

Phaeodactylum tricornutum as a source of value-added products: A review on recent developments in cultivation and extraction technologies

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1 ***Phaeodactylum tricornutum* as a source of value-added products: A review on**  
2 **recent developments in cultivation and extraction technologies**

3  
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13 **Abstract**

14 Microalgae are photosynthesizing organisms that produce high-value metabolites by  
15 using CO<sub>2</sub> as a feedstock, potentially promoting sustainable manufacturing solutions.  
16 They are indeed rich in polyunsaturated fatty acids, pigments, polysaccharides, and  
17 proteins, which are widely employed in the pharmaceutical, nutraceutical, cosmetic, and  
18 food sectors. Among marine diatoms, *Phaeodactylum tricornutum* is one of the most  
19 studied species and commercially suitable strain for large-scale cultivation thanks to its  
20 capacity to accumulate commercially relevant metabolites such as EPA,  
21 chrysolaminarin, and fucoxanthin. It can successfully operate phototrophy, but  
22 mixotrophy mode was revealed to enhance the growth rate and biomass concentration  
23 and quality. This review collects recent findings in cultivation strategies and extraction  
24 technologies tested on this species so far. The culture performances are discussed and a  
25 comparison between mixotrophic and phototrophic conditions is provided. Finally,

26 successful biorefinery extraction cascades experimented on this diatom are described to  
27 foster research in this direction.

28 **Keywords:** *Phaeodactylum tricornutum*; algal cultivation; extraction technologies;  
29 value-added products; biorefinery concept

## 30 **1. Introduction**

31 The climate change consequences that we are forced to face nowadays demand urgent  
32 change in society, especially in the manufacturing and transport sectors. There is a need  
33 to replace petroleum-based commodities and fuels with biobased ones before the  
34 consequence of industrialization will become overwhelming for our planet.

35 Recent political strategies have been implemented to facilitate the transition to a circular  
36 bioeconomy model, able of ensuring both economic growth and preservation of natural  
37 resources. Hope and effort in this sense have been placed on microalgae, key players in  
38 blue biotechnology because they can offer a significant contribution to the building of  
39 this sustainable economy model by providing both low-value and high-volume  
40 products, and high-value and low-volume products.

41 Beyond the several microalgae strains that are currently studied at a research level,  
42 industrial-scale cultivations have also arisen to exploit the intrinsic capacity of some  
43 strains to accumulate specific metabolites, like  $\beta$ -carotene from *Dunaliella salina* or  
44 astaxanthin from *Haematococcus pluvialis*, not to mention the long-established use of  
45 algae as a food ingredient in the Asiatic continent.

46 The present review proposes a literature collection on *Phaeodactylum tricornutum*, a  
47 model diatom, which has been already examined at a laboratory scale and whose  
48 industrial potential has been revealed. The ultimate scope is to provide the optimal  
49 cultivations conditions that have arisen from numerous studies conducted through the

50 years and how these influence the final biochemical composition and its consequent  
51 applicability.

## 52 **1.1 Diatoms overview**

53 Diatoms are single-celled algae belonging to the group of eukaryotic phytoplankton that  
54 have existed for more than 180 million years. Present in many areas of the world, they  
55 are particularly widespread at high latitudes and in coastal ecosystems rich in nutrients  
56 (Malviya et al., 2016). They can have radial symmetry, in the case of centric diatoms, or  
57 bilateral symmetry as for pennate diatoms. These unicellular photosynthetic organisms,  
58 sizing from 1  $\mu\text{m}$  to 200  $\mu\text{m}$ , undertake significant roles in the major biogeochemical  
59 cycles, such as carbon sequestration, oxygen production, and nutrient recycling,  
60 especially nitrogen, carbon, and silicon. About 20% of global carbon fixation can be  
61 indeed ascribed to diatoms (Hockin et al., 2012) which produce, through their  
62 photosynthetic apparatus, more than 20% of the world's oxygen production.  
63 Furthermore, they provide a large amount of organic matter, about 25% of global  
64 biomass production, thus supplying nourishment to most other aquatic organisms (Not  
65 et al., 2012).

66 Given their global distribution, diatoms present high variability and diversification.  
67 Researchers often attempted to estimate the numbers of diatom species through time:  
68 the latest values are from 1800 planktonic species to 200000, more than any other single  
69 algal class. Because of their complex evolutionary history and genomic reorganization,  
70 diatoms have developed a range of potentially useful features, such as a rigid silicified  
71 cell wall called frustule, vacuoles for nutrient storage, fast reactions to changes in  
72 environmental conditions, resting stage formation, ice-binding proteins, and a urea cycle  
73 (Malviya et al., 2016).

74 The frustules are constituted of silicic acid and other organic materials, whose  
75 morphology may vary according to environmental factors and cell condition. The  
76 presence of silica in diatoms has also been related to the formation of diatomite, which  
77 has been commercially mined for many uses. Indeed, along geological periods, diatoms'  
78 death and decomposition entail the formation of sediments of silicon on the seafloor,  
79 which constitutes this diatomaceous earth or diatomite (Branco-Vieira et al., 2018).

80 Other valuable and interesting metabolites can be found in diatom chloroplasts, such as  
81 chlorophyll *a*, *c1*, and *c2*, and a complement of accessory pigments, including  
82 xanthophylls and carotenes. The light-harvesting pigments chlorophyll *a*, chlorophyll *c*,  
83 and fucoxanthin together constitute the complex fucoxanthin-chlorophyll protein (FCP),  
84 which has the function of harvesting the light necessary for diatoms. The pigment  
85 fucoxanthin is also responsible for the golden-brown color of these microalgae, being  
86 the most abundant pigment. Nowadays, seaweeds represent the main source of  
87 fucoxanthin at industrial scale. Nevertheless, marine diatoms accumulate 5-10 fold  
88 higher amounts (2.24 to 26.6 mg g<sup>-1</sup> DW) and thereby are regarded as a potential source  
89 of this valuable pigment (Pereira et al., 2021).

90 Carotenoids instead are involved in cell photoprotection when the variability in light  
91 intensity, typical of the marine ecosystem, threatens to assimilate overmuch light energy  
92 and, consequently, may compromise photosynthetic productivity through a mechanism  
93 called photoinhibition. This process, occurring when light energy is absorbed beyond  
94 the photosynthetic capacity of the cell, involves the production of reactive oxygen  
95 species (ROS) like hydroxyl radicals (OH•), superoxide anions (O<sub>2</sub><sup>-</sup>), hydrogen  
96 peroxide (H<sub>2</sub>O<sub>2</sub>), and singlet oxygen (<sup>1</sup>O<sub>2</sub>), that can provoke the oxidization of any  
97 pigment, lipid, or protein nearby, thus damaging the photosynthetic apparatus (Jallet et

98 al., 2016). To prevent photoinhibition diatom cells have developed an assortment of  
99 defence tools, such as the diadinoxanthin cycle associated with diadinoxanthin de-  
100 epoxidation and diatoxanthin epoxidation. The latter is indeed directly involved in the  
101 dissipation of energy through the action of Non-Photochemical Quenching (NPQ),  
102 which allows the photoacclimation of the light-harvesting system (Kooistra et al.,  
103 2007). The presence of diadinoxanthin, diatoxanthin, and additionally violaxanthin,  
104 antheraxanthin, and zeaxanthin can be noted especially in cells subjected to excessive  
105 light energy (Kuczynska et al., 2020).

106 Lipids and carbohydrates instead represent energy storage metabolites. In particular,  
107 carbohydrates can be sorted into insoluble and soluble. The first ones are structural  
108 carbohydrates that are frustule-associated sugars, such as sulphated glucoromannans,  
109 callose, and chitin; whereas, soluble carbohydrates are chrysolaminarin,  
110 exopolysaccharides or extracellular polysaccharides (EPS), and free sugars (Caballero  
111 et al., 2016). Among polysaccharides, chrysolaminarin constitutes the preferred energy  
112 storage for diatoms and normally accounts for 10-20% of the total carbon in cells at the  
113 exponential stage (Kroth et al., 2008). It is constituted of a backbone of  $\beta$ -1,3-D-glucose  
114 units linked together, with a degree of branching of 0-0.2 at position 6 and a degree of  
115 polymerization of 5-60. Its accumulation occurs in vacuoles inside the cell during the  
116 photosynthetically active light periods, while it is consumed in the dark to fuel  
117 heterotrophic metabolism (Caballero et al., 2016).

118 Regarding the lipidic fraction, different types of lipids can be detected in these  
119 microalgae: fatty acids, polar lipids, non-polar lipids, triacylglycerides (TAGs), steroids,  
120 and oxylipins. Diatoms' cell membrane is predominantly composed of fatty acids,  
121 which comprise between 15 and 25% of their biomass. Common in diatoms are the

122 saturated fatty acid myristic acid (C14:0) and palmitoleic acid (C16:1), belonging to  
123 monounsaturated fatty acids (MUFAs). Polyunsaturated fatty acids (PUFAs) are instead  
124 composed of 18-22 carbon chains with two or more double bonds. Stearidonic acid  
125 (SDA, 18:4),  $\alpha$ -linolenic acid (ALA, 18:3), eicosapentaenoic acid (EPA, 20:5) and  
126 docosahexaenoic acid (DHA, 22:6) belong to  $\omega$ -3 PUFAs group; while  $\omega$ -6 PUFAs are  
127 for example arachidonic acid (ARA, 20:4) and  $\gamma$ -linoleic acid (GLA, 18:3). PUFAs,  
128 particularly the  $\omega$ -3 category, have gained great visibility through the years thanks to  
129 their role as bioactive substances and as essential nutrients promoting human health.  
130 The relative amounts of storage carbon that accumulate in lipids and carbohydrates can  
131 be modified by altering the cell's physiological condition as a result of the breakdown  
132 of some biosynthetic pathways and the activation of new ones, involving increased  
133 production of lipids or carbohydrates. For example, nitrogen depletion has been a  
134 widely tested strategy to manipulate algal metabolism with the intent of favoring lipids  
135 production. It was also argued that a boosted production of TAG can be stimulated by  
136 blocking the polysaccharides metabolism through genetic engineering, as the resulted  
137 unplaced storage carbon would be relocated to other storage metabolites, in this case,  
138 lipids. (Caballero et al., 2016).

