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## The Potential of Microalgae for the Production of Bioactive Molecules of Pharmaceutical Interest

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- 2 PHARMACEUTICAL INTEREST
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7

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19

20 **ABSTRACT**

21 Through the photosynthetic activity, microalgae process more than 25% of annual inorganic carbon dissolved in  
22 oceans into carbohydrates that ultimately, serve to feed the other levels of the trophic networks. Besides,  
23 microalgae synthesize bioactive molecules such as pigments and lipids that exhibit health properties. In addition,  
24 abiotic stresses, such as high irradiance, nutrient starvation, UV irradiation, trigger metabolic reorientations  
25 ending with the production of other bioactive compounds such as  $\omega$ -3 fatty acids or carotenoids. Traditionally,  
26 these compounds are acquired through the dietary alimentation. The increasing, and often unsatisfied, demand  
27 for compounds from natural sources, combined with the decrease of the halieutic resources, forces the search for  
28 alternative resources for these bioactive components. Microalgae possess this strong potential. For instance, the  
29 diatom *Odontella aurita* is already commercialized as dietary complement and compete with fish oil for human  
30 nutrition. In this contribution, the microalga world is briefly presented. Then, the different types of biologically  
31 active molecules identified in microalgae are presented together with their potential use. Due to space limitation,  
32 only the biological activities of lipids and pigments are described in details. The contribution ends with a  
33 description of the possibilities to play with the environmental constrains to increase the productivity of  
34 biologically active molecules by microalgae and by a description of the progresses made in the field of alga  
35 culturing.

36

37 **KEYWORDS:** bioactive compounds, algae, pigment, lipid, health benefit, abiotic stress, metabolic

38 reorientation, diatom

39

40 **INTRODUCTION**

41 More than 70% of Earth is covered with water, in which the most dominant group of living organisms is that of  
42 algae. Algae belong to the plant phylum. They are mostly living in water while they have colonized every type of  
43 ecological niche. The preferences of individual algal species, which determine their geographical distribution,  
44 are based on their environmental tolerance and their responses to abiotic interaction. On the other hand, natural  
45 populations are morphologically, physiologically and biochemically diverse because of genetic variability and  
46 abiotic conditions [1].

47 Algae have a tremendous impact on the sustainability of the marine ecosystem as being the primary producers  
48 [2] and, therefore, a food source for other marine organisms. Their potential is not restricted to this point as  
49 through feeding of other organisms placed at higher levels in the food chain can take benefit from particular me-  
50 tabolites such as photoprotective compounds [3]. On the basis of their constituting number of cells, algae can be  
51 grouped as unicellular or pluricellular organisms, these terms being often taken as synonym for microalgae or  
52 phytoplankton and macroalgae, respectively. Algae represent a few percentage among the total number of  
53 species described so far (Fig. S1) even though the number of species is probably largely underestimated [4]. This  
54 is especially true for microalgae. The use of algae as fertilizers and food is established since the antiquity. Con-  
55 sidering the increasing need of food, bioenergy, pharmaceutical and cosmetic compounds, a particular attention  
56 has been paid for the last decade to sustainable resources that do not compete with usual food resources. Mi-  
57 croalgae are pretty good candidates for such a purpose and their long evolutionary and adaptive diversification  
58 has led to a large and diverse array of biochemical constituents. Amazingly, the development of industrial pro-  
59 cesses using algae remains weak ( $15 \cdot 10^6$  T produced/year) when compared to the field production ( $4 \cdot 10^9$  T pro-  
60 duced/year) [4], probably because of their typical weak growth rate compared [5]. Therefore, the improvement of  
61 culturing performances constitutes the best way to make alga cost-competitive. This can be achieved through a  
62 deep knowledge of algal biochemistry and physiology and obviously through optimization of bioreactors. Never-  
63 theless, numerous new molecules are isolated, described at the atomic level and tested for their biological activi-  
64 ties, as testified by the increasing number of publications on this topic found in databases (total number of papers  
65 published between 1964 and 2011 = 705) (Fig. S2). This amount remains however very small when compared  
66 with the number of papers published about molecules originating from higher plants (> 13000) [1, 6-10]. Until  
67 recently, it was thought that the metabolism of algae is close to that of higher plants. However, the interpretation  
68 of sequenced genomes established the originality of the algal metabolism and will bring information about pri-  
69 mary and secondary metabolisms, and the presence of key molecules (e.g., [11]).

70 In this contribution, the microalga world **is first** briefly overviewed. Then the different types of biologically  
71 active molecules identified in microalgae **are** presented together with their potential use. Due to space limitation,  
72 only the biological activities of lipids and pigments are **discussed** in details. The contribution ends with a  
73 description **of** the possibilities to play with the environmental constrains to increase the productivity of  
74 biologically active molecules by microalgae and of the progresses made in the field of alga culturing. The data  
75 presented in this manuscript **are** limited to the eukaryotic microalgae producing molecules with a biological  
76 activity. Molecules isolated from macroalga or dealing with other usages will not be covered here and the  
77 interested reader is invited to read the excellent papers published on these topics (*e.g.*, [3,6-7,12-14]).

