


# Coproduction of EPA and Fucoxanthin with *P. tricornutum* – A Promising Approach for Up- and Downstream Processing

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Eicosapentaenoic acid (EPA) and fucoxanthin, a carotenoid, provide a broad variety of health benefits in human nutrition. In this study, an up- and downstream process for the coproduction of EPA and fucoxanthin using the diatom *Phaeodactylum tricornutum* in flat-panel airlift photobioreactors is proposed. The approach represents a promising alternative to conventional sources for both compounds, viz. marine fish and macroalgae. The productivity as well as the biomass-specific product content were optimized during cultivation. Subsequently, both compounds were extracted, separated and purified using pressurized liquids.

**Keywords:** Eicosapentaenoic acid, Carotenoids, Diatom, Light availability, Pressurized liquid extraction

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## 1 Introduction

*Phaeodactylum tricornutum* is a unicellular, marine pennate diatom, constituting a potential source for several high-value, bioactive compounds like polyunsaturated fatty acids (PUFA) as well as carotenoids [1]. For this reason, the biomass as well as fractions thereof are of increasing interest for the application in human nutrition and are widely used as a feedstuff, e.g., in aquacultures [2]. *P. tricornutum* has already been cultivated in various types of photobioreactors, e.g., open ponds, bubble columns and airlift reactors [3–5]. Most attention in the majority of studies concerning the cultivation of diatoms has been given on a high biomass productivity, although it is already described that diatoms have a remarkable capability to change their macromolecular composition as a response to different environmental conditions, e.g. by acclimatization [6–8]. Thus, cultivation conditions such as the light availability in the bioreactor, nutrient concentration in the culture media, as well as the design of the reactor system do not only influence the biomass productivity, but can also have a significant impact on the biomass-specific product content [9–11]. However, this fact has yet been more or less neglected even though the product content can play a crucial role concerning the economic feasibility of a production process.

Fucoxanthin is one of the most abundant carotenoids in diatoms [12, 13]. During photosynthesis, the oxygen-containing carotenoid (so called xanthophyll) acts as a light harvesting pigment, which is bound to fucoxanthin-chlorophyll a/c-proteins (FCP), located in the thylakoids of the

cells [14]. Fucoxanthin and chlorophyll c are responsible for the absorbance of blue and green light from the solar spectrum, which is of importance especially in aquatic environments [15]. Fucoxanthin has a unique structure compared to other carotenoids like beta-carotene due to its allenic bond, conjugated carbonyl group, 5,6-monoepoxide and carbonyl group. Because of its structure, fucoxanthin has anti-inflammatory, anti-carcinogenic, anti-obesity and anti-diabetic properties [12, 16–21]. Therefore, fucoxanthin is supposed to prevent and alleviate several chronic diseases. For an extensive review please see Peng et al. [12] or Zarekarizi et al. [24].

The total amount of fucoxanthin in diatoms was found to range from 6.2 to 59.2 mg g<sup>-1</sup> of total dry weight depending on the specific production strain and solvent used for

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extraction [11, 22–24]. Thus, the fucoxanthin content of microalgae can be up to 10 times higher compared to conventional sources for this carotenoid like macroalgae [25, 26]. However, the fucoxanthin levels of diatoms can change readily according to cultivation conditions and its origin [27, 28]. In particular diatoms like *P. tricornutum* are known to adapt their pigment content to light stress in order to avoid intracellular damage of the photosystem and to improve their capacity for light harvesting at light-limiting conditions [6, 7, 29].

Next to fucoxanthin, diatoms are known for a high content of eicosapentaenoic acid (EPA), a polyunsaturated fatty acid [30]. As EPA is part of galacto- and phospholipids constituting a major part of the thylakoid membrane, it can be assumed that the EPA content in the biomass is affected by environmental conditions, such as the available light, as well [31]. Nowadays, EPA is mainly derived from fish oil, which typically contains a mixture of EPA and docosahexaenoic acid (DHA). In contrast, some diatoms like *P. tricornutum* contain almost exclusively EPA, thus, providing this specific PUFA in a preferential pure form [3]. Both, EPA and DHA have anti-inflammatory effects and can provide health-beneficial effects, e.g., on the human blood cholesterol level [32]. Biomass-specific EPA contents (dry weight) of 3–5 wt % are reported for *P. tricornutum*, covering around one-third of the total fatty acids [3, 10, 30].

For a future production process, not only the production of EPA- and fucoxanthin-rich biomass is crucial, but also suitable downstream processes have to be evaluated, e.g., for the solid-liquid extraction of both compounds. Several extraction methods, like supercritical fluid extraction (SFE) and pressurized solvent extraction (PLE), are described to extract lipids like carotenoids and fatty acids from green microalgae [33–35]. Nevertheless, these extraction methods cannot be easily transferred to diatoms due to the presence of species-specific lipid classes and differences in the respective cell wall composition. Therefore, existing extraction methods as well as cell disruption procedures, which are mandatory for an efficient extraction, must be adapted to diatoms. So far, no comprehensive up- and downstream process is described for the coproduction of EPA and fucoxanthin based on diatoms yet. However, it is worth mentioning that recent publications show the possibility to influence the product content as well as the volumetric productivity of diatoms by adjusting specific operating parameters during cultivation. Gao et al. [36] and Wang et al. [37] reported an effect of the nitrogen level, the light intensity and the salinity on the specific product content and volumetric productivity of EPA and fucoxanthin using *P. tricornutum*. An approach to extract both compounds as a lipid fraction with “green” organic solvents from *P. tricornutum* was demonstrated by Delbrut et al. [38]. In our study, both the cultivation as well as the subcritical pressurized liquid extraction (PLE) from disrupted biomass of *P. tricornutum* were optimized and an overall approach for the coproduction of both compounds is given. PLE constitutes a very promising

emerging technology for the extraction of lipids from microalgae [39]. For PLE, a broad variety of organic solvents providing different polarities can be used for extraction [35, 40]. Recent publications indicate the high potential of this extraction method, especially for the extraction of polar lipids like fucoxanthin and EPA [30].

## 2 Materials and Methods

### 2.1 Organism, Precultures and Small-Scale Experiments

*P. tricornutum* UTEX 640, UTEX 642 and UTEX 646, also known as SAG 1090-1b, SAG 1090-1a and SAG 1090-6, were obtained from the Culture Collection of Algae at Göttingen University (SAG). The screening experiments in order to evaluate a suitable production strain were performed in a screening photobioreactor platform (HD10, Cell-DEG, Germany) using 10-mL incubators on an orbital shaking device at 250 rpm and 20 °C. The photobioreactor platform was described previously [41]. A constant photon flux density of  $10 \mu\text{mol m}^{-2}\text{s}^{-1}$  was supplied to the cultures. Starting at a cell density of  $OD_{750} = 1$ , a HRTT sensor (HRTTS, Cell-DEG, Germany) for a noninvasive direct cell density measurement was used to determine the specific growth rate for a cultivation time of four days.