139 Hence, it is evident how diatoms can be a potential feedstock for numerous  
140 biotechnological purposes and an extensive line of applications in industry. Besides  
141 their interesting biochemical composition and ability to produce valuable metabolites,  
142 they exhibit a high capacity to thrive in both fresh and marine water and do not require  
143 fertile lands, if compared to biomass produced by terrestrial crops. Moreover, they  
144 rapidly adapt to changing nutrient conditions and provide high biomass production  
145 rates. Despite these advantages, their commercialization and industrialization still lack

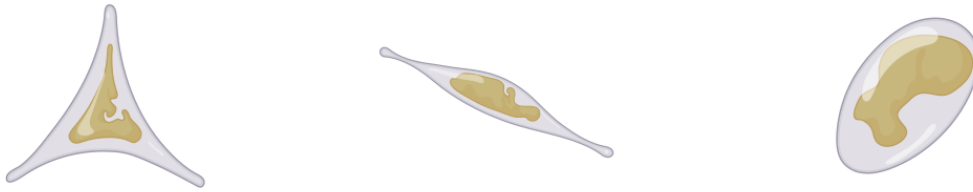
146 to be standardized at a large scale, mainly due to regulatory and technological barriers,  
147 such as the high water, energy, and nutrients consumption during the cultivation step  
148 and some technical hindrances to biomass recovery. Currently, the industrial production  
149 of microalgae for commercial purposes is related to a cost of 3.4 € kg<sup>-1</sup> for a  
150 hypothetical Spanish production plant (Penhaul Smith et al., 2020). However,  
151 production costs are subjected to variations depending on different factors, including the  
152 cultivation system (photobioreactors or open ponds), costs associated with workforce,  
153 operating costs, etc. Therefore, the gaps in the realization of large-scale cultivation,  
154 such as plant capability and dimension, and standardization of cultivation methods must  
155 be filled. The limits in the commercialization of high-value products need to be  
156 overcome by testing cheap and sustainable extraction technologies. Moreover, further  
157 studies are essential in the optimization of downstream processing leading to multiple-  
158 compound production in a biorefinery concept, which could ultimately enhance the  
159 economic viability.

## 160 **1.2 Properties and Potential of *Phaeodactylum tricornutum***

161 Among marine diatoms, *Phaeodactylum tricornutum* is one of the species examined to  
162 the highest extent and whose genome has been completely sequenced in 2008. It is a  
163 marine pennate diatom and belongs to the brown-algae division of Bacillariophyceae of  
164 Heterokontophyta. It contains about 36.4% of proteins, 26.1% of carbohydrates, 18.0%  
165 of lipids, and 15.9% of ash on dry weight, under ordinary cultivation conditions (Zhang  
166 et al., 2018). *P. tricornutum*, growing in brackish to saline water in several locations of  
167 the world, owns the peculiar feature to be polymorphic, thus exhibiting three principal  
168 morphotypes: oval, triradiate, and fusiform (**Figure 1**). Evidence is also present that a

169 fourth rarer morphotype exists, called cruciform, favoured by low temperatures, and  
170 converting to the oval type through the degeneration of arms (He et al., 2014).  
171 As a consequence of fluctuations in growth conditions, especially culture medium,  
172 salinity, pH, and temperature, the cells are subjected to reversible morphological  
173 conversions from one form to another. For instance, suboptimal conditions such as low  
174 temperature, hyposalinity, low light intensity, and nutrient limitation resulted to  
175 stimulate oval cell formation. Song et. al (2020) characterized ten strains of *P.*  
176 *tricornutum* and reported that Mann and Myers' medium favours morphotype transition  
177 from fusiform to oval, while the prevalence of fusiform cells was observed in f/2  
178 medium. Biochemical analysis proved that oval morphotype accumulated higher protein  
179 and pigments content, whereas lipids and carbohydrates were more abundant in  
180 fusiform cells. However, the latter exhibited a higher maximum specific growth rate  
181 ( $0.76 \text{ d}^{-1}$  against  $0.43 \text{ d}^{-1}$ ; doubling time  $0.92 \text{ d}$  against  $1.65 \text{ d}$ ) (Song et al., 2020).  
182 Thereby this study opens up the possibility of selectively growing a certain morphotype  
183 when specific final characteristics are desired for a particular industrial application.  
184 Oval cells are also characterized by harder walls (Young modulus equal to 500 kPa)  
185 compared to the other types (100 kPa) (Francius et al., 2008) and by siliceous frustules  
186 when silicic acid is included in the growth medium. Nonetheless, silicified substances  
187 are not indispensable for *P. tricornutum*, which can effectively reproduce also in  
188 absence of silica. Differences have been noted when comparing silicified fractions of  
189 the triradiate and fusiform with those of the oval type: fusiform and triradiate cells are  
190 characterized by silica bands; while only the oval ones hold silica valves typical of  
191 diatoms (Tesson et al., 2009).  
192

193



194

195 **Figure 1.** Morphotypes of *Phaeodactylum tricornutum* from left to right: triradiate,

196 fusiform, oval. (Created with Biorender)

197

198 Among other properties, *P. tricornutum* presents a high ability for the Non-  
199 Photochemical Quenching (NPQ) that dissipates the excess light in form of heat. This  
200 capacity was tested by using a sinusoidal light scheme for its cultivation and the  
201 outcome was that this diatom exhibited minimal indications of photoinhibition. Damage  
202 by photoinhibition can be measured as photosynthetic capability by the maximal  
203 quantum yield ( $F_v/F_m$ ) of photosynthetic system II (PSII), where  $F_m$  indicates  
204 the maximal fluorescence level and  $F_v$  is the variable fluorescence obtained by  
205 subtracting  $F_o$  (minimal fluorescence) to  $F_m$  (Jallet et al., 2016; Murchie and Lawson,  
206 2013). The study of Huete-Ortega et al. (2018) shows that light irradiance above 400-  
207 500  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  saturated PSII activity of *P. tricornutum*, independently of  
208 nitrogen-replete or -deplete conditions. Moreover, the NPQ response was not activated  
209 until approximately 200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  irradiance; therefore, an optimal light  
210 intensity of 213  $\mu\text{mol m}^{-2} \text{s}^{-1}$  was found for *P. tricornutum* in N-replete mode (Huete-  
211 Ortega et al., 2018).

212 An industrially relevant feature of *P. tricornutum* is that it is a fast-growing species,  
213 which makes it suitable to be cultivated at a large scale. To date, in Europe, it is  
214 cultivated by at least eight companies reaching a yearly production of 4 tons of dry  
215 biomass (Araújo et al., 2021). Among them, *P. tricornutum* is exploited for fucoxanthin  
216 production by AlgaTechnologies (Israel) and for EPA production by Simris (Sweden).  
217 Moreover, *P. tricornutum* extract and EPA-rich oil extract were proposed for  
218 authorization as novel food to the European Union, whose evaluations are still ongoing.  
219 This diatom presents a strong adaptation to a wide assortment of culture media,  
220 including Walnes, f/2, COMBO, M&M, and enriched seawater. It tolerates high  
221 pH/light intensities albeit growing also under poor light conditions (Butler et al., 2022).

222 In addition, it can be genetically transformed, and the employment of different genetic  
223 manipulations showed promising results in increasing lipid content. However, the  
224 exploitation of diatoms' lipids for biofuel production is far to be economically  
225 competitive on the market; thereby, priority should be given to multiple high-value  
226 compound production. The valorisation of all biomass components by extracting  
227 multiple algae metabolites, can improve the economic feasibility of this value chain and  
228 promote their industrial application (Branco-Vieira et al., 2020).

229 Even if less commercially competitive, healthful substances obtained from microalgae  
230 own specific advantages over standard synthetic alternatives: from a chemical point of  
231 view, synthetic molecules are only present in specific isomers, which are normally  
232 much less effective than natural versions for certain applications, such as in artificial  
233 milk, dietary supplements or fish pigment enhancers (Enzing et al., 2014). PUFAs such  
234 as EPA and DHA have been shown to improve eye health, and brain development, and  
235 perform cardiovascular and inflammatory disease prevention, anti-aging function, and  
236 treatment of psychiatric disorders. Currently, deep ocean fish oil represents the primary  
237 feedstock for EPA and DHA production; however, unpleasant smell, environmental  
238 impacts to the marine ecosystem due to overfishing, heavy metals toxicity, and  
239 considerable purification needs demanded sustainable fatty acids production alternatives  
240 (Kadalag et al., 2021). Moreover, only 0.2 million tons of EPA/DHA are obtained from  
241 fish sources, in the face of 1.3 million tons required for the world population, given the  
242 500 mg/day intake recommended by the World Health Organization (Wang et al.,  
243 2021). Hence, further studies to increase fatty acids accumulation in microalgae  
244 biomass are needed to facilitate their industrial application. Fucoxanthin also exerts  
245 multiple pharmacological bioactivities including anti-inflammatory, anti-obesity, anti-

246 diabetic effects, anti-cancer, and antioxidant (Song et al., 2020). Anti-tumor  
247 bioactivities were determined also in chrysolaminarin (Zhang et al., 2018).