78

### 79 **THE MICROALGA WORLD: A BRIEF OVERVIEW**

80 Algae is a generic term used to designate eukaryotic organisms sharing photoautotrophy (most of the species)  
81 and the absence of land plant characteristics such as trachea. From the evolution point of view, alga is a  
82 polyphyletic group of taxons, all deriving from the internalization of a cyanobacterium-type organism into a  
83 eukaryotic heterotrophic cell. **This** explains why actual chloroplasts are surrounded by two envelopes [15-17].  
84 On the basis of the chloroplast pigments, three lineages are currently considered as distinct evolutionary clusters  
85 of taxa [15-17]:

86 - *The blue lineage of primary endosymbionts* in which chlorophyll *a* (Chl *a*) is the only Chl-type of molecule  
87 and the chloroplast still contains a peptidoglycan cell wall typical of cyanobacteria. These organisms being  
88 not eukaryotes, this lineage is not presented here.

89 - *The red lineage of primary endosymbionts* in which Chl *a* is **also** the only Chl-type of molecule. Belong to  
90 this lineage more than 6,000 species, mostly unicellular and marine, including many notable seaweeds, of red  
91 algae or Rhodophyta. Subcellular and phylogenetic analyses revealed that red algae are one of the oldest  
92 groups of algae [18-19]. The oldest fossil eukaryote **so far identified** is a red alga and was found in rocks  
93 dating to 1,200 **million** years ago [20].

94 - *The green lineage of primary endosymbionts* in which Chl *a* is associated to Chl *b*. Belongs to this lineage the  
95 green algae or Chlorophyta (more than 6,000 species), **from** which the higher plants emerged. Chlorophyta  
96 forms a paraphyletic group of unicellular, colonial, coccoid, caenobial and filamentous forms as well as  
97 seaweeds.

98

99 To explain the presence of additional membranes around the chloroplasts, a secondary endosymbiotic act is  
100 usually invoked. The members of the red lineage of secondary endosymbionts constitute a very diverse group of  
101 organisms, the most important from the pharmaceutical point of view being the diatoms (Heterokonta) and the  
102 dinoflagellates (Alveolata).

103

#### 104 **Diatoms**

105 With 250 orders and more than  $10^5$  species, the diatom taxon is one of the most diverse group of microalgae [21].  
106 Diatoms are thought to contribute as much as 25% of the Earth primary productivity [22]. Diatoms have the  
107 unique property to have a siliceous cell wall and are characterized by a typical pigment composition: chlorophyll  
108 c as accessory Chl molecule and fucoxanthin as the main carotenoid [23-24]. Diatoms are used in aquaculture to  
109 feed mollusks whereas several intracellular metabolites such as lipids (eicosapentaenoic acid (EPA),  
110 triacylglycerols) and amino acids are extracted and used by pharmaceutical and cosmetic industries [25-26].  
111 Beside these compounds, diatoms may excrete toxins, pigments and antibiotics.

112

#### 113 **Dinoflagellates**

114 It is a large group of photosynthetic organisms thought a large fraction are in fact mixotrophic cells *i.e.*  
115 combining photosynthesis with ingestion of prey [27]. Some species live in symbiosis with marine animals  
116 (called zooxanthellae). As diatoms, dinoflagellates use Chl c as an accessory pigment. Dinoflagellata are mostly  
117 known for red tides and the neurotoxins released during such a phenomenon.

118

### 119 **MICROALGAE: NATURAL FACTORIES FOR BIOLOGICAL MOLECULES IMPORTANT FOR** 120 **HEALTH**

121

#### 122 **Toxins**

123 Toxic compounds are mostly produced by dinoflagellates and diatoms, although not every specie produces this  
124 type of compound. For instance, the dinoflagellates *Alexandrium* sp., *Karenia brevis* (previously *Gymnodinium*  
125 *breve*) produces paralytic shellfish toxins saxitoxin (**1**) [28] and brevetoxin-B (**2**). This last toxin is responsible  
126 for neurologic disorders [29]. **A single** taxon can synthesize several toxins (Table S1). The blooms may cause hu-  
127 man irritation of eyes and throat in the coastal area. Occasionally, the consumption of contaminated shellfishes re-  
128 sults in human poisoning, the prominent symptoms being gastrointestinal disorders. Beside these toxins, the di-



129 noflagellate *Amphidinium klebii* produces different groups of macrolides such as amphidinol-7 (**3**) [30] exhibit-  
130 ing extremely potent cytotoxicity against L1210 cells *i.e.* mouse lymphocytic leukemia cells and antifungus ac-  
131 tivity [29]. *Goniodoma pseudogonyaulax* excretes antimicrobial and antifungal substances such as goniodomin-  
132 A (**4**) [31-32]. In addition, goniodomoin A has been shown to inhibit angiogenesis [33]. *Prorocentrum lima* and  
133 *Dinophysis* sp. synthesize okadaic acid, a protein dephosphorylation inhibitor and *Gambierdiscus toxicus* pro-  
134 duces ciguatoxin and maitotoxin that cause diarrhetic disturbances (Table S1). *Gambierdiscus toxicus* also pro-  
135 duces fungus growth inhibitors, the gambieric acids [29] (Table S1).

