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# Systematic solvent selection enables the fractionation of wet microalgal biomass

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ABSTRACT

Wet microalgal biomass was recently proposed as a feedstock to circumvent the energy-intensive drying step in biorefineries. However, solvents commonly applied to extract valuable target compounds from dried biomass are usually less effective when applied on wet biomass. In the present study, we investigated the potential of systematic solvent selection to increase pigment and lipid yields for the extraction of wet *Phaeodactylum tricornutum* biomass. The solvent selection was guided by a large-scale computational screening approach. Experiments revealed, that 2-butanol – a non-toxic, partially water-miscible solvent – extracted 99.4 wt% of lipids and 82.6 wt % of carotenoids from wet biomass. By using only 2-butanol and water as benign solvents, we developed a biorefinery approach that effectively fractionates wet microalgal biomass under ambient conditions into proteins, carbohydrates, carotenoids and lipids without the need for energy-intensive biomass drying.

## 1. Introduction

Microalgae are a promising feedstock for the production of food, feed, chemicals, fuels, pigments, and other high-value products [1,2]. Their ability to withstand harsh conditions, such as high salinity and high light irradiation, enables cultivation on non-arable land, thus reducing the competition with land use for agriculture or renewable energy [3]. Furthermore, microalgae have higher growth rates than terrestrial plants [4]. These advantages render microalgae a very appealing feedstock for a circular economy within planetary boundaries.

In the early 2000s, there was growing interest in microalgae cultivation for the production of biodiesel. In conventional microalgae-based biodiesel processes, cultivation was followed by the harvesting stage, typically involving techniques such as centrifugation or filtration [5]. The resulting algal paste was dried to minimize the moisture content [6–8]. Subsequently, the neutral lipid fraction mainly containing triacylglycerides was extracted by organic solvents and was further converted to biodiesel by transesterification [6,8,9]. However, algal lipid conversion to biodiesel has not reached economic feasibility to date [10,11]. The energy-intense drying step was identified as the major bottleneck for the downstream process economics [7]. Another disadvantage was the resource-inefficient biomass utilisation. Depending on the algal strain and cultivation conditions, the triacylglycerides used for conversion to biodiesel comprise only 10–50 wt% of the overall biomass dry weight [12], whereas the residues were treated as waste in biogas plants [13,14]. Further concerns were related to the environmental, health and safety (EHS) properties of the solvents employed for lipid extraction. Despite ecotoxicity and health hazards [15–17], n-hexane, a fossil-based solvent, remains a prevalent choice for lipid and pigment extraction from microalgae to date [18–20].

Since then, the utilisation of wet algal biomass in a biorefinery framework has been proposed as a way to eliminate the energy demand for drying, and to increase resource efficiency. In the envisioned "zero-waste" process, all biomass fractions are converted to multiple marketable compounds [21–23]. The wet algal paste obtained after the harvesting stage is directly used as a feedstock for biomass fractionation, eliminating the need for the energy-intensive and costly drying step. *Phaeodactylum tricornutum* represents an excellent feedstock for biorefineries due to its balanced biomass composition, with 18 – 54 wt% proteins, 3 – 31 wt% carbohydrates, 14 – 54 wt% lipids and pigments, depending on the cultivation conditions[24–26]. Additionally, the diatom produces the high-value compounds eicosapentaenoic acid (EPA), a polyunsaturated fatty acid, and the red carotenoid fucoxanthin [27,28]. However, extracting and separating multiple target compounds is still challenging [29,30], additionally complicated by the high moisture content comprising up to 85 wt% of the biomass after harvest

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[31–33]. Therefore, no industrial-scale *P. tricornutum* biorefinery was established to date.

On the lab scale, the extraction and separation of proteins, carbohydrates, pigments and lipids is usually achieved by i) solid–liquid extraction of one or multiple target compounds and ii) further separation strategies, such as liquid–liquid extraction, precipitation, or filtration. These approaches rely heavily on the use of solvents and stand in stark contrast with the principles of green chemistry, stating that the use of solvents should be prevented whenever possible. Although biorefinery processes carry tremendous potential to contribute to a chemical industry within planetary boundaries, the use of unsuitable solvents could have detrimental effects on their overall sustainability. In fact, the solvent use contributes to more than half of the life cycle emissions in chemical and pharmaceutical processes [34,35], rendering solvent selection a crucial decision in early stages of the biorefinery design.

In recent studies, solvents that effectively extracted high-value lipids from dried microalgae, such as alkanes, were less efficient when used on wet, untreated biomass [32,33]. So far, predominantly cell disruption methods were explored to enhance the lipid yield [32,36,37]. Cell disruption releases the lipids from the wet biomass, thus enhancing the contact between the nonpolar solvent and the lipids. In particular cell disruption with pulsed electric fields is advantageous for biomass fractionation since they allow for targeted release of cellular compounds from undried biomass [38]. However, cell disruption methods are less effective for species with resistant cell walls. In addition to cell disruption, solvent selection has high potential to increase the yield of the target compounds and allows for innovate separation strategies in biorefineries.

In our recent works, we developed a computational solvent screening framework for biorefineries [39,40] based on COSMO-RS solubility predictions [39,41]. The screening framework is applicable to various types of biomass and was in particular experimentally validated for microalgae [39] and lignocellulose [40]. Additionally, the EHS properties for each solvent are predicted by computational models and are taken into account for the solvent selection [42]. In this way, a comprehensive database comprising more than 8000 potential solvents is automatically evaluated for biomass fractionation in microalgal biorefineries. Previously, the screening framework identified partially water-miscible (PWM) solvents which outperformed the toxic solvent n-hexane in terms of yield of lipophilic compounds. This study aims to i) expand the computational screening to water-miscible (WM) and non-water miscible solvents (NWM), and ii) provides a detailed experimental analysis of extracted lipids and pigments. Based on the computational and experimental findings, we investigate new routes for the fractionation of wet *P. tricornutum* biomass.

## 2. Results and discussion

### 2.1. P. tricornutum: Biomass composition and potential biorefinery products

*P. tricornutum* was cultivated in a flat-panel photobioreactor under artificial light irradiation (SI for details on cultivation). The biomass was harvested at a cell density of 4.81 g $L^{-1}$ with a composition of 39.9 wt% proteins, 19.9 wt% carbohydrates, 9.7 wt% lipids, 5.5 wt% chlorophylls, and 2.1 wt% carotenoids on a dry matter basis (Fig. 1 a). A detailed composition of fatty acids, chlorophylls, and carotenoids is given in the SI.

Each biomass component of *P. tricornutum* can be converted to a broad range of products. For example, water-soluble carbohydrates could serve as feedstock for biopolymer production [43], for fermentation to biofuels, or as food ingredients. For the analysis of potential biorefinery products and an estimation of their economic value, we allocated all biomass components to the most profitable application (see methods for details).

In line with other studies, fucoxanthin, a red carotenoid, was identified as the main carotenoid (1.5 wt%, dry matter). Carotenoids can be marketed in the health industry or as nutritional supplements due to their antioxidant properties at prices of 900 € $kg_{prod}^{-1}$ [44]. The carotenoids comprise 70 % of the overall economic biomass value despite their low abundance, with fucoxanthin as the largest contributor (Fig. 1 b). Chlorophylls can be used as natural colorants (E141) [45].