## 248 **2. Cultivation strategies**

249 Microalgae are generally considered to be obligate photoautotrophs, by using sunlight  
250 and converting CO<sub>2</sub> to reduced carbon, thus producing oxygen and chemical energy  
251 through photosynthesis (Penhaul Smith et al., 2020). This encompasses a series of  
252 biochemical redox reactions which convert light energy into chemical energy in the two  
253 photosystems, PSI and PSII. The ATP and the reducing power (NADPH), resulting  
254 from the electron transport chain, are then metabolized in the Calvin–Benson–Bassham  
255 cycle (CBB) by the Rubisco enzyme (Villanova et al., 2017). Nevertheless, many  
256 microalgae can assimilate organic nutrients and grow in the dark by respiration or  
257 fermentation of exogenous sugars through *heterotrophy*. If the species can switch  
258 between heterotrophy and *phototrophy*, or employs both respiration and photosynthesis,  
259 then the cultivation is carried out in *mixotrophy*. However, the provision of an organic  
260 carbon compound will induce a variation in the cell carbon partitioning with relative  
261 consequences on metabolic pathways, growth phase, and final biochemical composition  
262 (Penhaul Smith et al., 2020).

263 Even if microalgae production through phototrophy could be a cheap process since only  
264 light energy is exploited, this cultivation mode entails some limitations such as the  
265 scarce maximum biomass production achievable because of self-shading effects and the  
266 subsequent drawbacks in optimizing temperature control, gas/liquid ratio, and light  
267 penetration. On the other hand, mixotrophy is considered a viable alternative to obtain  
268 high biomass productivity, considering the lower demand for light penetration.  
269 Moreover, by employing cheap and easily accessible organic carbon sources, including

270 wastewaters, the economic feasibility of the process can be even further enhanced  
271 (Villanova et al., 2021). The selection of the cultivation mode, and subsequently of the  
272 carbon sources in the case of mixotrophy and heterotrophy, depends on the species and  
273 the bioproducts of interest. Indeed, if PUFAs production through heterotrophy is  
274 feasible, the same can not be achieved for pigments since the production of  
275 photosynthesis-related compounds is strictly associated with the presence of light and it  
276 is thereby nullified in heterotrophic cultures (Cerón-García et al., 2013, 2005). In  
277 addition, some species could not be able to operate heterotrophic growth through dark  
278 sugar fermentation, which is the case of *P. tricornutum* which can be grown  
279 heterotrophically only upon metabolic engineering (Villanova et al., 2021).

## 280 **2.1 Phototrophic cultivation of *Phaeodactylum tricornutum***

281 Several photobioreactors (PBRs) types such as flat-plate, bubble-columns, and tubular,  
282 have been designed and tested to cultivate *P. tricornutum* phototrophically. Among  
283 these, flat-panel configurations have proven to accomplish low shear stress and  
284 adequate irradiation which ultimately resulted in higher biomass, EPA, and fucoxanthin  
285 productivities. Illumination constitutes a primary affecting element in biomass  
286 production and biochemical composition, together with light wavelength and light:dark  
287 cycle. The increase of light intensity usually involves the rise of the growth rate until it  
288 reaches a saturation value. Nonetheless, above this saturation value, the growth is  
289 inhibited by the production of harmful substances (i.e., ROS), involved in the  
290 photoinhibition process, which eventually cause a decrease in biomass productivity  
291 (Villanova et al., 2021). Generally, the photosynthetic efficiency in microalgae grown in  
292 phototrophic conditions is lower than theoretically achievable values, due to the  
293 complex nature of the culture system, in which various parameters influence differently

294 the growth and the biochemical composition, resulting in countertrend effects. (Butler et  
295 al., 2020). Maximum biomass productivities achieved with *P. tricornutum*, and the  
296 productivity of different target compounds obtained in phototrophic cultures are  
297 reported in **Table 1** and **Table 2**, respectively.  
298

Strain	Medium	Photobioreactor design	Maximum biomass productivity	Reference
UTEX 640 and UTEX 646	f	Green wall panel III ( $\leq 40$ L) outdoor	$17.80 \text{ g m}^{-2} \text{ day}^{-1}$ (nitrogen-replete culture)	(Gilbert-López et al., 2015)
FACHB-Collection	f/2	Indoor bubble columns photobioreactors (3 L)	$227.09 \text{ mg L}^{-1} \text{ day}^{-1}$	(Rodolfi et al., 2017)
UTEX 640	ASW	Outdoor parallel photobioreactors (51 L)	$> 0.3 \text{ mg L}^{-1} \text{ day}^{-1}$	(Song et al., 2014)
		PVC circular outdoor pond	$\sim 0.15 \text{ mg L}^{-1} \text{ day}^{-1}$	
EPSAG-Collection	f/2	Greenhouse PBR phytobags (3000 L)	$7.62 \text{ g m}^{-2} \text{ day}^{-1}$	(Silva Benavides et al., 2013)
CCAP 1055/1	Cell-Hi JWP	Indoor Airlift glass tubular photobioreactor (ALR) PhycoLift (8 L)	$1.57 \text{ g L}^{-1} \text{ day}^{-1}$	(Wang et al., 2018)
		Outdoor Airlift glass tubular photobioreactor (ALR) PhycoLift (8 L)	$0.93 - 1.13 \text{ g L}^{-1} \text{ day}^{-1}$	

299

300 **Table 1.** Biomass productivity of *P. tricornutum* strains in various photosynthetic  
301 systems.

302

Product class	Product	Strain	Product yield / productivity	Operational conditions	Reference
Lipids	EPA	EPSAG-Collection	280 mg m <sup>-2</sup> day <sup>-1</sup> 3.68% DW	Greenhouse PBR phytobags (3000L)	(Butler et al., 2022)
		CCAP 1055/1	3.31% DW	Airlift glass tubular photobioreactor (8 L)	(Wang et al., 2018)
	TAG	UTEX 640	58.5 mg L <sup>-1</sup> day <sup>-1</sup> 45% DW	Outdoor green wall panel III (≤40 L)	(Butler et al., 2022)
Carbohydrates	Chrysolaminarin	CAS	94 mg L <sup>-1</sup> day <sup>-1</sup> 14% DW	Indoor flat-plate (50 L)	(Rodolfi et al., 2017)
Pigments	Fucoxanthin	CAS	4.7 mg L <sup>-1</sup> day <sup>-1</sup> 0.7% DW	Indoor flat-plate (50 L)	(Gao et al., 2017)
		CCAP 1055/1	1.33% DW	Airlift glass tubular photobioreactor (8 L)	(Gao et al., 2017)

303

304 **Table 2.** Productivity of different target products from *P. tricornutum* strains in  
305 phototrophic cultures.

306

### 307        **2.1.1 Influence of culture parameters on lipids production and composition**

308    The content of bioactive molecules in microalgae can be remarkably affected by the  
309    culture conditions and the biochemical composition of biomass is subjected to  
310    fluctuations during the culture period. Qiao et. al (2016) performed phototrophic  
311    laboratory cultures of *P. tricornutum* Bohlin, from the Microalgae Culture  
312    Center (MACC), Ocean University of China, in f/2 medium in 500 mL flasks, irradiated  
313    by LED lamps working on a 14:10 h light:dark photoperiod and shaken at 100 rpm. The  
314    authors studied the effect of the variation in salinity (15; 20; 28; and 35 ppt), nitrogen  
315    concentration (1.24; 12.35; 24.70; and 49.40 mg L<sup>-1</sup>), light intensity (photosynthetic  
316    photon flux density PPF) (50, 100 and 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  with 14:10 h light:dark  
317    photoperiod) and temperature (15; 20; 25°C) over fatty acid composition. Under N-  
318    limitation conditions, *P. tricornutum* produced greatly MUFAs and saturated fatty acids  
319    at the expenses of PUFAs. However, this nutrient starvation also led to a significant  
320    decrease in biomass concentration. The lowest salinity caused the lowest total FAs  
321    production, but no variation in FAs composition was observed as a function of salinity.  
322    DHA accumulation was increased by rising irradiance, while DHA, EPA, and PUFAs  
323    decreased with increasing temperature. N-starvation, low salinity, and low temperature  
324    promoted the production of DHA at the expense of EPA. Ultimately, the best biomass  
325    productivity was achieved for 28 ppt of salinity, 12.35 mg L<sup>-1</sup> concentration of nitrogen,  
326    50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity, and 20°C (Qiao et al., 2016).

327    Even if nitrogen limitation can improve lipid contents in microalgae, higher biomass  
328    productivity can be reached in higher nitrogen concentrations; an optimum of 32.09  
329    mg/L NaNO<sub>3</sub> was found to be most favorable for algal biomass productivity (Yodsuwan  
330    et al., 2017).

331 An indoor flat-plate photobioreactor (50 L) was used to test and study the biochemical  
332 composition of *P. tricornutum* obtained from the Institute of Oceanography, Chinese  
333 Academy of Sciences. Two different nitrogen concentrations were assayed: 14.5 mmol  
334 L<sup>-1</sup> (HN) and 2.9 mmol L<sup>-1</sup> (LN) of KNO<sub>3</sub>. Continuous illumination provided by cool  
335 white fluorescent lamps at an irradiance of 300 μmol m<sup>-2</sup> s<sup>-1</sup>, 25 ± 1°C and air  
336 bubbling (enriched with 1% of CO<sub>2</sub>) were the operative conditions for the 12 days of  
337 growing. The maximum biomass concentration was obtained under HN amounting to  
338 4.05 g L<sup>-1</sup>. However, lipid accumulation was accelerated under the LN condition,  
339 which achieved 42.48% DW of total lipids (TLs), compared to 36.69% in HN.  
340 Regarding the lipids profile, at first, glycolipids were the components more abundant of  
341 TLs under both HN and LN. Neutral lipids, mainly TAGs, increased as culture time  
342 proceeded and amounted to 63.84% and 75.7% of TLs in HN and LN conditions. It was  
343 also noted that as the growth period was protracted, the proportion of PUFAs  
344 experienced a decrease, while saturated fatty acids increased.  
345 Arachidonic acid (C20:4), EPA (C20:5), oleic acid (C18:1), linoleic acid (C18:2),  
346 palmitic acid (C16:0), palmitoleic acid (C16:1), and myristic acid (C14:0) were the fatty  
347 acids detected in most abundance. C16:1 increased from 15.55% to 38.67% of total  
348 fatty acids in HN treatment and from 15.40% to 40.96% in LN. C20:5 content  
349 drastically declined with culture time (Gao et al., 2017).  
350 Besides medium composition, important design considerations for the cultivation  
351 system should be accounted for. For example, when designing a photobioreactor  
352 system, a parameter to be considered as influencing growth performances is the Gas-  
353 Liquid Ratio (GLR). A certain value of gas-liquid ratio can assure the proper culture  
354 mix, thus preventing sedimentation, increasing the mass transfer, facilitating the

355 assimilation of nutrients by the cells, and forcing them to shift from dark to light zone.  
356 However, an excessive degree of the GLR may damage cells due to mechanical shear  
357 stress and cause the evaporation of the culture medium. *P. tricornutum* from the  
358 freshwater algae culture collection of the Institute of Hydrobiology in China (FACHB-  
359 Collection) was grown testing different GLR values to find the optimum experimental  
360 condition. A GLR of 1.5 vvm allowed to reach 0.5 d<sup>-1</sup> as specific growth rate and  
361 227.09 mg L<sup>-1</sup>d<sup>-1</sup> as biomass productivity, which were the highest values obtained along  
362 with the highest lipid productivity of 48.48 mg L<sup>-1</sup>d<sup>-1</sup>. (Song et al., 2014).