136 Several diatom species have been reported to synthesize domoic acid (**5**) (Table S1) [34], a tricarboxylic acid an-  
137 tagonist of the neuroexcitatory glutamate insecticidal properties [25] that can be fatal after accumulating in shell-  
138 fish, some of which being able to retain high level of this compound [35]. Domoic acid was found to be very ef-  
139 fective in expelling ascaris and pinworms [29].

140

141 **Pigments:** As mentioned earlier, most of the algae are photoautotrophs. Consequently, their chloroplasts are rich  
142 in pigmented molecules such as tetrapyrroles and carotenoids. The molecules are able to absorb light thanks to  
143 their characteristic conjugated double bonds. Each photosynthesizing microalga contains at least the close  
144 tetrapyrrole Chl *a* (**6**). Except in red algae, in which Chl *a* is accompanied by the open tetrapyrroles  
145 phycoerythrin, phycocyanin and allophycocyanin, green and brown algae contain another type of Chl molecule  
146 (Table 1). The set of light harvesting molecules is complemented with several carotenoids (Car) (Table 1). As it  
147 will be explained below in details, these molecules have a great health and therapeutic potential.

148 The diatom *Haslea ostrearia* synthesizes and excretes a hydrosoluble blue pigment, the so-called  
149 marrenine, responsible for the greening of oyster gills [7]. This pigment exhibits an antiproliferative effect on  
150 lung cancer model [36] and has potential antiviral and anticoagulant properties [37].

151

152 **Amino acids:** Beside the universal functions of amino acids in proteins, they are important for skin hydration,  
153 elasticity, photoprotection (see below) and are included in cosmetics [7]. Amino acids from diatoms exhibit  
154 dermatological properties [38].

155

156 **Photoprotectants:** The best known photoprotectants synthesized by microalgae are mycosporine-like amino  
157 acids (MAAs) (Fig. S3). MAAs act as sunscreens to reduce UV-induced damage and also as ROS scavengers  
158 [39]. Mycosporine-like amino acids have been found in more than 380 marine species, including microalgae

159 [40]. A database referencing the studies in microalgae, cyanobacteria and macroalgae is available at the Univer-  
160 sity of Erlangen, Germany ([http://www.biologie.univ-erlangen.de/botanik1/html/eng/mar-](http://www.biologie.univ-erlangen.de/botanik1/html/eng/mar-database.htm)  
161 [database.htm](http://www.biologie.univ-erlangen.de/botanik1/html/eng/mar-database.htm)). A recent study reported the screening of 33 different species belonging to 13 classes of microal-  
162 gae for MAAs [40]. The highest concentrations were found in dinoflagellates whereas diatoms contained only  
163 low amounts. MAAs have the potential to replace or supplement today's available sunscreens and particularly  
164 those based on petrochemical products. More recently, other photoprotective molecules such as pyropheophytin  
165 *a* (*Eicenia bicyclis*: [41]), fucoxanthin (Fuco) (*Hijikia fusiformis*: [42]) have been isolated from brown macroal-  
166 gae [3,29]. Because these molecules are also present in microalgae, they have been also considered here. Jeffrey  
167 *et al.* [43] have reported the occurrence of such compounds in 206 strains of 152 microalgae. In many microal-  
168 gae, the cell is made more resistant to UV by the accumulation in the cell wall of sporopollenin [44], a Car-poly-  
169 mer absorbing UV light.

170

171 **Lipids:** In animal cells, essential fatty acids and specifically **polyunsaturated fatty acids (PUFAs)** are  
172 incorporated into lipid membranes in which they increase the fluidity and exchanges between extra and  
173 intracellular compartments. Numerous studies have demonstrated that dietary  $\omega$ 3 PUFAs have a protective effect  
174 against atherosclerotic heart disease [45-48]. The two principal  $\omega$ 3 fatty acids in marine oils, eicosapentaenoic  
175 acid (EPA; 20:5 $\omega$ 3) (7) and docosahexaenoic acid (DHA; 22:6  $\omega$ 3) (8), have a wide range of biological effects.  
176 Both EPA and DHA are known to influence lipoprotein metabolism, platelet and endothelial function,  
177 coagulation, and blood pressure. More specifically, EPA performs many vital functions in biological membranes,  
178 and is a precursor of several lipid regulators involved in the cellular metabolism. In addition, the effect of  $\omega$ 3  
179 fatty acids may depend, to some extent at least, on the presence of underlying disorders such as dyslipidemia,  
180 hypertension, diabetes mellitus, and vascular diseases [48]. DHA is a major component of brain, eye retina and  
181 heart muscle, it has been considered as important for brain and eye development and also good cardiovascular  
182 health [49].  $\omega$ 3 fatty acid supplementation in animals and humans results in substantial increases in the plasma  
183 and tissue levels of EPA and DHA, as well as variable incorporation of the phospholipid classes in various  
184 tissues. These differences may be important for the subsequent use and metabolism of EPA and DHA. Although  
185 both fatty acids are thought to be biologically active, most studies have focused on the relative importance and  
186 effects of EPA, primarily because of its predominance in marine oils and fish species. Because animal cells are  
187 unable to synthesize these molecules, they must be acquired through the diet. So far, the main source for PUFAs,  
188 free or methyl ester derivatives, fatty alcohols, fatty amines and glycerol is fishes. However, fish oil depends on