The lipid fraction spans a diverse range of potential applications and contributes to 20 % of the overall economic biomass value. The polyunsaturated fatty acid EPA (2.6 wt% dry matter) is sought-after as a food additive or a nutritional supplement [46]. With a price of 200 € $kg_{prod}^{-1}$, EPA is the highest-value component of the lipids fraction. The largest economic potential for the lower-value lipids lies in the conversion to biolubricants, with an estimated sales price that is twice as high as that of biodiesel.

The water-soluble storage carbohydrate chrysolaminarin (2.78 wt%, dry matter) is a natural plant-protection agent [47] and is known for its antioxidant properties [48]. The selling price of chrysolaminarin is 20 € $kg_{prod}^{-1}$ which is much lower compared to that of EPA and the carotenoids. Therefore, chrysolaminarin valorisation plays a rather subordinate role in the economic profitability of the biorefinery process. Other water-soluble carbohydrates (11.78 wt%, dry matter) can be marketed as food or feed.

Bio-based feedstocks play an important role in the defossilisation of the polymer industry. The water-insoluble proteins (21.72 wt%, dry matter) and carbohydrates (5.34 wt%, dry matter) of *P. tricornutum* could serve as a feedstock for biopolymer production.

Water-soluble proteins of *P.tricornutum* (18.13 wt%, dry matter) have emulsifying properties and could serve as functional ingredients in

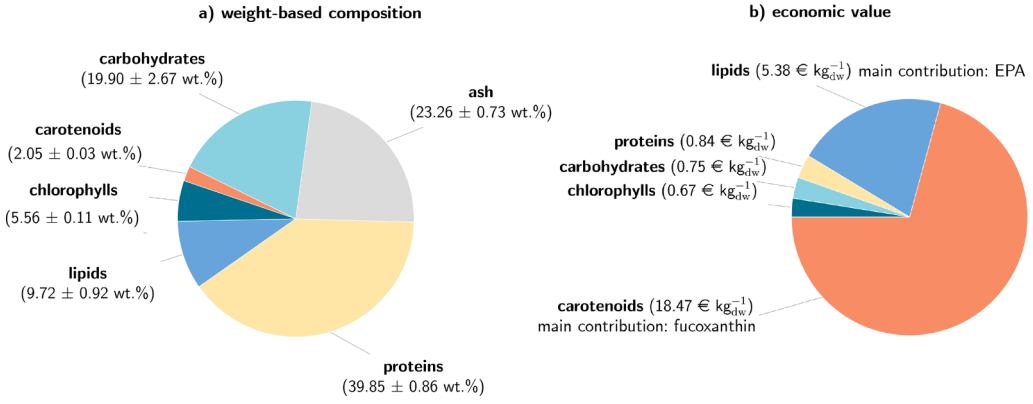


**Fig. 1.** Biomass composition of moisture-free *P. tricornutum* on a) weight basis and b) based on economic value. The high-value components, EPA and fucoxanthin, render the lipid and pigment fraction the most valuable biomass components.

the food industry[49].

*P. tricornutum* has a high ash content (23.3 wt%) in comparison with other species, however, the ash fraction has not been commercially used to date. Depending on the cultivation conditions, *P. tricornutum* ash contains biosilica which could be applied in biosensing or energy applications [50]. However, silica isolation from biomass requires high temperatures, oxidising agents, or strong acids, compromising the integrity of other biomass compounds [50,51]. Ash is also applied as an additive for biochar production [52].

In an ideal biorefinery scenario, assuming loss-free separation of all marketable products of the analysis above, *P. tricornutum* biomass has an overall value of 26.11 € $kg^{-1}$ (dry matter) which is comparable to other microalgae [44]. Fucoxanthin and EPA were identified as compounds with the highest economic value. Therefore, a profitable *P. tricornutum* biorefinery requires efficient extraction and separation of EPA and fucoxanthin. As both high-value compounds together account for only 4.1 wt% of the overall biomass, the remaining biomass fractions should be valorized using inexpensive separation techniques to increase resource efficiency and economic profitability. In this manner, the whole potential of the biomass can be harnessed.

## 3. Solvent selection and evaluation of EHS properties

Solvent extraction of wet algal paste can be seen as a solid–liquid extraction process influenced by solvent interactions with the moisture, the cell wall, and the target compounds [32,53,54]. First, the solvent is brought in contact with the biomass by mixing. Subsequently, the solvent diffuses through the cell wall into the inner cell. In the case of *P. tricornutum*, the cell wall is composed of sulfated α-mannan with glucuronic residues, proteins, and long-chain polyamines [55]. Polymers can swell and change their conformation upon solvent contact [56]. Therefore, the type of solvent might facilitate the transport through the cell wall. Cell wall disruption methods, such as sonication or milling, facilitate access to the cell contents. After penetrating the cell wall, the solvent diffuses to the organelles containing the target compounds. Some compounds are chemically bound to the target organelles. The pigments, for example, are bound to the fucoxanthin-chlorophyll-protein complexes (FCPs) *via* hydrogen bonds, located within the thylakoid membranes of the chloroplast [57]. Therefore, the selected solvent must be capable of disintegrating the target compound from the target organelle and subsequently dissolving the compound. The cells both contain moisture and are enveloped by it. Due to its polarity, the moisture influences all steps of the extraction process and should be taken into account during solvent selection.

In our previous study, we used the COSMO-RS-based screening approach to identify PWM solvents with benign EHS criteria for the fractionation of wet *P. tricornutum* [39]. In this study, the solvent screening approach was expanded to identify WM and NWM solvents. In brief, from a database containing more than 8000 potential solvents, unsuitable molecules were subsequently eliminated (Fig. 2). Only solvents with a net zero formal charge and suitable melting point and boiling point ranges ($T_m \leq 25$ °C, 40 °C $\leq T_b \leq 120$ °C) were considered. For each potential solvent, several EHS properties (see SI) were predicted using the QSAR models implemented in VEGA [42]. The model results were used to predict the EHS score according to Linke et al. [58] The EHS score is a metric describing the risk of the solvent use for the environment, human health, and safety [39,41,58]. An EHS score of 0 indicates harmful EHS properties, whereas a score of 1 suggests benign EHS properties. All solvent candidates with an EHS score below that of the benchmark solvent n-hexane were eliminated. All potential solvents passing these screening steps were subject to COSMO-RS solubility predictions for pigments, and neutral and polar lipids. These biomass fractions were represented by several model molecules (see SI for a detailed representation of the model molecules). A benchmark solvent was assigned to each biomass fraction (ethanol for pigments and polar lipids, n-hexane for neutral lipids). In case that a solvent candidate could not outperform at least one of the benchmark solvents, it was removed from the screening. To determine the degree of water-miscibility of the solvents, the liquid–liquid equilibria of water, mimicking the biomass moisture, and the solvents were predicted using COSMO-RS ($T = 25$ °C, atmospheric pressure). All potential solvent/water mixtures without liquid–liquid-equilibrium (LLE) were classified as WM solvents. Solvents with a water content of $0.1 < x^{org}_{H_2O} < 0.9$ in the organic phase were classified as PWM, and with $x^{org}_{H_2O} \leq 0.1$ as NWM. In both cases, a low solvent concentration in the aqueous phase is desired which was therefore limited to $x^{aq}_{solv} < 0.1$. A summary of all representative molecules and reference solvents is provided in the SI. Finally, 79 WM, 328 PWM, and 292 NWM solvents were identified by the computational screening approach (Fig. 2).