### 363 **2.1.2 Influence of culture parameters on fucoxanthin production**

364 Fucoxanthin accumulation in *P. tricornutum* was studied as influenced by several  
365 experimental factors. In particular, it was found to be decreasing as the culture time was  
366 prolonged, and the decline was accelerated by nitrogen deficiency (Gao et al., 2017). In  
367 contrast, low light intensities have been found to promote higher production of  
368 fucoxanthin. For instance, a light intensity of 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$  on *P. tricornutum* (CS-  
369 29) supplied by the Australian National Algae Culture Collection (ANACC), cultured in  
370 5 L photobioreactors with f/2 medium at  $25 \pm 3$  °C, resulted in a greater specific  
371 fucoxanthin concentration ( $42.8 \pm 19.5 \text{ mg g}^{-1}$ ) than at  $210 \mu\text{mol m}^{-2} \text{s}^{-1}$  ( $9.9 \pm 4.2 \text{ mg}$   
372  $\text{g}^{-1}$ ). This behaviour can be explained by considering that the production of this light-  
373 harvesting pigment is boosted when low light conditions are applied. Conversely, as the  
374 light intensity increases, fucoxanthin's content decreases on behalf of photoprotective  
375 pigments. In addition, the composition of growth medium was examined as affecting  
376 the growth and fucoxanthin accumulation, after choosing a working irradiance of 150  
377  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . For this purpose, besides f/2 standard recipe, two other media were  
378 prepared:  $10 \times \text{f/2}$ , where all nutrients' concentrations were increased by 10-fold, and

379 f/2 with only nitrate concentration increased by 10-fold. Cells cultivated with the first  
380 enriched medium resulted in a 2-fold final dry cell weight ( $0.59 \pm 0.10 \text{ g L}^{-1}$ ) compared  
381 to standard f/2 medium ( $0.29 \pm 0.03 \text{ g L}^{-1}$ ), but a lower specific growth rate.  
382 Fucoxanthin concentration was remarkably increased by the medium with nitrate  
383 supplementation reaching the maximum of  $59.2 \pm 22.8 \text{ mg g}^{-1}$  dry algae, against  $23.2 \pm$   
384  $7.0 \text{ mg g}^{-1}$  produced in f/2 medium and  $26.7 \pm 13.1 \text{ mg g}^{-1}$  in  $10 \times \text{f/2}$  medium. These  
385 results clearly indicate that fucoxanthin accumulation in *P. tricornutum* is promoted by  
386 supplementation of nitrate in culture medium, even if further investigations are required  
387 to understand the processes by which this happens (McClure et al., 2018). Yet, it is  
388 worth emphasizing that cellular growth and fucoxanthin production are not directly  
389 related as the best culture conditions do not always lead to the highest fucoxanthin  
390 production (Gómez-Loredo et al., 2016).

### 391 **2.1.3 Influence of culture parameters on carbohydrates production**

392 In all algae species, carbohydrates represent the first photosynthetic product exiting the  
393 Calvin–Benson cycle, acting as precursors for all cell components. Initially,  
394 carbohydrates' production is significant and then decreases due to their conversion into  
395 other metabolites required by cells. The evolution of total carbohydrate and  
396 chrysolaminarin contents in *P. tricornutum* was studied by Gao et. al (2017), who  
397 concluded that major amounts of carbohydrates are obtained when cells are in their  
398 exponential growth phase, rather than in cells entering the stationary phase. Besides, N-  
399 deplete conditions have proven to induce a fast accumulation of these metabolites.  
400 The maximum carbohydrate and chrysolaminarin amounts, under  $14.5 \text{ mmol L}^{-1}$  of  
401 nitrogen, were 21.2% and 17.1% of DW, respectively (Gao et al., 2017).

402 Carbohydrates' accumulation is favoured also by higher light intensities since their  
403 degradation takes place in the dark. This was confirmed also by Chauton et al. (2013)  
404 who found that carbohydrate content in *P. tricornutum* (CCMP 2561) increased with  
405 prolonged illumination or extended light phase considering that the phase in which they  
406 are produced is longer, while the phase associated with carbohydrates consumption  
407 (darkness) is reduced. Moreover, carbohydrates concentration was increased in cells  
408 grown in N-depletion rather than in P-depletion. (Chauton et al., 2013). The study of  
409 daily fluctuation in biomass composition of *P. tricornutum* CCAP 1055/1 performed by  
410 Jallet et al. (2016) allowed also to state that carbon dislocation between diverse  
411 metabolites varies remarkably during the light: dark cycle. For instance, biomass was  
412 found richer in TAGs and carbohydrates at the end of the light phase if compared to its  
413 start, while the reverse condition resulted in proteins. These daily trends should be  
414 accounted for when selecting the right moment to harvest, extract a specific metabolite,  
415 or for measuring daily productivity (Jallet et al., 2016). Similar results were obtained on  
416 the same strain by Caballero et al. (2016) who presented an experimental procedure to  
417 simultaneously characterize and resolve insoluble, soluble reducing, and soluble  
418 nonreducing carbohydrates from *P. tricornutum*, to study the carbon partitioning during  
419 nitrogen replete and nitrogen deplete conditions. They confirmed, through  
420 compositional and structural analysis, the hypothesis that chrysolaminarin belongs to  
421 the soluble nonreducing carbohydrate portion.

422 Moreover, by comparing laminarin and chrysolaminarin structures, they observed that  
423 *P. tricornutum*'s chrysolaminarin is smaller and lightly branched. During nitrate  
424 starvation, chrysolaminarin was produced to store only 4.9–6.0% of cellular carbon,  
425 which is definitively lower if compared to the TAG accumulation amounting to 43–

426 50%. This result led the authors to the conclusion that chrysolaminarin is not the  
427 preferred storage product during nitrogen limitation. Ultimately, nutrient replete  
428 conditions and high irradiance allowed high accumulation of chrysolaminarin in *P.*  
429 *tricornutum*, which occurs at night in a 12:12 light:dark cycle, while its consumption is  
430 completed by dawn (Caballero et al., 2016).

## 431 **2.2 Mixotrophic cultivation of *Phaeodactylum tricornutum***

432 Mixotrophic cultivation mode, occurring when an organic carbon source is supplied  
433 concurrently with the use of light, allows benefiting from the main advantages of both  
434 phototrophy and heterotrophy. This cultivation procedure can hence lead to high  
435 biomass productivity while enriching microalgal biomass with the profitable  
436 photosynthesis-related metabolites, which are normally missing in heterotrophic mode  
437 (Cerón-García et al., 2013).

438 It is noteworthy that mixotrophy demands proper uptake proteins that enable the  
439 metabolism of the chosen carbon source. If their upregulation and expression employ  
440 too much time, a lag phase in population growth may result (Penhaul Smith et al.,  
441 2020). Nevertheless, compared to phototrophic cultures, growth rates, and biomass  
442 production increase thanks to the synergy established between light and the organic  
443 source. In mixotrophic cultures, the cellular composition varies according to the period  
444 that cells spend in dark and enlightened areas, or, in other words, the respective  
445 contribution of the heterotrophic and phototrophic metabolisms to the total growth.  
446 In high-density cultures, occurring for high cell concentrations, the light fails to reach  
447 efficaciously all cells so that the contribution of the phototrophy to the overall growth  
448 rate decreases and the consumption of the organic carbon source becomes sustaining the  
449 growth. Even if mixotrophy is a promising alternative culture method to improve

450 biomass productivity, its potential and applicability can vary according to the target  
451 metabolite. An optimal balance exists indeed between phototrophic and heterotrophic  
452 growth for the optimal productivity of every specific compound (Cerón-García et al.,  
453 2013, 2005, 2000). Recent studies tried to unravel the molecular function of mixotrophy  
454 by concluding that the optimized photosynthetic efficiency in diatoms is achieved  
455 through cooperation between mitochondria and plastids. Actually, the synergic actions  
456 of these organelles, through exchanges of ATP and NADH, enable close coordination  
457 between respiration and photosynthesis, thus simultaneously improving the uptake of  
458 light and organic carbon during mixotrophy (Villanova et al., 2017).