189 fish quality and fish resources, which are declining and fish tends to accumulate poisonous substances *via* the  
190 food chain. Therefore, alternate sources have to be exploited. Microalgae present an excellent potential for this  
191 purpose because (i) their fatty acid profile is simpler than that of fish oil, (ii) the production condition can be  
192 controlled and last but not least, (iii) the algal species can be selected according to the PUFA required (see  
193 below). In contrast to EPA, molecules from fish oil products are unstable and exhibit a poor taste, EPA esters  
194 from microalgae are of better quality and more stable [50]. Importantly, selected PUFA can be favored through  
195 choosing culture conditions. Some species, such as *Phaeodactylum tricornutum* produce mainly EPA [51].  
196 Among the lipids, arachidonic acid (Ara), an essential fatty acids, is produced by some algae such as *Nitzschia*  
197 *conspicua* [52]. Ara is also a precursor of prostaglandins and leukotrienes and, is also a component of mature  
198 human milk [53]. All these molecules can be used for different activities such as nutrition (human and animal),  
199 pharmaceuticals, cosmetics, aquaculture and biodiesel production.

200

### 201 **Polysaccharides**

202 Best producers of polysaccharides of interest are brown and red seaweeds. Among the different types of  
203 polysaccharides synthesized by these algae and also synthesized by red microalgae such as *Porphyridium* sp.,  
204 those that are highly sulfated present an antiviral activity [54-55].

205

### 206 **Miscellaneous**

207 In addition to their used in flavor and fragrance industries, monoterpenes have drawn increasing commercial  
208 attention because of their putative action as natural insecticides and antimicrobial agents [56]. Little is known  
209 about the production of these molecules in microalgae but their use as biotransformant has been reported [56].

210 Water extract of the marine diatom *Haslea ostrearia* exhibited anticoagulant activity [37].

211 Due to space limitation for this review and the availability of the data, only the lipids and pigments, as molecules  
212 with biological activities, are detailed in the next section.

213

## 214 **LIPIDS AND PIGMENTS, TWO TYPES OF BIOLOGICALLY ACTIVE COMPONENTS** 215 **SYNTHESIZED BY MICROALGAE**

216

### 217 **Lipids**

218 Fishes and marine microalgae are the primary producers of  $\omega$ 3 PUFA. While microalgae synthesize  $\omega$ 3 PUFA,  
219 fishes usually obtain EPA *via* bioaccumulation in the food chain. So far, two of the questions that have been  
220 addressed are: (i) is it cheaper to produce  $\omega$ 3 fatty acids from algae is than from fishes? and (ii) are  $\omega$ 3 fatty  
221 acids obtained (EPA and DHA in particular) from microalgae as effective as those obtained from fish oil?  
222 Regarding the first question, it was shown that  $\omega$ 3 fatty acid production from microalgae would indeed be less  
223 expensive than the one from fishes. In addition, unlike fish oil, microalgal  $\omega$ 3 fatty acid extracts have no odour,  
224 are less susceptible to be contaminated by heavy metals, and do not contain cholesterol [57]. Finally, when  
225 microalgae are grown under controlled conditions, the composition of the fatty acids shows no seasonal variation  
226 [58]. As fish oil fails to meet the increasing demand for purified PUFA, alternative sources are being sought,  
227 especially from microalgae. Microalgae contain lipid levels between 20-50% (Table 2), but in stress conditions  
228 such as N-deprivation or an irradiance or temperature increase, some species of microalgae are able, to  
229 accumulate up to 80% of their dry weight in fat [59-60], including large quantities of high-quality  $\omega$ 3 PUFAs  
230 (Table 2). Thus, algae are gaining increasing attention because of their important values for human health as well  
231 as for aquaculture.

232 So far several algae are already used as dietary supplements. *Chlorella* sp., a freshwater unicellular green alga, is  
233 known to be a good source of proteins, lipid soluble vitamins, pigments, choline, and essential minerals in a  
234 bioavailable form. The administration of *Chlorella* affects some biochemical and physiological functions [71].  
235 As algal sources of DHA come the brown alga *Schizochytrium* sp. (40% DHA, 17% docosapentaenoic acid  
236 (DPA)), the green alga *Ulkenia* sp. and the red alga *Cryptocodinium cohnii* (40-50% DHA) [72]. The  
237 production from the latter species is especially well described [73] and marketed by Martek company. DHA  
238 produced from microalgae is mainly used for child and adult dietary supplements [74]. Moreover, *C. cohnii* have  
239 effects in aquaculture [75]. It has already been showed that algal oils rich in DHA are nutritionally equivalent to  
240 fish oils in several tests [76-77], suggesting that algal oils could constitute a substitution to fish oils. In addition,  
241 new algal sources for the  $\omega$ 3 very long chain PUFAs (VLCPUFA) are being examined. These include the  
242 production of EPA from other strains such as marine diatoms. *P. tricornutum*, a marine diatom, has been widely  
243 used as a food organism in aquaculture and considered as a potential source for EPA production [77]. The sole  
244 marine microalga known to be rich in EPA used as a dietary supplement is the marine diatom *O. aurita*. It has  
245 been shown that extracts of this microalga have an anti-proliferative effect on cultures of bronchopulmonary and  
246 epithelial cells [78]. Different experimental models are used to conduct studies in relation with use of  $\omega$ 3 fatty  
247 acids from microalga sources. Using freeze-dried microalgae, animals and specifically murine models are often