The screening approach identified $C_2$- and $C_3$-alcohols, and acetone as promising WM solvents. As PWM solvents, $C_4$-alcohols, esters of carboxylic acids, and 2-methyl tetrahydrofuran (2-MeTHF) were identified. The COSMO-RS approach predicted generally high solubilities of polar lipids (PLs) and mediocre solubilities for carotenoids (Fig. 3) for the WM and PWM solvents, and the solubility of carbohydrates, protein and neutral lipids (NLs) was low. Only for the identified NWM solvents, which all belong to the class of ethers, high NL solubilities were predicted. The solubility of PLs and pigments was predicted to be lower in NWM than in the WM and PWM solvents. In general, these results agree with the chemical intuition that more polar solvents dissolve more polar compounds better nonpolar compounds, and vice-versa. However, the

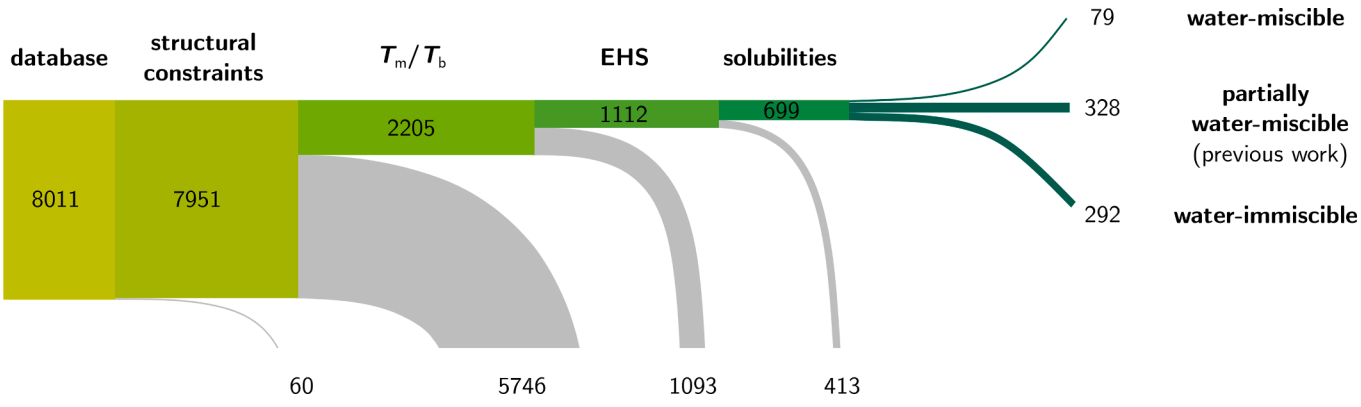


**Fig. 2.** Solvent screening procedure. A database containing more than 8000 solvents was screened for solvents with suitable structural features, melting and boiling points, and EHS properties. Solvents not meeting the criteria were subsequently eliminated (gray streams). Suitable solvents candidates were subject to COSMO-RS solubility predictions of pigments, NLs and PLs. Solvent candidates with high solubilities were finally classified as water-miscible, partially water-miscible or water-immiscible based on COSMO-RS liquid–liquid-equilibrium predictions ($T = 25$ °C, atmospheric pressure).

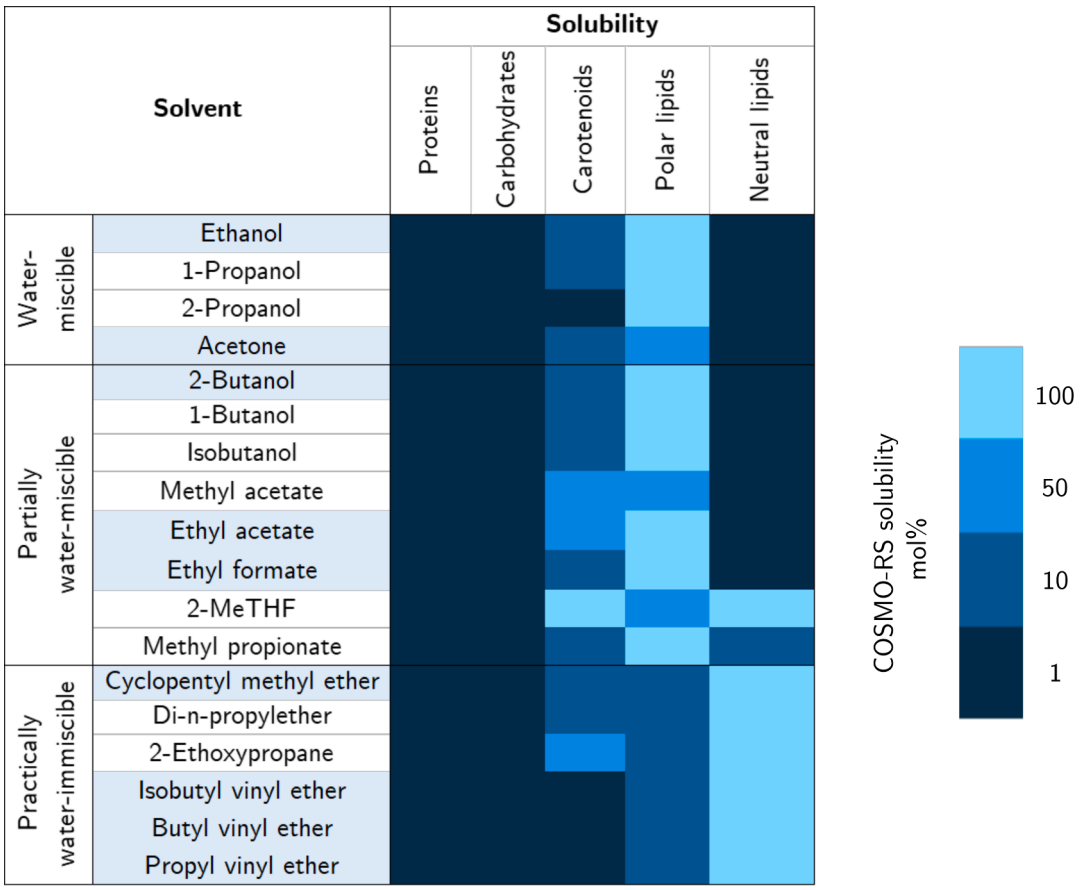


**Fig. 3.** COSMO-RS predicted solubilities ($T = 25$ °C, based on molar fraction) of proteins, carbohydrates, pigments, polar and neutral lipids for the computationally identified solvent candidates. Solvents selected for experimental tests are highlighted in blue.

computational results cannot explain that nonpolar solvents lead to low lipid yields when applied on wet microalgal biomass. Therefore, we selected solvents from each class for further experimental investigations. The final choice of solvents was based on their EHS properties, commercial availability, and price ($\leq$ 100 € per 10 g). We selected ethanol (EtOH) and acetone as WM solvents due to their low price, benign EHS properties and ease of recovery. To test a broad range of water-miscibility, 2-butanol (2-BuOH), ethyl acetate, and ethyl formate were selected. The amount of water in the organic phase $x_{H_2O}^{org}$ is the highest for 2-BuOH, followed by ethyl acetate and ethyl formate. Furthermore, we selected isobutyl vinyl ether (IBVE), butyl vinyl ether (BVE), propyl vinyl ether (PVE) and cyclopentyl methyl ether (CPME) as highly promising NWM solvents. These solvents were predicted to have lipid solubilities comparable to n-hexane. The COSMO-RS predicted sigma-profiles of the selected solvents, providing information about a solvent's polarity and hydrogen bonding behaviour, are included in the SI.