459 Several organic compounds such as glycerol, acetate, glucose, fructose, starch, and  
460 glycine can be used to cultivate the model diatom *P. tricornutum*, and some studies  
461 tested this capacity through the years. **Table 3** shows maximum biomass productivities  
462 and concentrations arising from the use of different organic sources on *P. tricornutum*  
463 under mixotrophic conditions. In **Table 4** the maximum productivities of diverse  
464 compounds obtained from *P. tricornutum* in mixotrophy conditions are collected.  
465

Strain	Organic carbon source	Maximum biomass productivity	Maximum biomass concentration	Reference
<b>UTEX 640</b>	Glucose 5 g L <sup>-1</sup>	10.70 mg L <sup>-1</sup> h <sup>-1</sup>	2.01 g L <sup>-1</sup>	(Cerón-García et al., 2005)
	Acetate 0.05 M	13.2 mg L <sup>-1</sup> h <sup>-1</sup>	1.15 g L <sup>-1</sup>	
	Glycine 0.01 M	11 mg L <sup>-1</sup> h <sup>-1</sup>	2.46 g L <sup>-1</sup>	
	Starch 1 g L <sup>-1</sup>	11.3 mg L <sup>-1</sup> h <sup>-1</sup>	1.79 g L <sup>-1</sup>	
	Lactic acid 0.005 M	7.73 mg L <sup>-1</sup> h <sup>-1</sup>	2.18 g L <sup>-1</sup>	
	Glycerol 0.1 M	17.5 mg L <sup>-1</sup> h <sup>-1</sup>	2.99 g L <sup>-1</sup>	
<b>CCAP 1055/1</b>	Glycerol 0.05 M	-	11.55 g L <sup>-1</sup>	(Cerón-García et al., 2005)
<b>UTEX 640</b>	Fructose 0.02 M	6.8 mg L <sup>-1</sup> h <sup>-1</sup>	8.2 g L <sup>-1</sup>	(Villanova et al., 2021)
	Glycerol 0.1 M	14 mg L <sup>-1</sup> h <sup>-1</sup>	14 g L <sup>-1</sup>	

466

467 **Table 3.** Maximum biomass productivity and concentration of *P. tricornutum* on

468 different organic sources under mixotrophic cultures.

469

Product	Strain	Organic carbon source	Product yield / productivity	Reference
EPA			9.51 ± 0.13 mg L <sup>-1</sup> d <sup>-1</sup>	
Fucoxanthin	CCAP 1055/1	Glycerol	1.97 ± 0.34 mg L <sup>-1</sup> d <sup>-1</sup>	(Butler et al., 2022)
FAMEs			51.96 ± 0.61 mg L <sup>-1</sup> d <sup>-1</sup>	
EPA		Glycerol	8.56 mg L <sup>-1</sup> d <sup>-1</sup>	
		Glycerol 0.1 M + Urea	11.53 mg L <sup>-1</sup> d <sup>-1</sup>	
Total Pigments	UTEX 640	Lactic acid	4.45% DW	(Cerón-García et al., 2005)
Chlorophylls		Lactic acid	3.79% DW	
Carotenoids		Starch	1.04% DW	

470

471 **Table 4.** Productivity of different target products from *P. tricornutum* strains in

472 mixotrophic cultures.

473

### 474        2.2.1 Glycerol

475        The effect of glycerol on *P. tricornutum* metabolism has been assessed via  
476        transcriptomics, metabolomics, metabolic modelling, and physiological studies. Results  
477        point out that carbon-storage, central-carbon, and lipid metabolism are all affected by  
478        the presence of this compound. In metabolic pathways of *P. tricornutum*, glycerol  
479        converts to glycerol phosphate, through the action of glycerol kinase, which constitutes  
480        the precursor for TAG synthesis, and to dihydroxyacetone phosphate, through the action  
481        of glycerol-3-phosphate dehydrogenase, in the glycolysis and pentose phosphate  
482        pathway (PPP), thus influencing the central-carbon metabolism. In the case of  
483        phototrophy, the diatom's carbon requirement is instead satisfied by the consumption of  
484        the inorganic carbon source occurring in the Calvin cycle, characterized by higher  
485        enzymatic and light energy costs. The supply of glycerol simulates the characteristic  
486        effects of N-starvation on lipid production, contributing to the triacylglycerol and fatty  
487        acid accumulation. Nevertheless, unlike nitrogen-deplete conditions which affect  
488        biomass productivity, the presence of glycerol neither decreases photosynthetic ability  
489        nor diatom's growth (Villanova et al., 2021, 2017).

490        Cerón-García et al. (2000) reported the EPA production from *P. tricornutum* UTEX 640  
491        strain under mixotrophic conditions using glycerol and studied the effect of different  
492        glycerol concentrations (0.005; 0.01; 0.05; and 0.1 M) and subsequent additions of  
493        glycerol and ammonium chloride on growth kinetics, biomass productivity, and fatty  
494        acid profiles. The authors used one-liter flasks with enriched seawater medium that  
495        were sparged with filter-sterilized air (0.22µm Millipore filter) at 0.1 v/v min<sup>-1</sup> to mix  
496        the suspension. The cultures were continuously illuminated at an irradiance of 165 µmol  
497        photons m<sup>-2</sup> s<sup>-1</sup> at a temperature of 20 ± 0.5°C. The optimal initial concentration of

498 glycerol was determined as 0.1 M, which resulted in maximum biomass productivity  
499 and concentration of  $17.5 \text{ mg L}^{-1} \text{ h}^{-1}$  and  $2.4 \text{ g L}^{-1}$ , respectively. However, a lag phase  
500 lasting for the first 160 h of growth was observed for cultures grown with higher  
501 glycerol concentrations; they indeed maintained a biomass concentration below that of  
502 the control (in absence of glycerol). No lag phases were observed for lower glycerol  
503 concentrations, which indicates that this physiological response was due to an  
504 adaptation period to a high glycerol environment. Once the optimal glycerol  
505 concentration was established, a fed-batch culture was performed by operating  
506 sequential additions of ammonium chloride and glycerol. In these conditions, the  
507 resultant EPA productivity equal to  $33.5 \text{ mg L}^{-1} \text{ d}^{-1}$  was recorded as the highest  
508 obtained (Cerón-García et al., 2000).

509 In another study, different pilot-scale photobioreactors systems were used for the  
510 cultivation of *P. tricornutum* UTEX 640 strain, grown in fed-batch and under  
511 mixotrophic conditions: outdoor vertical bubble column (BC), draft-tube airlift (DT),  
512 and split-cylinder (SC), each of 60 L working volume. The diatom was grown on a  
513 modified Ukele medium supplemented with an initial 0.1 M of glycerol, with  
514 subsequent additions of nutrients (glycerol, ammonium, urea), which instantaneously  
515 improved the growth rate. Best performance in terms of maximum biomass  
516 concentration was reached in SC photobioreactor with  $25.4 \text{ g L}^{-1}$ . The supplied organic  
517 nutrient did not significantly affect carotenoids and chlorophylls content since they  
518 remained at 0.4-0.8% and 1.5-2.0% DW respectively. EPA content raised to 3% DW  
519 reaching a productivity of  $56 \text{ mg L}^{-1} \text{ d}^{-1}$  (Fernández Sevilla et al., 2004).

520 Furthermore, *P. tricornutum* (CCAP 1055/3 axenic strain) was cultivated in a 250 mL  
521 Erlenmeyer using an artificial seawater medium enriched with extra nitrogen and

522 phosphorus (ESAW) to obtain 0.47 g L<sup>-1</sup> N and 0.03 g L<sup>-1</sup> P. Cultures were conducted  
523 in phototrophy with N-depletion (PHOT-N) shaken at 100 rpm and 20°C, under a light  
524 intensity of 40 μE m<sup>-2</sup> s<sup>-1</sup> with a 12h:12h light:dark cycle. Regarding mixotrophic tests,  
525 glycerol was supplemented to the medium to reach a final concentration of 50 mM in  
526 both N-replete and N-deplete tests. Glycerol enhanced biomass production by 2-fold  
527 compared to growth in PHOT medium, favoring nitrogen and phosphate consumption,  
528 and did not inhibit the photosynthetic ability. Moreover, its presence affected cellular  
529 lipid content by increasing TAG accumulation in both nitrogen-replete and starved  
530 cells. These results suggest that glycerol act as TAG-booster by providing the glycerol  
531 backbone and the acyl groups required for TAG assembly (Villanova et al., 2017).  
532 In a follow-up study, the optimized ESAW medium (added with sodium bicarbonate  
533 and extra nitrogen and phosphorous) was used to grow *P. tricornutum* (CCAP 1055/3  
534 strain) in mixotrophy with glycerol (4.6 g L<sup>-1</sup>) in a 2 L photobioreactor sparged  
535 continuously with air at a flow rate of 0.5 L min<sup>-1</sup> and under a controlled temperature  
536 of 20°C. Cultures were continuously illuminated by a variable light intensity of 70 to  
537 300 μmol m<sup>-2</sup> s<sup>-1</sup>. These fluorescence studies were conducted to determine the light  
538 dependency of NPQ: a value of 400 μE was found as sufficient to obtain the maximum  
539 value of fluorescence, without provoking damage to the photosynthetic system. The  
540 biomass concentration was found to increase by a factor of 9 in the optimized medium  
541 compared to the conventional enriched seawater medium. Moreover, both biomass  
542 quantity and quality were enhanced by the provision of the organic and inorganic  
543 (NaHCO<sub>3</sub>) carbon sources. Both respiration and photosynthesis performances were  
544 improved in these mixotrophic tests, confirming the energetic coupling of these two

545 metabolic processes. Ultimately, the optimized medium allowed to obtain higher  
546 productivity in biomass and lipids compared to other studies (Villanova et al., 2021).

### 547 **2.2.2 Other carbon sources**

548 Other carbon sources tested on *P. tricornutum* under mixotrophic conditions are acetate,  
549 glucose, fructose, starch, glycine, and lactic acid. Acetic acid is metabolized bonded to  
550 acetyl-CoA protein and carried in the glyoxysome, by entering the TCA cycle  
551 (tricarboxylic acid cycle) in the mitochondria, or directly used for fatty acid  
552 biosynthesis, which provides an increase in respiration and carbohydrate accumulation  
553 (Penhaul Smith et al., 2020).