248 used as previously described by several authors. Normal or modified (chemically and genetically) strains of mice  
249 and rats have been already used to study the effects of *Chlorella* sp. on myelosuppression induced by lead [79], on  
250 glycogenesis improvement in diabetic mice [71] and on dyslipidemia prevention in rats fed with high fat diet  
251 treatments [80]. The comparison of rats fed with freeze-dried *O. aurita* or with fish oil shows that the plasma  
252 triacylglycerol concentration in rats fed microalgae was lower than in the control group and also than in the fish  
253 oil group (Fig. 1). The plasma concentrations of HDL- and LDL-cholesterol were significantly higher by  
254 comparison with the control rats. For the rats fed with fish oil, LDL cholesterol was similar to the rats fed with  
255 control diet, while HDL cholesterol was higher than in the group of control rats. Nevertheless, the HDL/LDL  
256 cholesterol was statistically similar in both the control and microalga-fed groups of rats, whereas this ratio is  
257 greater in the rats fed with fish oil.

258 According to the use of microalga as an alternate to fish oil, differences in the enrichment of tissue in  $\omega$ 3 fatty  
259 acids and specifically in EPA were mentioned. Indeed, results reported in Fig. 2 show that the levels of EPA,  
260 obtained for each organ are significantly different from ones obtained in the two other groups (for all studied  
261 organs). In fact, whatever the organ considered (liver, heart or kidney), EPA levels were significantly higher in  
262 rats fed with the freeze-dried microalga diet than in those fed with fish oil or control diets. Moreover, significant  
263 higher amount of DPA was found in the liver and kidney total lipid of the rats fed with the diatom diet than in  
264 those from rats fed with fish oil or with the control diet. The n-6/n-3 ratio in liver, heart or kidney, were  
265 significantly different in the three experimental groups, the rats fed the control diet being systematically higher  
266 than in the two other groups. In addition, this ratio tends to be lower in the rats fed the freeze-dried microalga  
267 diet by comparison with those fed the fish oil one. These results showed that a freeze-dried *O. aurita* diet could  
268 be considered as an alternate source to fish oil in regulation of blood parameters involved in lipid metabolism  
269 and in the enrichment of tissue in  $\omega$ 3 fatty acids and specifically in EPA. This enrichment into EPA at the  
270 expense of Ara incorporation into total lipids of liver, heart and kidney could have beneficial effects in the  
271 cardiovascular disease prevention as described with fish oil. Moreover, when intact microalgae are used in diet,  
272 the effect of the  $\omega$ 3 fatty acid role could be potentiated with pigment content such as Fuco or other Cars. These  
273 results are in line with those published by Rao & Rao [81] and Micallef & Garg [82], who found a synergistic  
274 action between pigments, fatty acids and phytosterols on plasma lipid concentration decrease, on inflammatory  
275 response and thus on cardiovascular disease risk prevention. These molecules that are naturally contained in *O.*  
276 *aurita* make this organism a major actor in human nutrition as an alternate to fish oil.

277

278 **Pigments**

279 Three major classes of photosynthetic pigments occur among microalgae: Chls and derivatives, Cars (carotenes  
280 and xanthophylls) and phycobilins, which together represent hundreds of **molecule purification** [83]. Considering  
281 their high structural diversity and the possibility to pharmacomodulate these molecules, the potential of  
282 **microalga** pigments to obtain molecules of therapeutical interest is very high. Because of their lability and  
283 difficult purification, the biological activity of most molecules **remains** unstudied [27,59]. A large number of  
284 studies designed to purify and identify bioactive molecules from microalgae have lead to the isolation of  
285 pigments. These purified pigments usually have a high activity on pharmacological and cellular effectors, at very  
286 low concentrations.