Preparatory cultures of *P. tricornutum* UTEX 640 were cultivated in 250-mL Erlenmeyer flasks (100 mL culture volume) on an illuminated shaking device at  $60 \mu\text{mol m}^{-2}\text{s}^{-1}$  using modified Mann and Myers medium (described below). They were transferred to 5-L borosilicate bottles (Schott, Germany) when exceeding  $OD_{750} = 4$ . The bottles were equipped with a magnetic stir bar, additional carbon dioxide supply (2 vol %) and were mixed by a magnetic agitator. The cultures were diluted with culture medium once a week, up to a final volume of 4 L and  $OD_{750} = 3$ . These precultures served as an inoculum for experiments in the flat-panel airlift photobioreactor (FPA-PBR).

### 2.2 Culture Media and Substrates

A modified Mann and Myers medium was used as culture medium [42]. The culture medium is already described in Meiser et al. [3]. Additionally,  $20 \text{ mL L}^{-1}$  of a sterile trace element solution consisting of  $0.6 \text{ g L}^{-1}$  boric acid,  $0.22 \text{ g L}^{-1}$  iron(III) citrate,  $0.22 \text{ g L}^{-1}$  manganese(II) chloride  $\cdot 4 \text{ H}_2\text{O}$ ,  $33 \text{ mg L}^{-1}$  zinc sulfate  $\cdot 7 \text{ H}_2\text{O}$ ,  $0.7 \text{ mg L}^{-1}$  cobalt(II) nitrate  $\cdot 6 \text{ H}_2\text{O}$ ,  $0.2 \text{ mg L}^{-1}$  copper sulfate  $\cdot 5 \text{ H}_2\text{O}$ , and  $25 \text{ mg L}^{-1}$  sodium molybdate was added after autoclaving. Ammonium and phosphate were added separately depending on the experimental setup. For all precultures a concentration of  $90 \text{ mg L}^{-1}$  ammonium and  $60 \text{ mg L}^{-1}$  phosphate was added to the culture medium prior to inoculation. Ammonium bicarbonate and potassium dihydrogen phosphate were used to prepare the corresponding ammonium and phosphate stock solutions.

### 2.3 Cultivation of *P. tricornutum* in Flat-Panel Airlift Reactors

Experiments were conducted in FPA-PBR as previously described in detail by Bergmann et al. using artificial illumination [43, 44]. The FPA-PBR (see Fig. 1) was developed by the Fraunhofer Institute for Interfacial Engineering and Biotechnology IGB, Stuttgart, Germany, and is currently distributed by Subitec GmbH, Stuttgart, Germany [45]. Originally, it has been optimized for the outdoor cultivation of microalgae and provides a high degree of automation [46]. In comparison to conventional flat-panel bioreactors, the FPA-PBR is equipped with static mixers providing an optimized light distribution affecting all microalgae cells due to a controlled, circular movement of the cells from the unlit to the illuminated surface of the reactor [47]. Thus, photolimitation and photoinhibition are strongly reduced compared to other PBRs.



**Figure 1.** Flat-panel airlift photobioreactor (30 L) with artificial illumination and SPS control unit used for the cultivation of *P. tricornutum* at Fraunhofer IGB, Stuttgart.

For cultivation, a repeated fed-batch process was used with an initial biomass concentration of  $6 \text{ g L}^{-1}$ . Every 24 h, the culture was diluted to the initial biomass concentration according to the daily increase in biomass. For all experiments nitrogen- and phosphate was supplied to the cultures daily, ensuring no limiting effects on microalgae growth. A concentration for ammonia in between  $70\text{--}150 \text{ mg L}^{-1}$  and a phosphate concentration of  $60\text{--}120 \text{ mg L}^{-1}$  was kept constant during all experiments. Depending on the experiment, a constant biomass-specific light availability of 2, 5 or  $8 \mu\text{mol}_{(\text{photons})} \text{ g}^{-1}_{(\text{dry weight})} \text{ s}^{-1}$  within the PAR region (photosynthetic active radiation) was supplied according to equation (1).

$$I_{\text{spec}} = \frac{\text{PFD } A}{V c_{\text{d.w.}}} \quad (1)$$

with  $I_{\text{spec}}$  = specific light availability [ $\mu\text{mol}_{(\text{photons})} \text{ g}^{-1}_{(\text{dry weight})} \text{ s}^{-1}$ ], PFD = photon flux density on the reactor surface,  $A$  = illuminated reactor surface,  $V$  = reactor volume,  $c_{\text{d.w.}}$  = biomass concentration (dry weight).

The cultivation temperature was kept constant at  $20 \pm 1 \text{ }^\circ\text{C}$  and the pH value at  $7.3 \pm 0.1$ . Shifts in the pH value caused by daily nutrient supply were compensated by controlling the  $\text{CO}_2/\text{air}$  ratio within the air supply of 0.3 vvm. Cells were harvested during the cultivation process every day. Samples were washed twice with double-distilled water by centrifugation (4000 rpm, 5 min) to remove salts within the culture media. The cells were frozen immediately and subsequently freeze-dried. They were stored in the dark at  $-20 \text{ }^\circ\text{C}$  until further analysis. For experiments concerning downstream processing, larger amounts of *P. tricornutum* UTEX 640 biomass were obtained from the pilot-scale plant at Fraunhofer Center for Chemical-Biotechnological Processes CBP in Leuna. The biomass was produced in 180-L FPA-PBR using very similar cultivation conditions as described before [30].

### 2.4 Determination of Cell Dry Weight and Substrate Concentrations

Cell dry weight was measured every 24 h during cultivation. Therefore, 5 mL of the culture was filtered (MN 85/70, Macherey-Nagel, Germany) and washed twice with 5 mL  $\text{H}_2\text{O}$ . The filter paper was dried at  $105 \text{ }^\circ\text{C}$  (MA 30, Sartorius, Germany) and weighted (A7261 Delta Range, Mettler, Germany). During cultivation, ammonium and phosphate were controlled daily either with test stripes (Quantofix, Macherey-Nagel, Germany) using an automated reader (Quantofix Relax, Macherey-Nagel, Germany) or quantified with colorimetric cuvette tests (type LCK049 and LCK339, Hach-Lange, Germany) using a tabletop spectrophotometer (Lasa 100, Hach-Lange, Germany).

### 2.5 Cell Disruption, Lipid Extraction and Fractionation

Prior to the subcritical extraction of an EPA- and fucoxanthin-rich lipid fraction, *P. tricornutum* biomass was disrupted in a stirred ball mill as described in [30]. The disrupted biomass was extracted with ethanol, acetone and ethyl acetate at extraction temperatures between  $25\text{--}200 \text{ }^\circ\text{C}$  at a constant pressure of 100 bar using an Accelerated Solvent Extractor (ASE 350, Thermofisher, USA). Extraction cells with a volume of 5 mL were used for the extraction of 1 g of disrupted biomass. The static extraction time was 20 min. The method used for separation is described in [19] (Fig. 2).



**Figure 2.** EPA- and fucoxanthin-rich lipid fraction (right) extracted by PLE and purified fucoxanthin powder (>90 wt %) (left).