554 *P. tricornutum* UTEX-640 strain growth was tested on different substrates at diverse  
555 concentrations in fed-batch and discontinuous modes (fed-batch only for glycerol). The  
556 nutrients assayed were glucose (0.5 to 5 g L<sup>-1</sup>), acetate (0.005 to 0.1 M), glycerol (0.005  
557 to 0.1 M), lactic acid (0.005 to 0.1 M), starch (0.5 to 5 g L<sup>-1</sup>), glycine (0.005 to 0.02  
558 M). Filter-sterilized air at 0.1 vvm was used to mix and aerate the cultures. These were  
559 constantly irradiated at 165 μmol m<sup>-2</sup> s<sup>-1</sup> and maintained at 20 ± 0.5°C. The authors  
560 studied biomass productivity and biochemical composition. Experiments with acetate  
561 indicated that growth inhibition occurs above 5 mM: growth was decelerated, and  
562 biomass concentration and productivity declined. Contrastingly, growth was stimulated  
563 by all starch concentrations tested, and only a slight inhibitory response was reported  
564 for concentrations above 2 g L<sup>-1</sup> of starch. Lactate consistently promoted growth in  
565 most experiments, gaining the maximum biomass concentration for 5 mM; however, a  
566 possible inhibition was detected for 0.1 M of lactate which induced the decrease of final  
567 biomass concentration. All concentrations of glucose resulted in a stimulating effect for  
568 the growth, although not very intense. The study of the organic substrate as influencing

569 metabolites production led to varied results. If lactate, glycine, glycerol, and starch  
570 stimulated the biosynthesis of total pigments on one side, an opposite effect was  
571 observed for glucose. Same trends were found for chlorophyll contents. EPA  
572 productivity was remarkably influenced by nutrient source: lactate and glucose  
573 increased EPA content, while starch decreased it. Glycine had no significant influence  
574 on cellular EPA concentration (Cerón-García et al., 2005). Another study compared the  
575 mixotrophic cultivation of *P. tricornutum* UTEX-640 strain grown on fructose and  
576 glycerol. These cultures were performed in bubble photobioreactors (2 L) in fed-batch  
577 mode, with enriched seawater as medium, temperature maintained at  $20 \pm 0.5^\circ\text{C}$ , use of  
578  $\text{CO}_2$  for pH control, and a sparging air flow rate of 1.5 vvm. Diverse nutrient-supply  
579 procedures, along with a sequential increase of irradiance ( $280$  to  $750 \mu\text{E m}^{-2} \text{s}^{-1}$ ) were  
580 assayed. At first, the batch mode was employed to initiate the cultures; then, when cells  
581 enter the stationary stage, the nutrient supply and/or irradiance increment was executed.  
582 During the first part of the cultivation (117 h), the biomass concentration raised  
583 similarly to phototrophic control, and no fructose was consumed, indicating that high  
584 percentages of inorganic carbon ( $\text{CO}_2$  and  $\text{HCO}_3^-$ ) can force the diatom to grow  
585 phototrophically, which, in turn, inhibits the assimilation of organic carbon. The  
586 consumption of fructose was initiated when the  $\text{CO}_2$  injection for pH monitoring was  
587 discontinued; consequently, cell growth and biomass concentrations immediately raised  
588 upon the organic C source consumption. Despite this, final biomass concentrations of  
589 mixotrophic cultures with fructose were lower than the phototrophic control (Cerón-  
590 García et al., 2013).

### 591 **2.2.3 Waste effluents**

592 Recently, industrial, agricultural, and municipal wastewater effluents have been  
593 efficaciously tested for microalgae cultivation and production. There is no lack of  
594 studies testing the growth performances of *P. tricornutum* by adding wastewater  
595 effluents to the culture medium. The recovery of nutrients contained in these types of  
596 effluents can reduce the cost of fertilizers and has the advantage of reducing the amount  
597 of nutrient-rich digestate to dispose of, while eventually lowering the costs associated  
598 with microalgae cultivation thus promoting a circular economy approach (McDowell et  
599 al., 2020; Su et al., 2020). *P. tricornutum* batch cultures were grown mixotrophically  
600 with glycerol as carbon source and ultrafiltered digestate (UF) from a biogas plant as  
601 nitrogen source, testing different concentrations of glycerol (0.02; 0.03; 0.04 M). In  
602 their study, the strain SAG1090-1a was cultivated in a 0.5 L photobioreactor at 25°C, at  
603 a constant air flux of 10 L min<sup>-1</sup>, 312 μmol m<sup>-2</sup> s<sup>-1</sup>, and a 12h:12h photoperiod. CO<sub>2</sub>  
604 injection was used to control pH at 8 and the f/2 culture medium was added with UF  
605 and different concentrations of glycerol. The highest biomass concentration reached was  
606 4.96 g L<sup>-1</sup> corresponding to an addition of 0.04 M of glycerol. Biomass productivity  
607 increased by 1.29 and 1.60 times, compared to the control, with 0.03 M and 0.04 M of  
608 glycerol respectively. The balance study performed on nitrogen demonstrated that UF  
609 supplement increased nitrogen availability, thus promoting algal growth and protein  
610 accumulation. Regarding diatom's biochemical properties, the highest glycerol  
611 concentrations caused a decline in protein content due to N limitations, while lipid  
612 content did not show variability as UF and/or glycerol were provided. However, the  
613 fatty acid profile changed compared to the phototrophic control: under mixotrophic  
614 conditions, saturated fraction increased at the expense of ω-3 and PUFAs contents. The  
615 supply of glycerol was found to switch the metabolism to carbohydrate production,

616 whose content displayed a remarkable increase. Nevertheless, the authors reported that  
617 carbohydrates' increase and proteins' decrease were also a consequence of nitrogen  
618 starvation induced by microbial competition, which forced the diatom towards  
619 carbohydrates accumulation (Su et al., 2020).

620 Anaerobic digestate also represents a rich source of nitrate, ammonium, phosphorous,  
621 and trace micronutrients required for microalgae growth, although high concentrations  
622 of ammonium ( $> 80\text{-}100\text{ mg L}^{-1}$ ) can potentially inhibit it. Three AD plants were  
623 selected to investigate how their effluents affect the biochemical profile of *P.*  
624 *tricornutum*: (1) cow slurry and grass silage (CW); (2) pre-consumer food waste (FW);  
625 (3) pig slurry and grass silage (PW). 5 L photobioreactors irradiated by  $170\text{ }\mu\text{mol m}^{-2}$   
626  $\text{s}^{-1}$  at  $20 \pm 1\text{ }^{\circ}\text{C}$ , were used for the trials. *P. tricornutum* CCAP 1052/1B strain was  
627 cultivated in digestate media of different concentrations obtained by supplementing 1%,  
628 3%, or 6% v/v liquid digestate stream to seawater. All these media provided enough  
629 nourishment for *P. tricornutum* cells, whose growth rate was similar or higher to the  
630 ones reached in the f/2 control. Initial lag phases of cell adaptation were observed for  
631 higher concentrations of effluent. All concentrations of CW and FW resulted in a  
632 considerable enhancement of crude protein production compared to the control. CW 1%  
633 produced microalgae richer in  $\omega$ -3 and EPA, compared to PW 1%, but similar to  
634 control. Major quantities of fatty acids DW were obtained in microalgae grown in lower  
635 levels of digestate concentrations, being CW 1% and PW 1% the ones to produce the  
636 highest values of total fatty acids. Ultimately, CW 1% digestate has proved to be the  
637 best digestate in terms of the total fatty acids and proteins accumulation (McDowell et  
638 al., 2020).

639 The suitability of this diatom to grow also in municipal wastewater (MW) mixed with  
640 seawater (SW) in compositions MW:SW = 1:1 and MW:SW = 2:1 was tested  
641 successfully. *P. tricornutum* CCMP632 strain was grown at 18 °C and 120  $\mu\text{mol m}^{-2} \text{s}^{-1}$   
642 under a 12h:12h photoperiod. The final biomass increased by about 40% compared to  
643 f/2 control, indicating the possibility of using this type of effluents to grow the species.  
644 Growth performances were further enhanced in cultures supplemented with air aeration,  
645 which also facilitated lipids accumulation. MW: SW=1:1 and f/2 medium tests allowed  
646 to obtain the highest quantities of lipid (34.4% and 35.5%); furthermore, MW: SW=1:1  
647 condition accomplished also the highest lipid productivity (33.20  $\text{mg L}^{-1} \text{day}^{-1}$ ) (Wang  
648 et al., 2019). Besides the employment of wastewater effluents, the recycling of CO<sub>2</sub> flue  
649 gases for growing microalgae represents a further step towards the exploitation of waste  
650 resources in a circular economy perspective. Simonazzi et al. (2019) evaluated the  
651 viability of employing both waste CO<sub>2</sub> and a pre-treated digestate to cultivate *P.*  
652 *tricornutum* and optimize growth and  $\omega$ -3 productivity in the successive scale-up.  
653 Biomass productivity and accumulation of PUFAs were not altered when using waste  
654 CO<sub>2</sub>. *P. tricornutum* growth was resulted completely supported by the pre-treated  
655 digestate and waste CO<sub>2</sub>, without the risk of inhibition, to a final biomass density of 1-  
656 1.2  $\text{g L}^{-1}$ . The biochemical composition of resulted biomass after 15 days of culture  
657 was: proteins 36-37%, lipids 34-35%, and polysaccharides 5-6%. The authors reported  
658 no differences between biomass grown with flue gas and biomass grown with pure CO<sub>2</sub>.  
659 (Simonazzi et al., 2019).  
660 Other research has also proved the opportunity of growing diverse microalgae species  
661 on such effluents, thus potentially reducing the high costs necessary to sustain their  
662 industrial production, in terms of nutrient needs (representing 30–40% of operating

663 costs). At the same time, this solution would allow to purify wastewater effluents and  
664 eventually dispose of CO<sub>2</sub> off-gas thus also promoting climate change mitigation,  
665 through an economic and environmentally friendly approach. This would be  
666 advantageous also for saving money required to recycle and depurate waste streams by  
667 removing nutrients (Wang et al., 2019). However, it should be underlined that the  
668 chemical profile and complex composition of the effluent will strongly influence the  
669 resultant biochemical composition of microalgal biomass, thus influencing its  
670 downstream valorisation pathway and possible applications (McDowell et al., 2020).  
671 Moreover, with regards to CO<sub>2</sub> streams, the presence of the typical substances  
672 constituting flue gases such as NO<sub>x</sub>, SO<sub>x</sub>, and heavy metals, should be controlled due  
673 to the possibility of culture medium acidification, harmful to the cells, and the  
674 bioaccumulation of toxic compounds in the resulting biomass, which eventually  
675 compromises its quality and subsequent applicability (Simonazzi et al., 2019).