287

288 ***Antioxidant, anti-inflammatory and antimutagenic activities***

289 Oxidative stress is a major cause of inflammatory events implicated in a large number of diseases, such as  
290 cancer, neurodegenerative and cardio-vascular diseases, or diabetes. The antioxidant and anti-inflammatory  
291 activities of microalga pigments is widely demonstrated and evidenced in numerous *in vitro* free radical  
292 scavenging assays and *in vivo* assays. The antiradical capacity of metal-free Chl-derivatives such as chlorins,  
293 pheophytins, and pyropheophytins is much weaker than the corresponding metallo-derivatives. Protoporphyrin  
294 methyl ester and its magnesium chelated derivative, as well as pheophorbide *b* and pheophytin *b*, were also  
295 identified as strong antioxidant molecules [84]. The ability of the porphyrin ring to transfer electrons explains the  
296 antioxidant activity of **Chls** and derivatives. The high antioxidant activity of pheophorbide *b*, compared to  
297 pheophorbide *a*, suggests that the presence of the aldehyde function may also be critical to this activity [85]. **The**  
298 **antioxidant properties of Chls and Chl-derivatives disappear in the presence of light** [86]. Metal-free and  
299 metallo-Chl derivatives have also antimutagenic activities, as demonstrated using a bacterial mutagenesis assay  
300 [87-88]. Microalgal carotenoids (*e.g.*, zeaxanthin (Zea), astaxanthin (Asta) (**9**)) and epoxy-carotenoids (*e.g.*,  
301 neoxanthin) have strong antioxidant activities *in vitro* and *in vivo* in animal models. Particularly, Asta has a great  
302 potential to prevent cancer, diabetes and cardiovascular diseases [89-90]. The presence of the hydroxyl and keto  
303 endings on each ionone ring explains Asta unique features, such as the ability to be esterified [91], a higher  
304 antioxidant activity and a more polar configuration than other Cars [92]. Epidemiologic studies demonstrate an  
305 inverse relationship between cancer incidence and dietary Car intake or blood carotenoid levels, but intervention  
306 trials using a high dose of carotene supplements did not show protective effects against cancer or cardiovascular  
307 disease. Rather, the high risk population (smokers and asbestos workers) showed an increase in cancer cases in

308 these trials [93]. Phycocyanin *c* and phycoerythrin also exhibit antioxidant and anti-inflammatory activities [94-  
309 96]. As a conclusion, most **microalga** pigments exerts strong *in vitro* antioxidant activity, but additional  
310 intervention trials are required to precise their absorption, metabolism and potential as natural antioxidant, anti-  
311 inflammatory and antimutagenic compounds *in vivo*.

312

### 313 **Cytotoxicity**

314 A large number of studies performed in cancer cells grown *in vitro* clearly demonstrate the antiproliferative,  
315 cytotoxic and pro-apoptotic activities of Chl derivatives, Cars, and phycobilins [27]. Moreover, several studies  
316 designed to purify antiproliferative molecules from marine microalgae have led to the isolation of carotene (Zea)  
317 [83,91] and epoxyCars (*e.g.*, Fuco, violaxanthin (Viola) (**10**)) [78,92]. Fuco is the prototypical example of a  
318 microalgal cytotoxic pigment with an important therapeutic potential. Its strong antiproliferative, cytotoxic and  
319 pro-apoptotic **activities**, at concentrations inferior to 1  $\mu\text{M}$ , have been widely studied and demonstrated on a  
320 large number of human cancer cell lines from various tissular origin (lung, breast, prostate, lymphoma, gastric,  
321 uterine, neuroblastoma,*etc*) [98-102]. The molecular mechanisms involved in the cytotoxic activity of Fuco are  
322 not completely understood, but various cellular targets of Fuco have been identified. Because of its  
323 hydrophobicity, Fuco easily crosses and integrates cell membranes. It inhibits mammalian DNA-dependent DNA  
324 polymerases [103], protects against ROS and UV-induced DNA injury [99,104-107], down regulates cyclins and  
325 CDK expression, disturbs major transduction pathways controlling cell survival and transcriptional activation of  
326 genes involved in resistance to apoptosis and anticancer drugs in cancer cells. (MAPK, NF- $\kappa\text{B}$  [99,101],  
327 p21WAF/Cip1 CDK inhibitor [108], Bcl-xL [109-110]). Fuco also enhances Gap junction intracellular  
328 communication, an important process in the control of cell growth, differentiation, apoptosis induction and  
329 diffusion of anticancer drugs [111]. Intestinal absorption and metabolism of dietary Fuco into its major  
330 metabolite fucoxanthinol was demonstrated in mices, but not in humans. Absorption studies in humans indicated  
331 that less than 1  $\text{nmol.L}^{-1}$  is found in plasma after a 1 week diet containing Fuco- rich diet [112]. In the same way  
332 as Fuco, a large number of microalga pigments were identified as cytotoxic at very low concentrations in cancer  
333 cells. They belong to the epoxyCars class (*e.g.*, Viola [96], halocynthiaxanthin [100,103,113-114], peridinin  
334 [114-117]), to Chl derivatives (*e.g.*, Chl *a*, pheophytin *a*, pheophytin *b*, pheophorbide *a*) or to phycobilins (*e.g.*,  
335 phycocyanin) [92]. Moreover, for some of them, their anticancer activity was confirmed after *per os* absorption.  
336 As an example, in the pathogen-free ddY strain mice, the development of skin tumors induced by 12-*O*-  
337 tetradecanoylphorbol-13-acetateis suppressed when 1  $\mu\text{mol}$  peridinin is added in dietary water [118]. For most

338 molecules, intestinal resorption, bioavailability and metabolism are unknown. Besides, their effect in noncancer  
339 cells and immune cells is mostly unexplored. Understanding their pharmacological activity in human cells may  
340 allow to obtain potent selective anticancer pharmaceuticals.