## 2.6 Determination of Fatty Acid Profile and Fatty Acid Content

The fatty acid content of the *P. tricornutum* biomass (reference) as well as the fatty acid content of the extracted lipid fractions were analyzed according to the method of Lepage and Roy [48] with slight modifications as described in [3]. Prior to GC analysis, the extracts were diluted with methanol/acetyl chloride (20:1 v/v) for transesterification. Fatty acid methyl esters (FAME) were analyzed using a Supelco SBP-PUFA 30 m×0.32 mm×0.2 μm column (24314, Sigma-Aldrich, USA) in a gas chromatograph (7890A, Agilent, USA) equipped with an FID detector. Results were compared to a certificated C<sub>4</sub>–C<sub>24</sub> FAME mix (Supelco-18919-1AMP, Sigma-Aldrich, USA).

## 2.7 Determination of Fucoxanthin by HPLC

The carotenoid and chlorophyll content was determined according to the method previously described in [49] with slight changes. In brief, samples were analyzed with reversed-phase HPLC using a Suplex pKb 100 (5 μm, 250×4.6 mm) column (58934, Supelco, USA) and a Waters photodiode array detector. For separation of carotenoids a binary gradient with a flow rate of 1 mL min<sup>-1</sup> was applied. The samples were diluted five times and injected with a volume of 20 μL. The mobile phase A consisted of methanol/acetonitrile/2-propanol (54/44/2, v/v/v) and mobile phase B of mobile phase A/water (85/15, v/v). The gradient cycle started with a segment of 40 % A, followed by increasing A to 80 % at 10 min and to 100 % from 20 min to 28 min. From 29 to 36 min the gradient was changed to 40 % A and 60 % B. Fucoxanthin was detected at 450 nm at a retention time of 4.96 min. The concentration was calculated from calibration curves prepared with an analytical standard (16337, Sigma-Aldrich, USA).

## 2.8 Statistical Analysis and Illustrations

The software OriginPro 2019b (OriginLab Corporation, USA) was used for statistical analysis and to generate the corresponding diagrams. All data sets were evaluated using one-way ANOVA. Two sets of data were compared using the t-test ( $p \leq 0.05$ ). Small letters in the figures (see box plots in Fig. 3 and 4) describe significant differences between different sets of data.

## 3 Results and Discussion

### 3.1 Comparison of Different *P. tricornutum* Strains

Nowadays, a lack of profound knowledge concerning suitable microalgae production strains as well as a dearth of empiric data concerning the dependency of the EPA- and fucoxanthin content in the biomass from the culture conditions substantially hinders the production of both compounds in industrial photobioreactors. Nevertheless, current literature data show, that especially different strains of *P. tricornutum* can provide high EPA and fucoxanthin levels [24, 50]. Furthermore, *P. tricornutum* is in particular suitable for cultivation in closed photobioreactors [3]. To evaluate a promising production strain, the specific growth rate as well as the EPA and fucoxanthin content of three different *P. tricornutum* strains were compared in a small-scale photobioreactor platform (see Tab. 1). In these small-scale conditions, the strain *P. tricornutum* UTEX 646 provided the highest growth rate of  $0.31 \pm 0.1$  d<sup>-1</sup>. The biomass-specific fucoxanthin content of *P. tricornutum* UTEX 642 and UTEX 646 corresponds with data published previously [51, 52]. However, the results show that the fucoxanthin content of *P. tricornutum* UTEX 640 ( $19.1 \pm 1.0$  mg g<sup>-1</sup>) can be substantially higher than reported for this specific strain previously [53]. The EPA content of all three strains was in accordance to literature and no significant differences were observed [3, 54, 55].

### 3.2 Effect of Light Availability on the Fucoxanthin and EPA Content

As previously reported, the fucoxanthin content of *P. tricornutum* cells can change according to the light availability during cultivation [6, 7]. Nevertheless, the effect caused by photoadaptation mechanisms on the biomass-specific product content is not sufficiently quantified for diatoms in an industrial photobioreactor yet. Fig. 3 shows the volumetric fucoxanthin productivity and the biomass-specific fucoxanthin content of *P. tricornutum* UTEX 640 as a function of the specific light availability in a repeated fed-batch process using an FPA-PBR.

Due to a higher overall biomass productivity at high light settings ( $5$  and  $8 \mu\text{mol}_{(\text{photons})}\text{g}^{-1}\text{s}^{-1}$ ) compared to low light

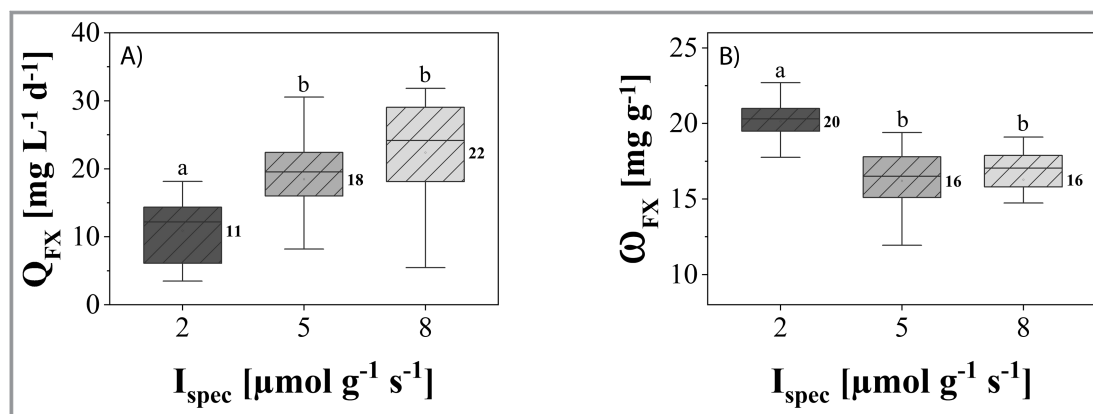
**Table 1.** Specific growth rate, biomass-specific fucoxanthin and EPA content of different *P. tricornutum* strains investigated in this study. Strains were cultivated in a Cell-DEG membrane photobioreactor at  $10 \mu\text{mol m}^{-2} \text{s}^{-1}$  starting from  $OD_{750} = 1$  as described before.

