conclusion

Of the tested algae species, *P. tricornutum* and *M. subterranea* are best suited for ß-glucan production. *Phaeodactylum tricornutum* showed the highest ß-glucan content and reached it in a shorter time compared to *M. subterranea*. *Monodopsis subterranea* showed a delayed response to N-depletion, but reached a higher maximal volumetric ß-glucan concentration compared to *P. tricornutum*. Furthermore, in contrast to *P. tricornutum*, *M. subterranea* continued to produce ß-glucan throughout the whole experiment and beyond. In comparison to the other two species tested, *C. fusiformis* showed a lower accumulation of energy storage molecules (ß-glucan and fatty acids) in response to N-depletion. For a production process, other valuable compounds should be considered besides ß-glucan. EPA content is still rather high during N-depletion, especially in *M. subterranea* and although FX content of *P. tricornutum* and *C. fusiformis* decreased during N-depletion, it could still be extracted in a cascaded extraction to increase value for these biomasses.

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Authors' contributions **KF**: Conceptualization, Methodology, Validation, Investigation, Data Curation, Writing—Original Draft, Visualization.

TE: Methodology, Visualization, Writing—Review & Editing.

USS: Conceptualization, Methodology, Resources, Writing—Review & Editing, Supervision, Project administration, Funding acquisition.

YCY: Formal analysis, Data Curation, Writing—Review & Editing.

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Data availability All data generated or analysed during this study are included in this published article. The data and materials used in this paper comply with field standards, all cultivations were conducted in biological triplicate.

Declarations

Conflicts of interest/Competing interests The authors have no competing interests.

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