According to the Sanofi, the Chem21 and the GSK solvent selection guides [15–17], n-hexane is rated as hazardous, and thus, its replacement was requested, whereas the alcohols, acetone, and ethyl formate are recommended for industrial use. The identified ethers are non-toxic but highly flammable [59] and potentially form explosive peroxides upon exposure to air [60]. In the following, the selected solvents are applied in lipid and pigment extractions from wet and dried *P. tricornutum* biomass.

### 3.1. Lipid extraction from wet algal biomass

We performed extraction experiments with wet *P. tricornutum* paste using the selected solvents to determine the lipid yield and the fatty acid composition. To investigate the influence of a cell disruption step, we compared the lipid yields of sonicated and non-sonicated samples (see SI microscopic images of untreated and disrupted samples). The lipids of *P. tricornutum* are composed of nonpolar NLs, mainly stored as triacylglycerides in the lipid droplets [61], and PLs, being the major component of cell membranes. The NL/PL ratio and the distribution of EPA between both lipid classes are highly dependent on the cultivation conditions. While Yang et al. reported a higher EPA distribution in the PLs [62], Remmers et al. showed a dynamic redistribution of EPA from the PLs towards the NLs during cultivation [63].

In our study, 18.9 wt% of all lipids were identified as PLs, and 81.1 wt% as NLs. 90 wt% of EPA was distributed in the NL fraction. To fully exploit the economic potential of the lipids, the solvents must be able to effectively extract the NLs.

Mixing the wet biomass with water-immiscible solvents leads to the formation of two liquid phases and emulsification, which may impede the solvent contact with the lipids. Therefore, we expected higher lipid yields for the WM and PWM solvents compared to the NWM solvents, which was confirmed by the experiments.

Despite the fact that pure lipids typically have lower solubilities in polar solvents, such as the tested WM and PWM, the lipid yield measured in these solvents was higher compared to the nonpolar NWM solvents, see Fig. 4. Even without cell disruption, 96 vol% ethanol ($EtOH_{96}$) extracted 96.0 wt% of the lipids, followed by 75 vol% 2-butanol (2-$BuOH_{75}$) and acetone, with a lipid yield of 92.6 wt% and 86.2 wt%, respectively. A lipid yield of 99.4 wt% was obtained by extraction with 2-$BuOH_{75}$ combined with sonication treatment. Based on these results, we suggest that target compound solubility is not entirely the governing factor for the efficient extraction of wet microalgal biomass. Furthermore, the lipid yields of the untreated and sonicated samples after $EtOH_{96}$, 2-$BuOH_{75}$, and acetone extraction were comparably high. We

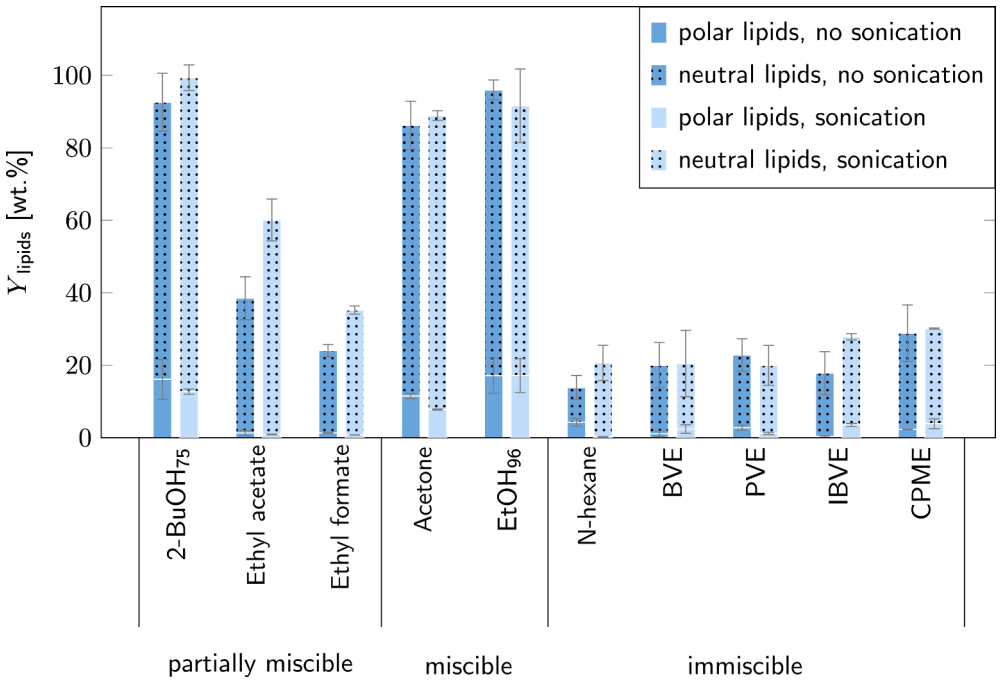


**Fig. 4.** Lipid extraction (90 min incubation) from wet *P. tricornutum* paste, with and without sonication treatment. Even without sonication, 75 vol% 2-butanol, 96 vol% EtOH and acetone extracted more than 86 wt% of the lipids. Hexane and all other practically water-immiscible solvents in contrast, achieved yields below 33 wt % with sonication.

suspect that these solvents could efficiently penetrate the untreated cell walls, e.g. by diffusion. Thus, in these solvents, cell disruption did not have a significant impact on the lipid yields.

The extractions with the PWM solvents ethyl acetate and ethyl formate resulted in lower lipid yields compared to that with 2-$BuOH_{75}$. Sonication increased the lipid yields from 38.5 to 60.1 wt% using ethyl acetate and from 22.7 to 35.2 wt% using ethyl formate. Notably, the lipid yields decreased in line with their water miscibility and effective polarity of the mixture (2-$BuOH_{75}$ > ethyl acetate > ethyl formate). Extractions using the NWM solvents resulted in a maximum lipid yield of 33.1 wt% for CPME and could be barely increased by sonication. Also other studies reported comparably low lipid yields for the wet extraction of *Nannochloropsis* sp. and *Chlorella pyrenoidosa* biomass, using ethyl acetate, hexane and CPME [32,33]. The lipid yields ranged between 20 to 40 wt% and could be improved to around 60 wt% by adding a more polar co-solvent such as methanol [32,33].

The amount of extracted PLs was low for all investigated NWM solvents as well as for the solvents with low water miscibility (ethyl acetate, ethyl formate). For all tested solvents, EPA contributed about 30 wt% of all extracted fatty acids.