Strain	Growth rate $\mu$ [ $\text{d}^{-1}$ ]	Fucoxanthin content [ $\text{mg g}^{-1}$ (dry weight)]	EPA content [ $\text{mg g}^{-1}$ (dry weight)]
<i>P. tricornutum</i> UTEX 640	$0.25 \pm 0.1$	$19.1 \pm 1.0$	$42.8 \pm 0.7$
<i>P. tricornutum</i> UTEX 642	$0.26 \pm 0.1$	$10.3 \pm 2.3$	$42.5 \pm 3.9$
<i>P. tricornutum</i> UTEX 646	$0.31 \pm 0.1$	$11.0 \pm 1.4$	$44.5 \pm 0.23$

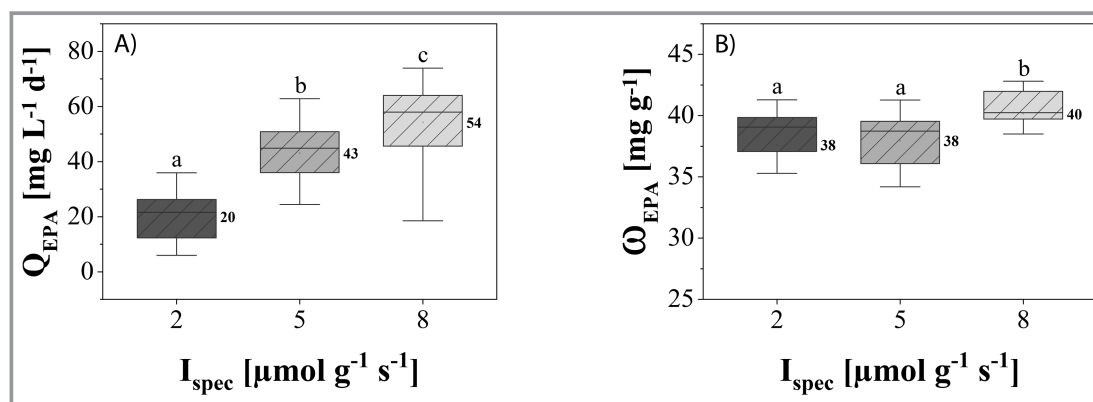
settings ( $2 \mu\text{mol}_{(\text{photons})} \text{g}^{-1} \text{s}^{-1}$ ) the volumetric fucoxanthin productivity increases. To the knowledge of the authors  $22.4 \pm 7.6 \text{ mg L}^{-1} \text{d}^{-1}$  is the highest volumetric productivity reported for the production of fucoxanthin with microalgae yet. Nevertheless, the results reveal that an increased biomass-specific product content (increase of around 20 %) is

achieved at low light settings. These findings correlate with previous observations made for *P. tricornutum* on a molecular level [7] and are in line with data published for the cultivation of diatoms at reduced light intensity previously [11, 37, 56]. Our data show for the first time that the phenomenon of light adaption is valid for this particular *P. tricornutum* strain (UTEX 640) and thus, it can be used for the production of fucoxanthin-rich biomass with a biomass-specific fucoxanthin content of up to  $20.1 \pm 1.6 \text{ mg g}^{-1}$ . An increased fucoxanthin content can be beneficial concerning downstream processing, since a smaller amount of biomass has to be disrupted and extracted subsequently.

However, unlike fucoxanthin, the maximum biomass-specific EPA content of  $40.2 \pm 2.2 \text{ mg g}^{-1}$  as well as the maximum volumetric productivity of  $54.2 \pm 15.3 \text{ mg L d}^{-1}$  were observed at high light conditions (see Fig. 4). Previous studies concerning the relation of EPA and available light do not supply clear evidence if either a low or a high light availability



**Figure 3.** Volumetric fucoxanthin productivity  $Q_{\text{FX}}$  (A) and biomass-specific fucoxanthin content  $\omega_{\text{FX}}$  (B) of *P. tricornutum* UTEX 640 at a specific light availability of 2, 5 and  $8 \mu\text{mol}_{(\text{photons})} \text{g}^{-1} \text{s}^{-1}$ . Box plots represent  $n \geq 15$  samples collected from  $n = 3$  cultivations. Different small letters indicate significant differences ( $p \leq 0.05$ ).



**Figure 4.** Volumetric EPA productivity  $Q_{\text{EPA}}$  (A) and biomass-specific EPA content  $\omega_{\text{EPA}}$  (B) of *P. tricornutum* UTEX 640 at a specific light availability of 2, 5 and  $8 \mu\text{mol}_{(\text{photons})} \text{g}^{-1} \text{s}^{-1}$ . Box plots represent  $n \geq 15$  samples taken from  $n = 3$  cultivations. Different small letters indicate significant differences ( $p \leq 0.05$ ).

is favorable in terms of a high EPA content. For instance, Qiao et al. did not observe a significant effect of different photon flux densities on the portion of EPA in the fatty acid profile of *P. tricornutum* [10]. In contrast, Liang et al. reported a decrease of EPA at an increased biomass concentration during batch cultivation and thus, a reduced specific light availability [57].

For EPA as well as fucoxanthin we determined that the conversion of light into product significantly increases at a low specific light availability. For example, in case of fucoxanthin a light yield of  $12.6 \pm 2.9 \text{ mg}_{(\text{fucoxanthin})} \text{mol}^{-1}_{(\text{photons})}$  at a light availability of  $2 \mu\text{mol}_{(\text{photons})} \text{g}^{-1} \text{s}^{-1}$  was obtained compared to  $7.5 \pm 2.7 \text{ mg}_{(\text{fucoxanthin})} \text{mol}^{-1}_{(\text{photons})}$  using  $8 \mu\text{mol}_{(\text{photons})} \text{g}^{-1} \text{s}^{-1}$ . This can be an important aspect concerning the economic feasibility of a production process, since energy input for light represents a main cost driver when using artificial illumination for cultivation [58].

### 3.3 Downstream Processing

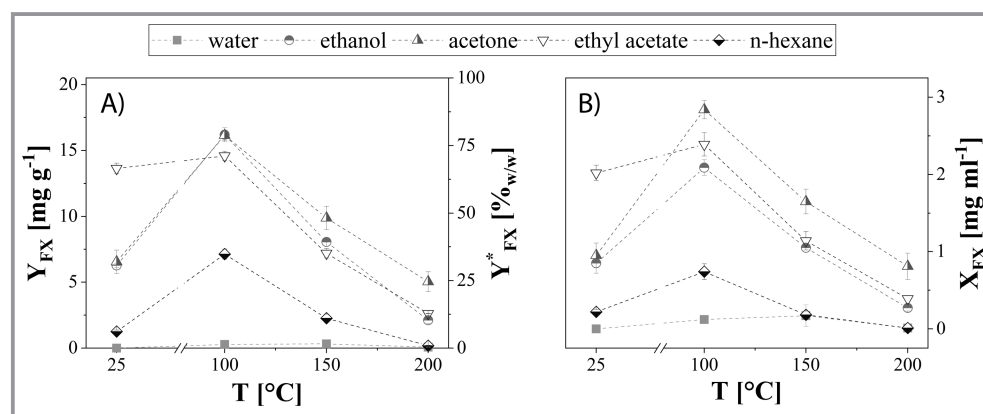
For the subcritical pressurized solid-liquid extraction of fucoxanthin from freeze-dried biomass the highest yields were observed with polar solvents like ethanol, ethyl acetate and acetone (see Fig. 5A). This corroborates the results published by Kim et al. operating at comparable extraction conditions [22]. The highest fucoxanthin yield of  $16.2 \pm 0.5 \text{ mg g}^{-1}$  was observed with ethanol at  $100^\circ\text{C}$  using a single extraction step. This extraction temperature is lower than extraction temperatures previously evaluated for the extraction of fucoxanthin by PLE from macroalgae [59].