### 676 **3. Extraction of value-added products from *Phaeodactylum tricornutum***

677 Among microalgae species, diatoms are indicated as an auspicious feedstock for the  
678 biotechnology sector, since multi-product and fractionation procedures would be able to  
679 launch new production chains in different industrial fields (Savio et al., 2020). Although  
680 the products recoverable from microalgae are highly profitable from an economic  
681 perspective, their industrialization still struggles to establish on the market. Among the  
682 reasons, high investments required for downstream purification steps contribute  
683 significantly. Moreover, these conventional processes making use of a huge quantity of  
684 organic solvents, produce a heavy impact on the environment. Lipid fraction from  
685 microalgal biomass has been usually extracted by employing toxic solvents such as  
686 chloroform, n-hexane, diethyl ether, methanol, and their mixtures. Therefore, extensive

687 studies have been conducted on the optimization of extraction technologies and  
688 especially on the design of new protocols excluding the use of toxic solvents to increase  
689 efficiency and sustainability, reduce waste production, and decrease energy  
690 consumption. Some green extraction techniques complying with these requirements are  
691 Supercritical Fluid Extraction (SFE), Pressurized Liquid Extraction (PLE), Ultrasound-  
692 Assisted Extraction (UAE), Microwave-Assisted Extraction (MAE), Pulsed Electric  
693 Field (PEF), Enzymatic-Assisted extraction (EAE), and alternative solvents like deep  
694 eutectic solvents (DES), and ionic liquids (ILs). Briefly, UAE and MAE make use of  
695 generally recognized as safe solvents (GRAS) such as water and ethanol, while  
696 enhancing and boosting their extractive capacity, also reducing operating temperature,  
697 extraction time, and solvent consumption. During ultrasonication, high-frequency  
698 acoustic waves cause a cavitation effect, which culminates in the cell disruption by high  
699 shear forces. MAE instead consists in transferring energy to the solution, through the  
700 concurrence of dipole rotation and ionic conduction mechanisms. Polar molecules, such  
701 as the water inside the cells, absorb the energy and heat up rapidly causing the pressure  
702 inside the cell to increase till the cell ruptures and releases cell compounds (Günerken et  
703 al., 2015; Zhou et al., 2019). SFE employs a determinate solvent (such as CO<sub>2</sub>) at  
704 pressures and temperatures higher than the critical point (31.2 °C and 73.8 bar for CO<sub>2</sub>)  
705 that improve its solvent capacity. This technology also offers the possibility to use a co-  
706 solvent to modify the overall polarity and selectively extract specific compounds. PLE  
707 applies high temperature (but lower than the critical point) and pressures sufficiently  
708 high to keep the solvent liquid. In this case, the solvent possesses augmented solubility  
709 and lower viscosity which in turn improves mass transfer and permeation into the  
710 biomass sample (Gallego et al., 2018). During PEF, an electrical potential throughout

711 the cell wall is originated by the application of an external electric field. In this case,  
712 cell disruption is accomplished by electromechanical compression (Günerken et al.,  
713 2015). In addition, ionic liquids are emerging as an interesting alternative to fractionate  
714 microalgal biomass. These are low melting salts that are generally used to perform soft  
715 and selective extractions from both non-disrupted and disrupted biomass while  
716 preserving structural integrity and bioactivity of metabolites (Eppink et al., 2021). The  
717 extraction of fucoxanthin from *P. tricornutum* biomass (provided by  
718 the Microalgae Collection of Ningbo University) was carried out to investigate the  
719 effects of a few factors on fucoxanthin's yield. These factors were the solvent used  
720 (methanol, ethanol, acetone, water, and ethyl acetate, ethanol-water mixtures from 60%  
721 to 100 % v/v), extraction time (15 to 120 min), extraction temperature (4 to 40 °C), and  
722 total extraction rounds. The decreasing order of the solvent's performance in terms of  
723 fucoxanthin extraction yield was the following: 1) methanol; 2) ethanol; 3) acetone and  
724 ethyl acetate. Thereby, better extraction performances were accomplished when using  
725 polar solvents. Even if methanol was revealed to be the most efficient for extracting  
726 fucoxanthin, also the greener mixture of 70% ethanol-water performed well. The  
727 quantity of fucoxanthin extracted was favored by the increase in operating time and  
728 temperature. Moreover, the first extraction was enough to obtain about 80% of the total  
729 fucoxanthin. Hence, the optimum experimental conditions to mildly extract fucoxanthin  
730 from *P. tricornutum* biomass were solvent/solid ratio equal to 40 mL g<sup>-1</sup>, operating time  
731 90 min, 25°C, 70% mixture ethanol-water, and one extraction round. These  
732 conditions allowed to have an extract containing both lipids and fucoxanthin. Therefore,  
733 this extract was further purified by an ethanol precipitation process at 25°C, which led  
734 to an extraction yield of 7.14 ± 0.11 mg g<sup>-1</sup> with a recovery percentage of 80.04 ±

735 1.18%. (Sun et al., 2022). Gilbert-López et al. performed a comparison between MAE  
736 and PLE technologies for extracting value-added compounds from *P.*  
737 *tricornutum* freeze-dried biomass provided by Fitoplancton Marino S.L. and studied the  
738 effects of temperature, solvent, and extraction time. A factorial Design of Experiment  
739 (DoE) sized  $3^2$  was conducted to investigate PLE performance and optimize  
740 experimental conditions. The investigated factors were temperature (50; 110; 170 °C)  
741 and ethanol in different percentages (0; 50; 100%). Whereas the  $3^3$  factorial design for  
742 MAE studied the following factors: extraction time (2; 11; 20 min), composition of  
743 ethanol (0; 50; 100%), and temperature (30; 100; 170 °C). The response variables for  
744 both DoE studies were extraction yield, antioxidant activity (indicated as Trolox  
745 equivalent antioxidant capacity TEAC), total carotenoids, chlorophylls, and total  
746 phenols content (TPC). For PLE, the temperature was found to have a positive effect on  
747 extraction yield, indicating that it improved the mass transfer, and the solvent solubility,  
748 while reducing its viscosity, thus promoting the solvent permeation ability. The solvent  
749 mixture water-ethanol (50:50) reached higher yields, compared to pure solvents.  
750 Whereas higher phenols content and antioxidant activity were found for pure  
751 ethanol. The content of carotenoids increased with a higher percentage of ethanol and  
752 decreased with the temperature. Regarding MAE process, the extraction time did not  
753 show any significant effect on all response variables. Like PLE, the temperature had a  
754 favorable influence on extraction yield also in MAE, and the same was observed when  
755 water was present in the solvent mixture; the lowest yields were indeed obtained with  
756 pure ethanol. Higher phenolic and TEAC content were found in pure ethanol. Finally,  
757 carotenoids were primarily extracted by 100% ethanol, but their content decreased with  
758 the temperature, indicating the possibility of compound degradation. Optimum

759 extraction conditions for PLE and MAE were modeled to maximize each response  
760 variable, all response variables, and all variables except the yield (**Table 5**). HPLC–  
761 APCI–MS/MS was used to characterize the extracts obtained at optimum conditions.  
762 Fucoxanthin was the most abundant compound; then diatoxanthin, other two  
763 carotenoids, and one carotenoid ester were detected. Other minor peaks in the  
764 chromatogram were attributed to chlorophylls, having the same absorbance  
765 spectrum. The amount of fucoxanthin recovered in PLE and MAE is also presented in  
766 **Table 5**. MAE optimum extracts were richer in phenols, carotenoids, and TEAC;  
767 however, higher extraction yields were obtained from PLE. Regarding fucoxanthin, its  
768 concentration was similar between MAE and PLE extracts, but its recovery was better  
769 with PLE technology thanks to the higher extraction yield (Gilbert-López et al., 2017).  
770

771

Optimum modeled extraction conditions	PLE	MAE	Recovered fucoxanthin [mg g <sup>-1</sup> algae]
Yield maximization	170 °C; 40% EtOH	170 °C; pure water; 20 min	-
TPC maximization	50 °C; 100% EtOH	30 °C; 100% EtOH; 2 min	7.73 (PLE)
TEAC maximization	50 °C; 100% EtOH	170 °C; 100% EtOH; 2 min	
Carotenoids maximization	170 °C; 100% EtOH	30 °C; 100% EtOH; 2 min	-
Yield, TPC, TEAC, and carotenoids maximization	170 °C; 97% EtOH	170 °C; 100% EtOH; 5.8 min	5.81 (PLE), 2.97 (MAE)
TPC, TEAC and carotenoids maximization	50 °C; 100% EtOH	30 °C; 100% EtOH; 2min	4.59 (MAE)

772

773 **Table 5.** Modeled optimum conditions for extraction of valuable compounds from *P.*774 *tricornutum* using PLE and MAE.