The results agree well with other studies and are likely transferable to other algal species. Liu et al. used the WM solvent 1,2-dimethoxyethane for the extraction of wet *Botryococcus braunii* biomass resulting in close to total lipid yield (determined using n-hexane on dry biomass). However, the present regulations limit the use of dimethoxyethane on industrial scale due to its health hazard and safety risks [64]. Derwenskus et al. performed pressurized liquid extraction on wet *Chlorella vulgaris* and *P. tricornutum* biomass [65]. For *P. tricornutum*, increasing yields were obtained for n-hexane < ethyl acetate < EtOH. Similarly, wet *C. vulgaris* was least efficiently extracted by n-hexane, however, the use of ethyl acetate led to higher lipid yields than EtOH.

### 3.2. Carotenoid and chlorophyll extraction from wet algal biomass

Next, we investigated the chlorophyll and carotenoid yields with the most promising solvents on wet *P. tricornutum* biomass. We selected the PWM solvent 2-$BuOH_{75}$, and the WM solvents $EtOH_{96}$ and acetone as these solvents were most effective in the lipid extractions. These solvents were compared against the NWM solvents CPME and hexane. As the vinyl ethers were ineffective for lipid extraction, and are prone to spontaneous polymerisation and peroxide formation [66], they were not considered for pigment extractions.

Similar to the lipid extraction experiments, $EtOH_{96}$, acetone, and 2-$BuOH_{75}$ most effectively extracted chlorophylls and carotenoids, see Fig. 5. Without sonication, a maximum carotenoid yield of 77.8 wt% was obtained by extraction with $EtOH_{96}$. 2-$BuOH_{75}$ obtained the maximum chlorophyll yield with 82.3 wt%. Surprisingly, among the WM and the PWM solvents, the carotenoid yields were lower than that of the lipids (see Fig. 4), although, conversely, the carotenoid solubility was predicted to be higher than the NL solubility. A potential cause for the reverse solubility-yield relationship could be related to the accessibility of the target organelles. The pigments are connected *via* hydrogen bonds to the FCPs, located in the thylakoid membranes of the chloroplast [57]. In contrast, the NLs are stored in lipid globules that float inside the cells [61]. As the lipid globules are likely easier accessible to the solvent than the FCPs, the lipid yield might be higher despite the lower solubility.

Sonication treatment increased both the carotenoid and chlorophyll yield for all tested solvents. $EtOH_{96}$ with sonication treatment led to the highest carotenoid yield of 95.0 wt%, followed by acetone. Extraction with 2-$BuOH_{75}$ with sonication resulted in complete chlorophyll extraction and a carotenoid yield of 82.6 wt%, indicating that 2-BuOH was efficiently transported to the FCPs but chlorophylls were more efficiently released.

The NWM solvents n-hexane and CPME led to the lowest pigment yields within this study. Here, we observed a stronger effect of sonication treatment on the carotenoid yields compared to the lipid yield. Sonication treatment increased the carotenoid yield from 7.9 to 40.8 wt % using n-hexane as extraction solvent, whereas the increase in lipid yield was less prominent. CPME combined with sonication obtained the maximum carotenoid yield among the NWM solvents. While n-hexane selectively extracted carotenoids over chlorophylls, CPME extracted both chlorophylls and pigments with similar efficiency.

In accordance with our results, Derwenskus et al. reported increasing carotenoid yields for pressurized liquid extraction of wet *P. tricornutum*

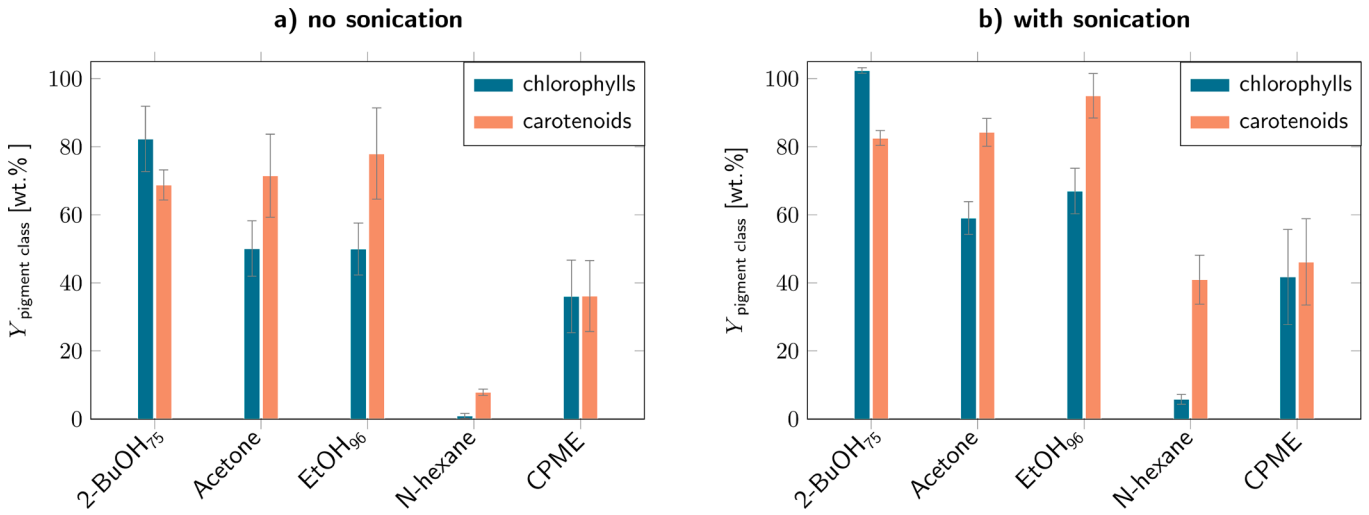


**Fig. 5.** Chlorophyll and carotenoid yields after 90 min extraction of wet *P. tricornutum* biomass (moisture content = 81-85 wt%) a) without sonication and b) with 2 min sonication treatment.

biomass, increasing in the order of n-hexane < ethyl acetate < EtOH [65].

### 3.3. Dry extraction

Finally, we performed extractions on lyophilized biomass (moisture content: 8.5 wt%) to compare the yields for the wet and the dry route. We selected 2-BuOH$_{75}$ and EtOH$_{96}$ as best best-performing representatives for the PWM and WM solvents, respectively. The NWM solvent n-hexane is commonly used as a solvent for lipid and pigment extraction on dry biomass and serves as a benchmark.

The most effective method for lipid extraction was using 2-BuOH$_{75}$ on dry biomass (Fig. 6), achieving a slightly higher lipid yield than the reference method (chloroform/methanol, 1/1, v/v). The highest carotenoid yield was obtained using EtOH$_{96}$ on dry biomass (reference method for pigment extraction, therefore, 100 wt% carotenoid yield). We also tested absolute EtOH for carotenoid extraction on dry biomass, however, with a slightly lower yield (data not shown). Wet extraction with EtOH$_{96}$ and sonication was slightly less efficient (within standard deviation), with a carotenoid yield of 95.0 wt%. 2-BuOH$_{75}$ (wet + sonication and dry route) and EtOH$_{96}$ (dry route) led to complete chlorophyll extraction.