Fig. 5A furthermore shows that extraction temperatures above  $100^\circ\text{C}$  significantly decreased the yield. Even though only little is known about the stability of fucoxanthin at high temperatures, apparently degradation of the molecule might be the reason for the decreasing yield [60]. Thus, the extraction temperature represents a trade-off criterion between a high extraction capability and the avoidance of fucoxanthin degradation. Further investigations revealed that the temperature optimum for PLE is around  $90^\circ\text{C}$  assuming an extraction time of 20 min (data not shown). In general the results

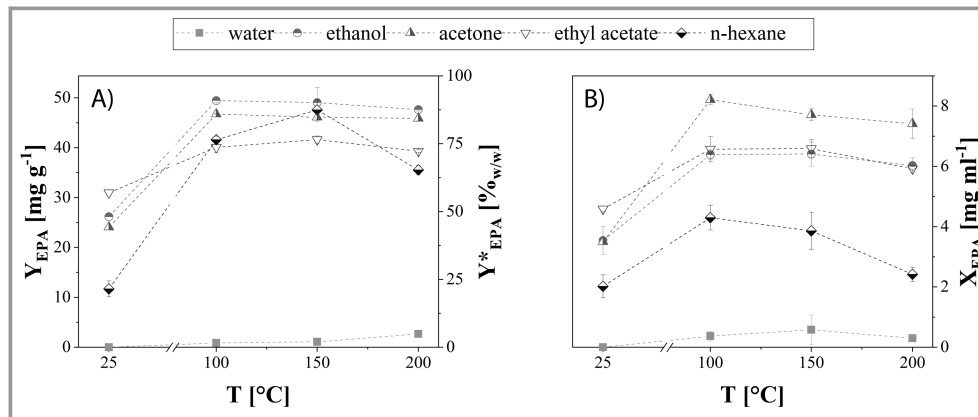
indicate that the use of subcritical liquids greatly reduced the amount of organic solvents compared to conventional extraction procedures like maceration [38]. In case of ethanol and ethyl acetate, for example,  $2.1 \pm 0.1 \text{ mg}_{(\text{fucoxanthin})} \text{mL}^{-1}_{(\text{ethanol})}$  and  $2.4 \pm 0.2 \text{ mg}_{(\text{fucoxanthin})} \text{mL}^{-1}_{(\text{ethyl acetate})}$  were extracted using PLE (see Fig. 5B). Thus, neglecting a reduced extraction efficiency using less extraction solvent, a solvent/biomass ratio of around 1:8 is hypothetically enough to completely solve the entire fucoxanthin present in the biomass during PLE. For maceration, solvent/biomass ratios of 1:10 were proposed for the sequential extraction of *P. tricornutum* in larger scales [38]. However, in contrast to a single extraction with PLE as described here, this suggestion refers to two solid-liquid extraction steps with an extraction time of at least 1 h.

Fig. 6 shows that the pressurized solid-liquid extraction of EPA using the same extraction conditions as for fucoxanthin is possible with both, polar solvents like ethanol as well as unpolar solvents such as *n*-hexane. However, the highest EPA-extraction yield of  $49.5 \pm 0.4 \text{ mg g}^{-1}$  was achieved at  $100^\circ\text{C}$  using ethanol, corresponding to an EPA extraction yield of around 90 wt%. Nevertheless, it is noteworthy that also *n*-hexane extracted a considerable amount of EPA. Considering the general principle „similia similibus solvuntur“ this is quite surprising since EPA is an integral part of the glyco- as well as phospholipids in the thylakoid membrane of *P. tricornutum* and both lipid classes are generally classified as polar [31]. This leads to the assumption that the extraction at temperatures of  $\geq 100^\circ\text{C}$  might cause a release of free fatty acids, which are subsequently being dissolved.

Summing up, PLE enables the co-extraction of an EPA- and fucoxanthin-rich lipid fraction. Using solvents like ethanol, both compounds can be extracted almost completely in a single extraction step with a contact time of 20 min. The ethanolic fraction ( $100^\circ\text{C}$ ) contains around 90 wt% of fucoxanthin and EPA present in the biomass, respectively. Based on those lipid fractions, both compounds can be separated and purified. For this purpose, methods described in literature are mainly based on multiple liquid-liquid extraction and silica gel chromatography [22, 61, 62] or, depending on the solvent used for extraction,



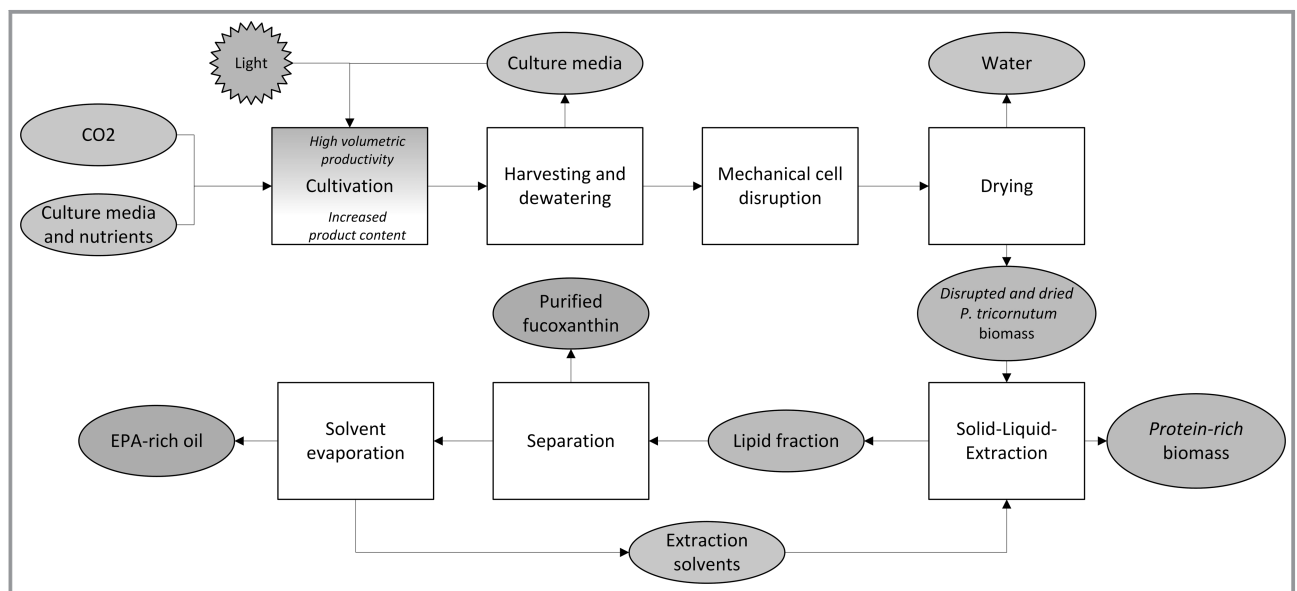
**Figure 5.** Extraction yield  $Y_{\text{FX}}$  ( $Y_{\text{FX}}^*$  compared to reference method) (A) and solvent loading of fucoxanthin  $X_{\text{FX}}$  (B) using different solvents and temperatures for PLE ( $p = 100 \text{ bar}$ ,  $t = 20 \text{ min}$ ). Error bars indicate the mean standard deviation ( $n = 3$ ).