341

342 **Ref 95**

343

#### 344 **Multi-drug resistance reversion in cancer cells**

345 Microalgae pigments may have interest to restore drug sensitivity or reverse multi-drug resistance in cancer  
346 cells, as some of them inhibit or down-regulate drug efflux pumps. As examples, neoxanthin increases  
347 rhodamine 123 accumulation in multi-drug resistance (MDR) colon cancer cells [113], inhibits the P-  
348 glycoprotein (P-gp) efflux pump and reverses MDR in doxorubicin-resistant MCF-7 cells and hmdr1- transfected  
349 L1210, at 4 and 40  $\mu\text{g}\cdot\text{mL}^{-1}$ , respectively [119]. Viola and violeoxanthin are effective MDR modulators in Colo  
350 320, at 4 and 40  $\mu\text{g}\cdot\text{mL}^{-1}$ , respectively [120]. Moderate P-gp inhibition by Viola was observed in hMDR1-  
351 transfected L1210 and MDA-MB-231 expressing the MRP1 pump (HTB26) at 20  $\mu\text{g}\cdot\text{mL}^{-1}$  [121-122]. In the  
352 same way, a significant reduction of P-glycoprotein expression in HepG2 cells, at both transcriptional and  
353 translational levels, was observed when cells were treated with pheophorbide *a* [123].

354

#### 355 **Antiangiogenic activity**

356 Fucoxanthin and its physiological metabolite fucoxanthinol have antiangiogenic effects, as demonstrated in the blood  
357 vessels and HUVEC tube formation assays. In SCID mice injected subcutaneously with  $10^7$  HUT-102 cells,  
358 fucoxanthinol did not affect tumor incidence, but significantly slowed tumor growth. It also significantly  
359 decreased microvessels outgrowth, in a dose-dependent manner, in an *ex vivo* angiogenesis assay.

360

#### 361 **Use as fluorescent probes**

362 The physicochemical characteristics of phycobilins, Chl and Chl catabolites make them suitable for use as  
363 fluorescent probes for cellular and tissular analysis (*e.g.*, cell sorting, cytofluorescence, flow cytometry,  
364 histofluorescence, binding assays, ROS detection, labeling of pathological or apoptotic cells, *etc.*). Phycocyanin  
365 or phycoerythrin-coupled antibodies are common reagents available for research and medical use, in which  
366 phycobilins act as powerful and highly sensitive fluorescent probes (for reviews, see [96]).

367



**Other preventive or therapeutic use**

Microalgal pigments have demonstrated their lack of toxicity and biological activity in a wide range of biological applications, including prevention of acute and chronic coronary syndromes, atherosclerosis, rheumatoid arthritis, muscular dystrophy, cataract and neurological disorders. They are also recommended to protect the skin and eyes against UV radiation [124-125]. Lutein is one of the major xanthophylls found in green microalgae. It selectively accumulates in the macula of the human retina, protects the eyes from oxidative stress, and acts as a filter of the blue light involved in macular degeneration and age-related cataract [112,126-127]. Fuco anti-allergic activity was recently evidenced using a rodent mast cells model [127]. It could also have interest to limit the risk of obesity [127,129]). Because of their antioxidant and anti-inflammatory activity, most microalga pigments have neuroprotective effects in cultured rat cerebellar neurons, and hepatoprotective effects in hepatocytes grown *in vitro* (e.g., phycocyanin, phycoerythrin) [96]. Besides, some studies have demonstrated antiviral and antifungal activities for some pigments (e.g., allophycocyanin, phycocyanin) [96, 130].

380

**Potential and obstacles to a possible pharmaceutical development of microalgae pigments and derivatives**

The lack of oral toxicity of microalgae pigments may be due to a weak intestinal resorption but also suggests a possible pharmaceutical development for these molecules (e.g. [24]). Most microalga pigments are labile molecules, sensitive to light and oxygen, and it is highly probable that their half-life in a physiological context is short [131]. This lability has interest when considering new applications, but is also a limit to their pharmaceutical development. It also explains the high price and low availability of pigments standards, necessary to set up intervention trials and clinical assays. Consequently, there is a lack of *in vivo* studies on absorption, metabolism and pharmacokinetics of microalga pigments [27]. Moreover, dozens of pigments and derivatives are unstudied because no standard is available. It is essential to carry on the development of economically viable industrial processes to obtain high amounts of pigments and derivatives (selection of over-producing species and strains, definition of physiological conditions giving the best production yields, optimization of eco-extraction and purification processes, development of chemical and chimioenzymatic synthesis). As an example, the average carotenoid concentration in dry microalgae is 0.1-2% (w:w). When grown under optimized conditions of salinity and light intensity, *Dunaliella* produces up to 14%  $\beta$ -carotene [72,130-132]. Purification from natural sources is much more expensive than chemical synthesis, but has the advantage of providing pigments in their natural isomer proportions (e.g., carotene) [72,130-132]