The experiments showed that the extraction of wet, undisrupted biomass was in general the least effective method for all analysed biomass fractions, supporting that the moisture content of the biomass has an influence on the yield of lipophilic compounds. Applying WM and PWM solvents on wet, untreated biomass resulted in lipid yields close to that of the dry route. Thus, a cell disruption step was less impactful. Surprisingly, the WM and PWM solvents were also more effective than n-hexane on dried biomass. Angles et al. reported a comparably low lipid yield of 27 wt% after extraction of dried *Nannochloropsis* biomass using the structurally similar solvent n-heptane which was attributed to limited wettability and dispersion of the biomass in the solvent.

Remarkably, carotenoid yields for 2-BuOH$_{75}$ and n-hexane

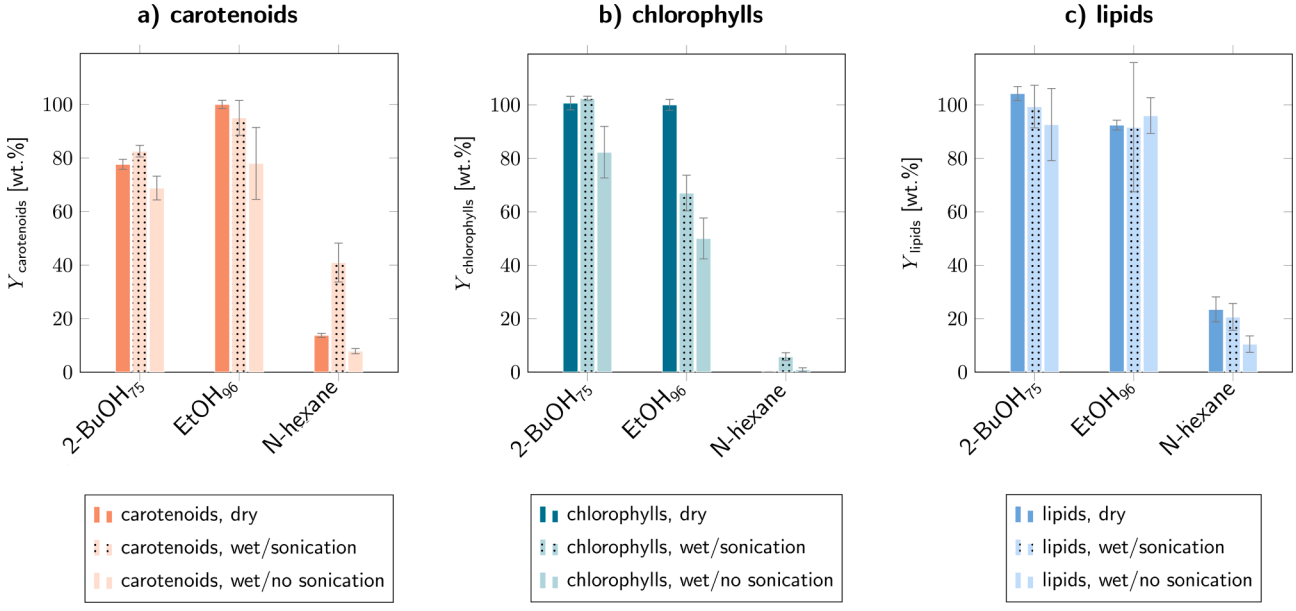


**Fig. 6.** Dry extractions of freeze-dried *P. tricornutum* biomass (moisture content = 8.5 wt%) compared to wet *P. tricornutum* paste (moisture content = 81-85 wt%). In both cases, the biomass was extracted for 90 min. The biomass (on a dry matter basis) to solvent ratio was equal for all extractions. The yields of carotenoids, chlorophylls and lipids are shown in a), b), and c), respectively.

extraction could be boosted beyond dry route levels by sonication. Higher carotenoid yields after wet extraction of disrupted *Chlorella thermophila* compared to oven-dried biomass was also reported by Sarkar et al. [67].

The yield differences between the wet and the dry route seem to be influenced by several factors, including solvent selection, and the cell wall. Therefore, a prudent solvent selection combined with efficient cell disruption methods is required to obtain pigment and lipid yields comparable to those of dried biomass.

### 3.4. Integrated extraction and separation of lipids and carotenoids by wet extraction with 2-butanol

The separation of lipids and carotenoids is crucial for the economic viability and the resource-efficiency of a *P. tricornutum* biorefinery process. In the literature, two strategies for this separation were reported: anti-solvent or temperature-induced precipitation of fucoxanthin in n-hexane followed by filtration [68], or liquid–liquid extraction and subsequent chromatographic separation [28]. Our experiments showed that lipid extraction from wet *P. tricornutum* biomass does not require cell disruption when solvents with high water miscibility are used. Pigment extraction without cell disruption was, in contrast, less effective. From wet, undisrupted biomass, 2-BuOH$_{75}$ extracted 92.6 wt% of lipids but only 68.8 wt% of carotenoids. Tailoring the operational parameters towards a lower carotenoid yield potentially leads to a more selective extraction of lipids, whereas the carotenoids preferably remain in the biomass. The remaining pigments could be extracted in a second extraction step. In this way, carotenoids and lipids would be efficiently separated. Since 2-BuOH is partially water-miscible, adding excess water triggers the formation of two liquid phases. In this way, hydrophobic compounds, (lipids and pigments) are directed to the organic phase, and hydrophilic compounds (proteins and carbohydrates) move to the aqueous phase. Therefore, we investigated the effect of the 2-BuOH/$H_2O$ ratio to modify the effective polarity of the mixture and the incubation time on the lipid and pigment yields.

First, we assessed the miscibility range of 2-BuOH and water which was experimentally determined by Lladosa et al. [69] ($T$=20 °C). The mass fraction of water in the organic phase at LLE was $w^{org}_{H_2O} = 36$ wt%. Therefore, to prevent phase separation during extraction, the water content in the 2-BuOH/$H_2O$-mixture during extraction ($w_{H_2O,\ extr}$) must be below this threshold. We varied $w_{H_2O,\ extr}$ between 5 and 32 wt%. A water content of 32 wt% corresponds to a 2-BuOH content of 75 vol% (2-BuOH$_{75}$) as used in previous experiments (please note the difference between weight- and volume-based expressions).

The water content $w_{H_2O,\ extr}$ also accounts for the mass of water originating from the moisture of the biomass $m_{moisture\ biomass}$ and is defined as

$$w_{H_2O,\ extr}\ [wt\%] = \frac{m_{moisture\ biomass} + m_{water\ added}}{m_{moisture\ biomass} + m_{water\ added} + m_{solv}} \bullet 100 \quad (1)$$

where the mass of solvent is given as $m_{solv}$ and the mass of water additionally to the solvent is denoted as $m_{water\ added}$.

Second, we performed extractions with varying $w_{H_2O,\ extr}$ on undisrupted, wet *P. tricornutum* paste for 90 min. In our experiments, the variation of the water content revealed a parabolic dependence of lipid and pigment yields on $w_{H_2O,\ extr}$(Fig. 7 a). The highest investigated water content led to the highest lipid (95.9 wt%), carotenoid (68.7 wt%) and chlorophyll yield (82.3 wt%). The selectivity of the carotenoid-lipid separation is described by the difference between carotenoid and lipid yield. A maximum selectivity towards lipids was reached at $w_{H_2O,\ extr}$ between 20 and 30 wt%, where ca. 80 wt% of the lipids were extracted and 80 wt% of the carotenoids remained in the biomass.