775

776 Tommasi et al. (2017) also explored the lipid extraction from *P. tricornutum* (furnished  
777 by Micoperi Blue Growth) by using dimethyl carbonate (DMC) solvent extraction (a  
778 nonvolatile, cheap, non-corrosive, non-toxic, and eco-friendly solvent) and supercritical  
779 CO<sub>2</sub> (scCO<sub>2</sub>). Microwaves (MWs) and deep eutectic solvents (DESs) and their  
780 combination were used as pretreatments for both extractions and their performances  
781 were evaluated. The authors tested the application of different DESs obtained by  
782 choline chloride and diverse hydrogen donors, concluding that DESs obtained by ChCl  
783 and carboxylic acids was the best solvent among the tested ones, as higher selectivity  
784 and an enhancement of total fatty acid (TFA) extraction performances of DMC were  
785 determined. Instead, the MW process alone did not enhance DMC extraction capacity,  
786 which remained scarcely effective or selective. The combination of DESs and MW  
787 pretreatments and the subsequent DMC extraction resulted in a TFA yield and fatty acid  
788 profile similar to those obtained by the conventional Bligh and Dyer procedure, together  
789 with a much-improved selectivity (88% against 35%). The combination DES-MW  
790 remarkably enhanced the extraction efficiency of scCO<sub>2</sub>, improving the TFA yield by  
791 20-fold and obtaining very purified extracts. Despite DES-scCO<sub>2</sub> extracting lower  
792 quantities of total fatty acids than DES-MW-scCO<sub>2</sub>, it resulted in the highest content of  
793 EPA (35%). Neither DES nor DES-MW pretreatments were found to degrade PUFAs  
794 and EPA, which hence can be effectively extracted employing DMC and scCO<sub>2</sub>  
795 procedures (Tommasi et al., 2017). No other extraction technologies have been  
796 proposed and tested for *P. tricornutum* so far, except for some studies concerning the  
797 combined production of diverse value-added compounds in a biorefinery approach that  
798 will be discussed in the next section. Hence, other, especially environmentally friendly  
799 techniques should be assessed on this interesting diatom both to identify the best

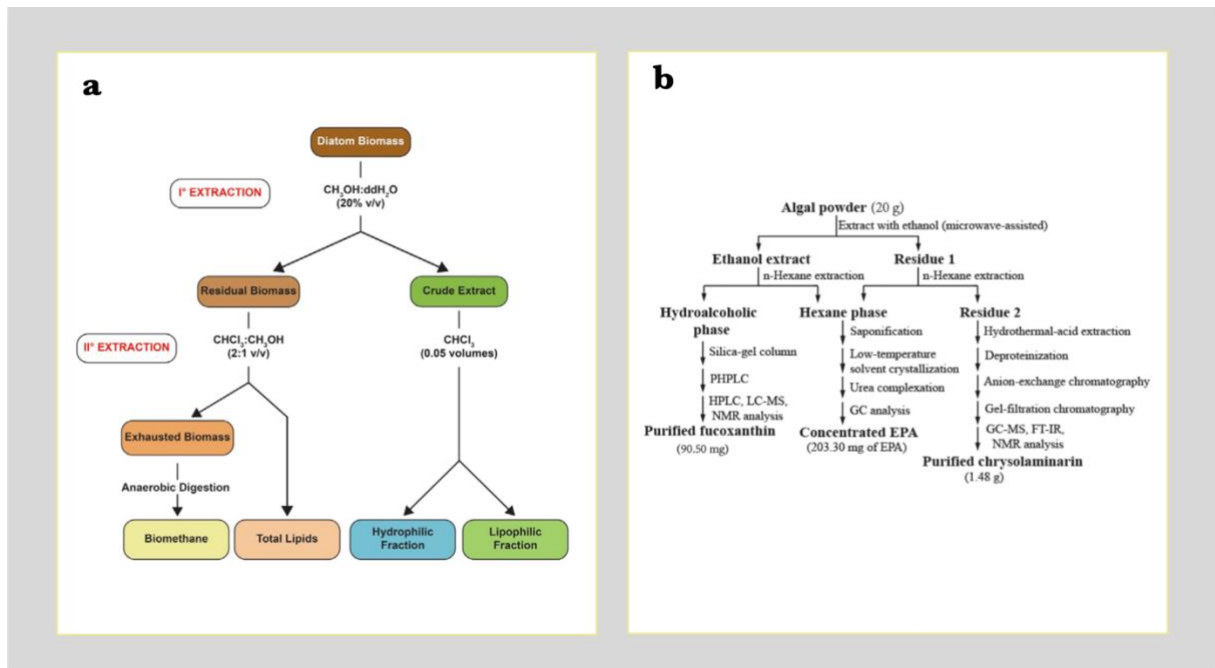
800 extraction conditions leading to the highest yields and to determine what is extracted  
801 and what remains in the residual if it is undamaged, thus exploring the possibility of a  
802 cascade extraction and purification of algae biomass.

### 803 **3.1 Biorefinery approaches**

804 Currently, at a commercial scale, just a single compound (e.g., astaxanthin,  $\beta$ -carotene)  
805 is produced from microalgae sources, while other conceivably fractions such as proteins  
806 and carbohydrates, are disposed of. Instead, to enhance the economic feasibility of  
807 bioactive compounds production from microalgae, all biomass fractions should be  
808 valorised, leading to a multi-product biorefinery (Bhattacharya and Goswami, 2020;  
809 Branco-Vieira et al., 2020). A microalgae-based biorefinery would then rely on an  
810 industrial chain producing microalgal biomass under optimal conditions and,  
811 subsequently, a facility generating a broad range of products, such as chemicals, food  
812 additives, health-enhancing compounds, and biofuels, exactly as occurs for petroleum-  
813 based processes (Gilbert-López et al., 2015). Some studies developed and tested  
814 cascade extractions on *P. tricornutum* to obtain multiple compounds production in a  
815 perspective of biorefinery. For example, a multi-step aqueous and non-aqueous  
816 extraction was developed for *P. tricornutum* biomass provided by Algosource Saint-  
817 Nazaire, as follows: high voltage electrical discharges (HVED: 1-8 ms; 40 kV cm<sup>-1</sup>) or  
818 high-pressure homogenization (HPH: 10 passes; 1200 bar) (first step); aqueous  
819 extraction to obtain carbohydrates, proteins, and water-soluble pigments (second step);  
820 liposoluble pigments extraction by 95% ethanol (third step); and lipids extraction by  
821 chloroform and methanol (fourth step). Characterizations of proteins, carbohydrates,  
822 ionic compounds, and hydro-soluble pigments were conducted to test the extraction  
823 performances of the two pre-treatments employed. The authors showed how HVED

824 performed better selective extraction of hydro-soluble compounds. However, the  
825 efficiency of HPH treatment in disrupting microalgal cells and extracting hydro-soluble  
826 compounds (carbohydrates and proteins) was higher; while the non-aqueous extraction  
827 (third and fourth steps) of pigments and lipids was more efficient when HVED was  
828 used. Overall, the pre-treatments allowed to improve the amounts of components of  
829 interest in the extracts, compared to the untreated samples (Zhang et al., 2020). **Figure**  
830 **2a** shows another stepwise extraction procedure to produce multiple-valued compounds  
831 from the same biomass of *P. tricornutum*, used as lyophilized biomass from  
832 Phytobloom Necton. A crude extract was first obtained using methanol (20% v/v);  
833 successively, chloroform and methanol (2:1 v/v) were adopted to obtain total lipids  
834 from the remaining biomass, and finally, the “leftover” material, was subjected to  
835 anaerobic digestion to assess its Biochemical Methane Potential (BMP). The crude  
836 extract obtained from the first step was further separated into a hydrophilic fraction and  
837 a lipophilic one by adding 0.05 volumes of chloroform. Obtained fractions from the  
838 extraction protocol were then characterized using NMR analysis, bioactivity test, and  
839 fatty acid profile analysis. The lipid content of the residue from step 2 was 17.09% ±  
840 1.50%. Final exhausted biomass provided an Organic Fraction content of 88.07 ± 0.50%  
841 and led to 164.8 ± 10.3 ml g<sup>-1</sup> of organic fraction as methane yield (Savio et al., 2020).  
842 Another study proposed a stepwise extraction process on *P. tricornutum* to obtain EPA,  
843 chrysolaminarin, and fucoxanthin in a biorefinery concept, utilizing different solvent  
844 solutions (**Figure 2b**). The algal powder was first subjected to a Microwave-Assisted  
845 Extraction with ethanol; then both extract and residue were treated with hexane and  
846 resulted in three fractions: hydroalcoholic phase, which was further purified to obtain  
847 fucoxanthin, hexane phase, which gave a concentrate in EPA after few other steps, and

848 a residue, from which chrysolaminarin was separated. The yields obtained were  $34.03 \pm$   
 849  $0.72\%$ ,  $23.00 \pm 0.29\%$ , and  $43.54 \pm 0.91\%$  DW, respectively. Moreover, the use of  
 850 ethanol and MW treatments of 1 min resulted in the highest fucoxanthin yield (Zhang et  
 851 al., 2018). Although these works are the first reports on the comprehensive utilization of  
 852 *P. tricornutum* biomass, the use of toxic solvents was still selected. Moreover, as  
 853 depicted in **Figure 2b**, the developed step-wise extraction is composed of numerous  
 854 steps, which could be hardly scaled up at the industrial level. Hence, it should be further  
 855 explored the possibility of designing a biorefinery based on *P. tricornutum* biomass  
 856 making ideal use of exclusively green extraction technologies and safe solvents.



857  
 858 **Figure 2.** Step-wise extraction of value-added compounds from *P. tricornutum* biomass  
 859 in a biorefinery perspective. **a)** (Savio et al., 2020) **b)** (Zhang et al., 2018)

860

861 **4. Conclusions**

862 As illustrated, *Phaeodactylum tricornutum* shows great potential in the microalgae  
863 sector to produce valuable compounds. Glycerol was confirmed as the best organic  
864 source for growing this diatom mixotrophically, enhancing both biomass concentration  
865 and growth rate. Beyond EPA, fucoxanthin, and chrysolaminarin, other valuable  
866 compounds worth to be studied could be present in extraction biomass residuals.  
867 Therefore, more effort should be addressed to the study of green extraction methods in a  
868 biorefinery concept, also exploring the possibility of using wet extraction methods thus  
869 saving the energy required for the drying step. Finally, techno-economic analysis and  
870 Life Cycle Assessment could be used to assess the effective potential of a microalgae-  
871 biorefinery.

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875 **6. References**

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