**Figure 6.** Extraction yield  $Y_{EPA}$  ( $Y^*_{EPA}$  compared to reference method) (A) and solvent loading of EPA  $X_{EPA}$  (B) using different solvents and temperatures for PLE ( $p = 100$  bar,  $t = 20$  min). Error bars indicate the mean standard deviation ( $n = 3$ ).

on the separation of fucoxanthin by precipitation and filtration [19]. Here, it was possible to separate the fucoxanthin from the EPA fraction by precipitation and filtration as a powder (see also Fig. 2) with a purity of about 90 wt % (data not shown). Nevertheless, for a scale-up of PLE from lab to industrial scale a general technical problem arises since a bigger volume of organic solvent has to be heated to the final extraction temperature. In lab scale, the preheating of the solvent takes place in the extraction chamber in presence of the extraction material while heat energy is supplied through the reactor jacket [63]. The investigations revealed that this approach is not reasonable in larger scales since especially fucoxanthin is very sensitive to heat and is exposed to high temperatures for a too long period of time during the heating process. To overcome this problem, it is recommended to preheat the solvent in an additional heating chamber connected to the extraction chamber before adding the preheated solvent to the biomass. Thus, the residence time of the biomass can be reduced.

In consideration of all results, a process approach for the coproduction of fucoxanthin and EPA is proposed as shown in Fig. 7. Depending on the specific light availability used for the cultivation of *P. tricornutum* in the FPA-PBR, either a higher volumetric productivity or a higher biomass-specific fucoxanthin content can be achieved. A high product content can be beneficial since less biomass has to be processed in subsequent downstream processing. On the other hand, a high volumetric productivity increases the overall output of a cultivation plant. Anyway, after a mechanical cell disruption step, which proved to be mandatory in order to efficiently extract both compounds, fucoxanthin and EPA can be extracted from dry *P. tricornutum* biomass almost completely using subcritical solvents, e.g., ethanol. It is worth mentioning, that the residual biomass after extraction of the lipids is rich in proteins. Even though the proteins might be degraded due to the high extraction temperatures, the residual biomass can still serve as an amino acid source for, e.g., animal feed. EPA and fucoxanthin contained



**Figure 7.** An approach for the up- and downstream processing of EPA and fucoxanthin using *P. tricornutum*.

within the lipid fraction can be separated either by a combination of multiple liquid-liquid extractions and silica gel chromatography or by precipitation and filtration [19,61]. Finally, two fractions, an EPA-rich oil as well as purified fucoxanthin can be produced within this biorefinery approach. In the near future, techno-economic assessments are necessary to reveal, if the suggested process can compete economically to the production of both compounds based on conventional sources, i.e., marine fish oil (EPA) and macroalgae (fucoxanthin). This scientific issue is currently addressed in related research projects at Fraunhofer IGB, Stuttgart, and information will be available soon.

## 4 Summary and Conclusion

The diatom *P. tricornutum* enables the coproduction of fucoxanthin and EPA in high quantities. It is very suitable for flat-panel airlift photobioreactors and can provide a high volumetric productivity and a high biomass-specific product content. During photoautotrophic cultivation, photoadaptation mechanisms can be used to shift the product content of the biomass and increase the fucoxanthin content. Nevertheless, a high biomass productivity and thus a high product productivity generally requires a high specific light availability. This is an economic issue since artificial illumination is a major cost driver for microalgae production processes.

The results show that it is possible to separate both compounds from the biomass by pressurized solid-liquid extraction using organic solvents, which can be used for food applications according to current legislations [64]. Compared to conventional extraction methods like maceration, PLE proved to be less solvent consuming and a yield of about 90 wt % for both compounds can be achieved within a single extraction step. However, fucoxanthin showed to be unstable at high extraction temperatures. After extraction both compounds can be separated from the corresponding lipid extracts into different fractions making them available for different fields of applications, e.g., food or cosmetic products.

Summing up, a comprehensive up- and downstream process is available to produce both compounds as separate fractions, but techno-economic assessments have to supply evidence that this process can compete with traditional methods relying on marine fish and macroalgae. In any case, the concept of using light availability to optimize productivity and product content enables the tailored production of algae biomass. Additionally, microalgae are cultivated in a controlled environment. This is an advantage compared to macroalgae, since there is always a risk that the biomass is charged with toxins. Furthermore, when using energy from renewable sources, it can be assumed that a process based on microalgae is more sustainable, since it can avoid the costly production of fish and supersedes the manual harvesting of macroalgae from the oceans. However, this assumption can only be confirmed by an

extensive life cycle assessment, which is already an essential part of ongoing research projects.

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## Symbols used

$I_{\text{spec}}$	$[\mu\text{mol g}^{-1}\text{s}^{-1}]$	specific light availability
$Q_{\text{EPA}}$	$[\text{mg L}^{-1}\text{d}^{-1}]$	volumetric EPA productivity
$Q_{\text{FX}}$	$[\text{mg L}^{-1}\text{d}^{-1}]$	volumetric fucoxanthin productivity
$X_{\text{EPA}}$	$[\text{mg mL}^{-1}]$	solvent loading for EPA
$X_{\text{FX}}$	$[\text{mg mL}^{-1}]$	solvent loading for fucoxanthin
$Y_{\text{EPA}}^*$	[%]	yield of EPA compared to reference method
$Y_{\text{FX}}^*$	[%]	yield of fucoxanthin compared to reference method
$Y_{\text{EPA}}$	$[\text{mg g}^{-1}]$	yield of EPA
$Y_{\text{FX}}$	$[\text{mg g}^{-1}]$	yield of fucoxanthin
$\omega_{\text{EPA}}$	$[\text{mg g}^{-1}]$	biomass-specific EPA content
$\omega_{\text{FX}}$	$[\text{mg g}^{-1}]$	biomass-specific fucoxanthin content

## Abbreviations

DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
FPA-PBR	flat-panel airlift photobioreactor
Fraunhofer CBP	Fraunhofer Center for Chemical-Biotechnological Processes
Fraunhofer IGB	Fraunhofer Institute for Interfacial Engineering and Biotechnology
PAR	photosynthetic active radiation
PLE	pressurized liquid extraction
PUFA	poly-unsaturated fatty acid
SAG	Culture Collection of Algae at Göttingen University
UTEX	University of Texas