The parabolic relationship between yields and $w_{H_2O,\ extr}$ is surprising. An increasing water content leads to a higher polarity of the 2-BuOH/water mixture. According to the "like dissolves like" principle, higher polarity of the solvent is associated with lower solubility of non-polar biomass compounds, which was also confirmed by COSMO-RS solubility predictions (see SI). Therefore, the experimental observations cannot be solely explained by the solubility of the target compounds. Interestingly, Ren et al. observed increased lipid yields when wet algal biomass was treated with water between two extraction steps with organic solvents [70]. The water treatment caused microscopically observable alterations in the cell wall structure which were attributed to the increased lipid yields. We assume that the combined effects of solvent-cell wall interactions, interactions between solvent and the target organelles, and solubility are influenced by water addition and likely contribute to the observed parabolic yield profile. However, the extent to which each of the phenomena influenced the yield remains unresolved within this study and represent interesting options for further research.

To study the kinetics of the 2-BuOH extraction, we performed experiments with varying incubation time at a constant water content of $w_{H_2O,\ extr}$ = 32 wt% (corresponding to 2-BuOH$_{75}$, Fig. 7 b). The lipids

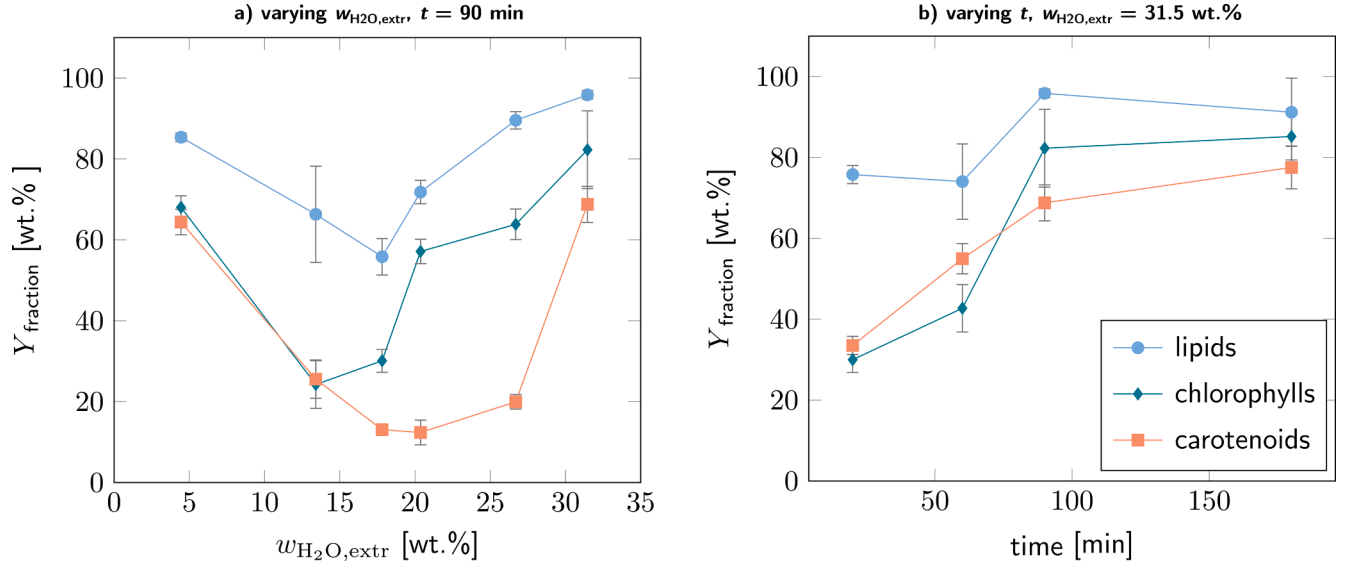


**Fig. 7.** Experiments investigating parameters for 2-BuOH extraction of wet *P. tricornutum* biomass. a) Influence of the water content in the system ($t$ = 90 min, no sonication). b) Influence of incubation time (water content $w_{H_2O,\ extr}$ = 31.5 wt%, no sonication).

were most rapidly extracted. After 20 min, already 75.8 wt% of the lipids were extracted whereas 66.5 wt% of the carotenoids remained in the biomass.

The observed polarity differences were exploited for a novel biorefinery strategy that integrates lipid-carotenoid separation into the extraction step (Fig. 8 a). We measured the extracted lipids, pigments, proteins and carbohydrates and used the data to model the mass flows (Fig. 8 b, the mass balance is provided in the SI). First, the biomass was extracted with 2-$BuOH_{79}$ ($t$ = 90 min). The resulting lipid-rich stream was further purified by phase separation which was triggered by water addition beyond the miscibility window. The organic phase was predominantly composed of lipids. The overall lipid yield was 79 wt% (based on the dry weight of the initial biomass). We observed an orange hue in the residual biomass due to carotenoid retention and partial extraction of the chlorophylls. After a cell disruption step, 2-$BuOH_{75}$ ($t$ = 90 min) extracts the residual pigments. Adding excess water to the pigment-rich stream leads to phase formation and carotenoid accumulation in the organic phase with an overall yield of 65 % (based on the dry weight of the initial biomass, note the orange hue of the extract). Carbohydrates were the main component in the aqueous phases of both phase separation steps. The biomass remaining after the second extraction step was predominantly composed of proteins. This novel approach represents a simple biorefinery approach for wet *P. tricornutum* biomass using 2-BuOH and $H_2O$ as green solvents under ambient conditions. The separation efficiency of carotenoid and lipids can be further optimized using design of experiments-approaches, including also the extraction rates. Mixing rates and biomass loading offer additional degrees of freedom for increasing the selectivity towards lipids. Furthermore, this approach should be tested for other microalgal species with a different cell wall composition.

### 3.5. Conclusions

High carotenoid and lipid yields drive the profitability of a *P. tricornutum* biorefinery. Our study clearly indicates that lipid extraction from wet microalgal biomass results in comparable yields to the dry route when the solvent is carefully selected. Combined with a cell disruption step, the carotenoid yields from the wet route could be boosted beyond dry route levels. The solvent selection was guided by a computational method, screening a database containing more than 8000

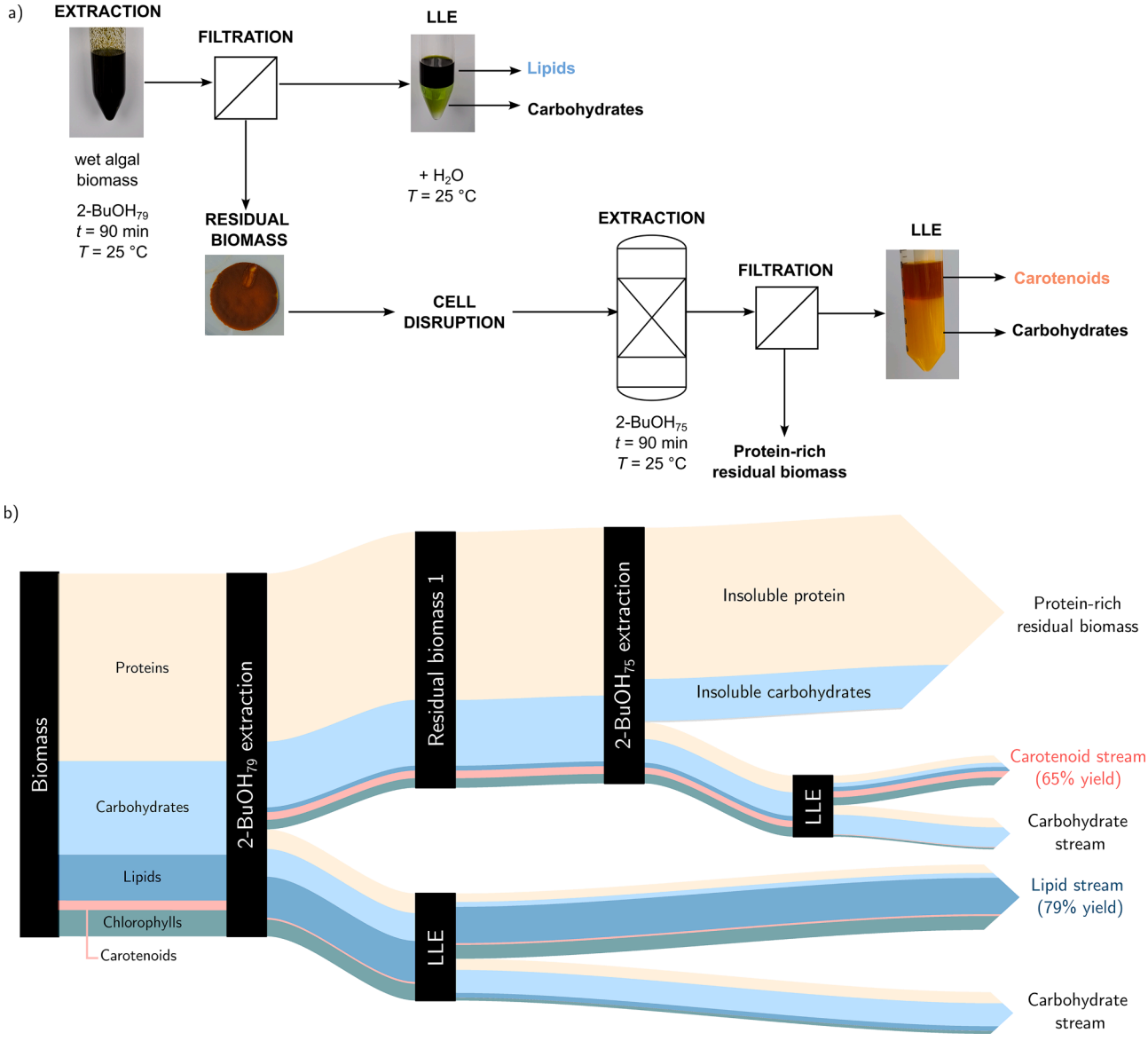


**Fig. 8.** Overview of the developed biorefinery process for *P. tricornutum*. a) Process flow diagram. b) Corresponding mass flows modelled on the basis of experimental measurements. The yields are based on the dry weight of the initial biomass.

solvents. Extraction experiments employing the computationally identified solvents showed that solvents with high water-miscibility, such as 2-BuOH, EtOH and acetone, obtained higher lipid yields despite having a lower NL solubility compared to nonpolar NWM solvents. When combined with a cell disruption step, the PWM solvent 2-BuOH achieved lipid and carotenoid yields of 99.4 and 82.6 wt%, on wet *P. tricornutum* biomass, respectively. However, even without cell disruption, lipid extraction with 2-BuOH was highly efficient (around 90 wt% yield). EtOH and acetone extracted carotenoids from wet *P. tricornutum* with carotenoid yields between 80 and 90 wt%.

By tuning the water content of 2-BuOH and exploiting its partial water-miscibility, we were able to fractionate wet *P. tricornutum* biomass into lipids, carotenoids, proteins and carbohydrates. The carotenoid-lipid separation was integrated into the extraction step. This integrated method represents a simple biorefinery approach for *P. tricornutum*, using only 2-BuOH and $H_2O$ as green solvents at ambient conditions. This approach uses wet biomass as a feedstock (moisture content ca. 81–85 wt%) and does not require energy-intensive biomass drying. The separation efficiency can be further increased by optimising the incubation time.

## 4. Methods

Details about computational methods, applied solvents, the cultivation and harvest of *P. tricornutum*, determination of the moisture, ash, lipid, carbohydrate and protein content, as well as lyophilisation are provided in the SI.

### 4.1. Extraction procedure for the wet and dry biomass

500 mg of wet algal paste (moisture content: 81-85 wt%) was weighed into 50 ml Pyrex tubes for wet extraction. For cell disruption, we added 2 ml of the solvent or solvent mixture and performed sonication for 2 min (amplitude = 80 %, cycle = 0.6, UIS250V combined with a LS24d7-L2 probe, Hielscher). After sonication, further 8 ml of solvent or solvent mixture was added to the sample. In experiments without sonication, a stir bar and 10 ml of the solvent system were directly added to the sample. For the extraction of dry biomass, 100 mg of lyophylized biomass (moisture content = 8.5 wt%), corresponding to the same mass of dry matter as in the wet extraction experiments, were used and 10 ml solvent or solvent mixture were added. The samples were incubated for 90 min on a magnetic stirrer (250 rpm). Subsequently, the samples were filtered through 0.2 µm PTFE syringe filters and 10 mg BHT was added. The extracts were stored at -20 °C until further analysis. The samples were protected from light during all steps. Lipid analysis was performed using gas chromatography (GC) [71], chlorophylls and carotenoids were quantified using high performance liquid chromatography (HPLC) [20]. The carbohydrate content was determined by the phenol–sulfuric acid method [72]. The proteins were quantified using the method of Lowry et al [73]. All details for the analyses are provided in the SI.

### 4.2. Yield

The yield $Y_{\text{fraction}}$ of the extracted biomass fractions is defined as

$$Y_{\text{fraction}}[\%] = \frac{m_{\text{fraction,extracted}}}{m_{\text{fraction,total}}} \bullet 100 \quad (2)$$

where $m_{\text{fraction,extracted}}$ denotes the mass of the fraction determined by the extraction experiments and $m_{\text{fraction,total}}$ represents the total mass of the fraction in the biomass (see Fig. 1 a for a detailed biomass composition).

### 4.3. Economic analysis

The economic value of each component *c* in the biomass was calculated as

$$\text{economic value}\,[€\,\text{kg}^{-1}_{\text{dry matter}}] = m_c \bullet \max(\text{price}_{\text{industy sector}}) \quad (3)$$

where $m_c$ represents the mass of component *c*. The model assumes the ideal separation of all biomass compounds and sales in the industry sector with the highest revenue. Therefore, the obtained economic value of the complete biomass reflects a benchmark. The model was parameterized based on a *Nannochloropsis* biorefinery [44] and adapted to *P. tricornutum* (see SI).

## Declaration of competing interest

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

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seppur.2024.129462

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