## References

- [1] *Microalgae Biotechnology* (Eds: C. Posten, S. Feng Chen), Vol. 153, Springer International Publishing, Cham **2016**.
- [2] M. Sørensen, G. M. Berge, K. I. Reitan, B. Ruyter, *Aquaculture* **2016**, *460*, 116–123. DOI: <https://doi.org/10.1016/j.aquaculture.2016.04.010>
- [3] A. Meiser, U. Schmid-Staiger, W. Trösch, *J. Appl. Phycol.* **2004**, *16* (3), 215–225. DOI: <https://doi.org/10.1023/B:JAPH.0000048507.95878.b5>
- [4] A. S. Mirón, M. C. C. García, A. C. Gómez, F. G. Camacho, E. M. Grima, Y. Chisti, *Biochem. Eng. J.* **2003**, *16* (3), 287–297. DOI: [https://doi.org/10.1016/S1369-703X\(03\)00072-X](https://doi.org/10.1016/S1369-703X(03)00072-X)
- [5] A. M. Silva Benavides, G. Torzillo, J. Kopecký, J. Masojídek, *Biomass Bioenergy* **2013**, *54*, 115–122. DOI: <https://doi.org/10.1016/j.biombioe.2013.03.016>
- [6] M. Janssen, L. Bahtke, J. Marquardt, W. Krumbein, E. Rhiel, *Int. Microbiol.* **2000**, *4* (1), 27–33.
- [7] M. Nymark, K. C. Valle, T. Brembu, K. Hancke, P. Winge, K. Andresen, G. Johnsen, A. M. Bones, *PLoS one* **2009**, *4* (11), e7743. DOI: <https://doi.org/10.1371/journal.pone.0007743>
- [8] M. Herbstová, D. Bina, P. Konik, Z. Gardian, F. Vácha, R. Litvin, *Biochim. Biophys. Acta, Bioenerg.* **2015**, *1847* (6–7), 534–543. DOI: <https://doi.org/10.1016/j.bbabi.2015.02.016>
- [9] I. Orefice, R. Chandrasekaran, A. Smerilli, F. Corato, T. Caruso, A. Casillo, M. M. Corsaro, F. D. Piaz, A. V. Ruban, C. Brunet, *Algal Res.* **2016**, *17*, 1–13. DOI: <https://doi.org/10.1016/j.algal.2016.04.013>
- [10] H. Qiao, C. Cong, C. Sun, B. Li, J. Wang, L. Zhang, *Aquaculture* **2016**, *452*, 311–317. DOI: <https://doi.org/10.1016/j.aquaculture.2015.11.011>
- [11] D. D. McClure, A. Luiz, B. Gerber, G. W. Barton, J. M. Kavanagh, *Algal Res.* **2018**, *29*, 41–48. DOI: <https://doi.org/10.1016/j.algal.2017.11.015>
- [12] J. Peng, J.-P. Yuan, C.-F. Wu, J.-H. Wang, *Mar. Drugs* **2011**, *9* (10), 1806–1828. DOI: <https://doi.org/10.3390/md9101806>
- [13] K. Miyashita, S. Nishikawa, F. Beppu, T. Tsukui, M. Abe, M. Hosokawa, *J. Sci. Food Agric.* **2011**, *91* (7), 1166–1174. DOI: <https://doi.org/10.1002/jsfa.4353>
- [14] K. Gundermann, M. Schmidt, W. Weisheit, M. Mittag, C. Büchel, *Biochim. Biophys. Acta, Bioenerg.* **2013**, *1827* (3), 303–310. DOI: <https://doi.org/10.1016/j.bbabi.2012.10.017>
- [15] V. Giovagnetti, A. V. Ruban, *Biochim. Biophys. Acta, Bioenerg.* **2017**, *1858* (3), 218–230. DOI: <https://doi.org/10.1016/j.bbabi.2016.12.005>
- [16] M. Abidov, Z. Ramazanov, R. Seifulla, S. Grachev, *Diabetes Obes. Metab.* **2010**, *12* (1), 72–81.
- [17] A. Gille, B. Stojnic, F. Derwenskus, A. Trautmann, U. Schmid-Staiger, C. Posten, K. Briviba, A. Palou, M. Bonet, J. Ribot, *Nutrients* **2019**, *11* (4), 796. DOI: <https://doi.org/10.3390/nu11040796>
- [18] E. Kotake-Nara, A. Asai, A. Nagao, *Cancer Lett.* **2005**, *220* (1), 75–84. DOI: <https://doi.org/10.1016/j.canlet.2004.07.048>
- [19] U. Neumann, F. Derwenskus, V. Flaiz Flister, U. Schmid-Staiger, T. Hirth, S. Bischoff, *Antioxidants* **2019**, *8* (6), 183. DOI: <https://doi.org/10.3390/antiox8060183>
- [20] U. Neumann, S. Louis, A. Gille, F. Derwenskus, U. Schmid-Staiger, K. Briviba, S. C. Bischoff, *J. Appl. Phycol.* **2018**, *30* (5), 2837–2846. DOI: <https://doi.org/10.1007/s10811-017-1352-7>
- [21] K. Yamamoto, C. Ishikawa, H. Katano, T. Yasumoto, N. Mori, *Cancer Lett.* **2011**, *300* (2), 225–234. DOI: <https://doi.org/10.1016/j.canlet.2010.10.016>
- [22] S. M. Kim, Y.-J. Jung, O.-N. Kwon, K. H. Cha, B.-H. Um, D. Chung, C.-H. Pan, *Appl. Biochem. Biotechnol.* **2012**, *166* (7), 1843–1855. DOI: <https://doi.org/10.1007/s12010-012-9602-2>
- [23] E. Ryckebosch, C. Bruneel, R. Termote-Verhalle, K. Muylaert, I. Foubert, *Algal Res.* **2014**, *3*, 36–43. DOI: <https://doi.org/10.1016/j.algal.2013.11.001>
- [24] A. Zarekarizi, L. Hoffmann, D. Burritt, *J. Appl. Phycol.* **2019**, *31* (1), 281–299. DOI: <https://doi.org/10.1007/s10811-018-1558-3>
- [25] M. K. W.-A. Airanthi, M. Hosokawa, K. Miyashita, *J. Food Sci.* **2011**, *76* (1), C104–C111. DOI: <https://doi.org/10.1111/j.1750-3841.2010.01915.x>
- [26] E. Susanto, A. S. Fahmi, M. Abe, M. Hosokawa, K. Miyashita, *Aquat. Procedia* **2016**, *7*, 66–75. DOI: <https://doi.org/10.1016/j.aqpro.2016.07.009>
- [27] S. M. Kim, S.-W. Kang, O.-N. Kwon, D. Chung, C.-H. Pan, *J. Korean Soc. Appl. Biol. Chem.* **2012**, *55* (4), 477–483. DOI: <https://doi.org/10.1007/s13765-012-2108-3>
- [28] J.-L. Mouget, P. Rosa, G. Tremblin, *J. Photochem. Photobiol. B.* **2004**, *75* (1–2), 1–11. DOI: <https://doi.org/10.1016/j.jphotobiol.2004.04.002>
- [29] J. Lavaud, B. Rousseau, A.-L. Etienne, *Biochemistry* **2003**, *42* (19), 5802–5808. DOI: <https://doi.org/10.1021/bi027112i>
- [30] F. Derwenskus, F. Metz, A. Gille, U. Schmid-Staiger, K. Briviba, U. Schließmann, T. Hirth, *GCB Bioenergy* **2019**, *11* (1), 335–344. DOI: <https://doi.org/10.1111/gcbb.12563>
- [31] B. Lepetit, R. Goss, T. Jakob, C. Wilhelm, *Photosynth. Res.* **2012**, *111* (1–2), 245–257. DOI: <https://doi.org/10.1007/s11120-011-9633-5>
- [32] J. Monteiro, M. Leslie, M. H. Moghadasian, B. M. Arendt, J. P. Alard, D. W. L. Ma, *Food Funct.* **2014**, *5* (3), 426. DOI: <https://doi.org/10.1039/c3fo60551e>
- [33] M. Cooney, G. Young, N. Nagle, *Sep. Purif. Rev.* **2009**, *38* (4), 291–325. DOI: <https://doi.org/10.1080/15422110903327919>
- [34] M. A. Islam, R. J. Brown, I. O'Hara, M. Kent, K. Heimann, *Energy Convers. Manage.* **2014**, *88*, 307–316. DOI: <https://doi.org/10.1016/j.enconman.2014.08.038>
- [35] S. Pieber, S. Schober, M. Mittelbach, *Biomass Bioenergy* **2012**, *47*, 474–482. DOI: <https://doi.org/10.1016/j.biombioe.2012.10.019>
- [36] B. Gao, A. Chen, W. Zhang, A. Li, C. Zhang, *J. Ocean Univ. China* **2017**, *16* (5), 916–924. DOI: <https://doi.org/10.1007/s11802-017-3174-2>
- [37] H. Wang, Y. Zhang, L. Chen, W. Cheng, T. Liu, *Bioprocess Biosyst. Eng.* **2018**, *41* (7), 1061–1071. DOI: <https://doi.org/10.1007/s00449-018-1935-y>
- [38] A. Delbrut, P. Albina, T. Lapierre, R. Pradelles, E. Dubreucq, *Molecules* **2018**, *23* (4), 874. DOI: <https://doi.org/10.3390/molecules23040874>
- [39] M. Herrero, P. J. Martín-Álvarez, F. J. Señoráns, A. Cifuentes, E. Ibáñez, *Food Chem.* **2005**, *93* (3), 417–423. DOI: <https://doi.org/10.1016/j.foodchem.2004.09.037>
- [40] M. Herrero, A. Cifuentes, E. Ibanez, *Food Chem.* **2006**, *98* (1), 136–148. DOI: <https://doi.org/10.1016/j.foodchem.2005.05.058>
- [41] L. Bähr, A. Wüstenberg, R. Ehwald, *J. Appl. Phycol.* **2016**, *28* (2), 783–793. DOI: <https://doi.org/10.1007/s10811-015-0614-5>
- [42] J. E. Mann, J. Myers, *J. Phycol.* **1968**, *4* (4), 349–355.
- [43] P. Bergmann, W. Trösch, *Algal Res.* **2016**, *17*, 79–86. DOI: <https://doi.org/10.1016/j.algal.2016.03.040>
- [44] P. Bergmann, P. Ripplinger, L. Beyer, W. Trösch, *Chem. Ing. Tech.* **2013**, *85* (1–2), 202–205. DOI: <https://doi.org/10.1002/cite.201200132>
- [45] U. Schmid-Staiger, R. Preisner, W. Trösch, P. Marek, *Chem. Ing. Tech.* **2009**, *81* (11), 1783–1789. DOI: <https://doi.org/10.1002/cite.200900079>

- [46] R. Münkler, U. Schmid-Staiger, A. Werner, T. Hirth, *Biotechnol. Bioeng.* **2013**, *110* (11), 2882–2893.
- [47] J. Degen, A. Uebele, A. Retze, U. Schmid-Staiger, W. Trösch, *J. Biotechnol.* **2001**, *92* (2), 89–94. DOI: [https://doi.org/10.1016/S0168-1656\(01\)00350-9](https://doi.org/10.1016/S0168-1656(01)00350-9)
- [48] G. Lepage, C. C. Roy, *J. Lipid Res.* **1984**, *25* (12), 1391–1396.
- [49] A. Gille, A. Trautmann, C. Posten, K. Briviba, *Int. J. Food Sci. Nutr.* **2016**, *67* (5), 507–513. DOI: <https://doi.org/10.1080/09637486.2016.1181158>
- [50] M. Petrushkina et al., *Algal Res.* **2017**, *24*, 387–393. DOI: <https://doi.org/10.1016/j.algal.2017.03.016>
- [51] U. Eilers, A. Bikoulis, J. Breitenbach, C. Büchel, G. Sandmann, *J. Appl. Phycol.* **2016**, *28* (1), 123–129. DOI: <https://doi.org/10.1007/s10811-015-0583-8>
- [52] B. Guo, B. Liu, B. Yang, P. Sun, X. Lu, J. Liu, F. Chen, *Mar. Drugs* **2016**, *14* (7), 125. DOI: <https://doi.org/10.3390/md14070125>
- [53] M. M. Reboloso-Fuentes, A. Navarro-Pérez, J. J. Ramos-Miras, J. L. Guil-Guerrero, *J. Food Biochem.* **2001**, *25* (1), 57–76. DOI: <https://doi.org/10.1111/j.1745-4514.2001.tb00724.x>
- [54] G. C. Zittelli, F. Lavista, A. Bastianini, L. Rodolfi, M. Vincenzini, M. R. Tredici, *J. Biotechnol.* **1999**, *70* (1), 299–312.
- [55] L. Rodolfi, G. Chini Zittelli, N. Bassi, G. Padovani, N. Biondi, G. Bonini, M. R. Tredici, *Biotechnol. Bioeng.* **2009**, *102* (1), 100–112. DOI: <https://doi.org/10.1002/bit.22033>
- [56] X. Lu, H. Sun, W. Zhao, K.-W. Cheng, F. Chen, B. Liu, *Mar. Drugs* **2018**, *16* (7), 219. DOI: <https://doi.org/10.3390/md16070219>
- [57] Y. Liang, J. Beardall, P. Heraud, *Bot. Mar.* **2006**, *49* (2). DOI: <https://doi.org/10.1515/BOT.2006.021>
- [58] W. Blanken, M. Cuaresma, R. H. Wijffels, M. Janssen, *Algal Res.* **2013**, *2* (4), 333–340. DOI: <https://doi.org/10.1016/j.algal.2013.09.004>
- [59] Y. F. Shang, S. M. Kim, W. J. Lee, B.-H. Um, *J. Biosci. Bioeng.* **2011**, *III* (2), 237–241. DOI: <https://doi.org/10.1016/j.jbiosc.2010.10.008>
- [60] D. Zhao, S.-M. Kim, C.-H. Pan, D. Chung, *Food Chem.* **2014**, *145*, 505–513. DOI: <https://doi.org/10.1016/j.foodchem.2013.08.045>
- [61] A. Kawee-ai, A. Kuntiya, S. M. Kim, *Nat. Prod. Commun.* **2013**, *8* (10), 1934578X1300801. DOI: <https://doi.org/10.1177/1934578X1300801010>
- [62] S. Xia, K. Wang, L. Wan, A. Li, Q. Hu, C. Zhang, *Mar. Drugs* **2013**, *11* (7), 2667–2681. DOI: <https://doi.org/10.3390/md11072667>
- [63] B. E. Richter, B. A. Jones, J. L. Ezzell, N. L. Porter, N. Avdalovic, C. Pohl, *Anal. Chem.* **1996**, *68* (6), 1033–1039. DOI: <https://doi.org/10.1021/ac9508199>
- [64] European Commission, *Directive 2009/32/EC*, **2009**.