397

398 **ABIOTIC STRESSES: A CONVENIENT WAY TO INDUCE THE METABOLIC REORIENTATION**  
399 **AND INCREASE THE PRODUCTION OF SELECTED BIOACTIVE COMPOUNDS.**

400 As microalgae play a major role in CO<sub>2</sub> uptake [2,22], numerous studies deal with effects of abiotic stresses on  
401 algal biology and metabolism. The main objectives of some of those studies were to predict how and what algae  
402 will cope with climatic change and increasing pollution. The commercial exploitation of the natural microalgal  
403 diversity for the production of carotenoids and PUFAs has already started up with few strains such as *Chlorella*  
404 *vulgaris* (Trebouxiophyceae), *Dunaliella salina* (Chlorophyceae), *Haematococcus pluvialis* (Chlorophyceae)  
405 [133-134] and *O. aurita* (Bacillariophyceae). In this section, the impacts of abiotic stresses such as light, UV-ra-  
406 diation, nutrient starvation, temperature and metals on microalgal metabolism and on the production of biologi-  
407 cal active compounds is reviewed.

408

409 **Light**

410 More than terrestrial plants, microalgae display a diversity of light harvesting pigments (Table 1) allowing  
411 photosynthesis at different depths according to pigment content. Photosynthetic apparatus of most microalgae  
412 acclimates to light level and light quality by optimizing pigment content and composition [135-141]. Microalgae  
413 are confronted with variations of light by movements in the water column and emersion for coastal benthic  
414 species. To cope with high sunlight intensities, microalgae have developed different photoprotective  
415 mechanisms. One of these, the xanthophyll cycle, consists in the reversible conversion of Viola, antheraxanthin  
416 and Zea in green algae and in the reversible conversion diadinoxanthin and diatoxanthin in brown algae [91,141-  
417 142]. Acclimation to low irradiance intensity or blue enriched light increases Car synthesis such as Fuco [140].  
418 The photoprotection or the low photoacclimation leads carbon to Cars whereas in nonstressfull conditions, C  
419 serves mainly to growth (cell wall edification). In the marine diatom *Haslea ostrearia*, C fixation by  $\beta$ -  
420 carboxylation is almost equal to that in the C<sub>3</sub> pathway whereas under low irradiation C<sub>3</sub> fixation dominates  
421 [144]. Light intensity has also an impact on lipid synthesis, PUFAs: EPA, was significantly higher under low  
422 light, and saturating fatty acids and DHA levels were higher under high light in *Pavlova lutheri* [140]. EPA and  
423 DHA are now recognized as having a number of important nutraceutical and pharmaceutical applications.

424

425 **UV-radiation**

426 Microalgae experience high levels of UV-radiation in shallow areas, low turbide habitats or during low tides  
427 when they are deposited on intertidal flats. Several authors have shown that UV exposure increases carotenoid

428 content [145-146] and, in some Bacillariophyceae, MMAs synthesis [147-148]. Guihéneuf *et al.* [149] have  
429 shown that a 8-day exposure to UV decreases the PUFA content, EPA by 20% and DHA by 16% in *Pavlova*  
430 *lutheri* but not in *Odontella aurita* in which the PUFA content remains unchanged. As other environmental  
431 stresses, UV radiation stimulates the intracellular ROS production [150-151] triggering antioxidative defence  
432 such as antioxidative enzyme activities and antioxidant compounds (glutathione,  $\alpha$ -tocopherol, ascorbate, *etc.*).

433

#### 434 **Nutrient starvation**

435 The reorientation of the metabolism induced by nutrient starvation is illustrated by the accumulation of Asta in  
436 *H. pluvialis* under N-limitation and P- or S-starvation [133,152-153]. Asta accumulation is related to a massive  
437 increase in carbohydrate content up to 63% of the cell dry weight [154] and an increase of lipid content in the  
438 cytoplasm. In the Cryptophyceae *Rhodomonas* sp., N-starvation triggers a rapid decline in N-containing  
439 compounds causing an almost complete loss of phycoerythrin [155]. Riyahi *et al.* [156] have shown that the  
440 production of  $\beta$ -carotene in *Dunaliella salina* was increased with nitrate 1 mM and salinity 30%, On the other  
441 hand, in the microalga *Tradydiscus minutus* (Eustigmatophyceae), N-starvation brings about a nearly 50% drop  
442 in triacylglycerols (TAGs) containing EPA, and also a decrease of TAGs containing Ara, while P-starvation has a  
443 sizable effect on those TAGs that contain two or three Ara [157]. Many microalgae promote a shift in lipid  
444 metabolism by producing substantial amount (20-50% of dry weight) of TAGs as lipid storage during the  
445 stationary phase when nitrate becomes depleted [158]. The amount of EPA partitioning into TAGs varies  
446 according to strains and also during the different phases of growth within a species.

447

#### 448 **Metals**

449 Some metals such as Cu, Fe, Zn are essentials for cell metabolism since they are components of electron  
450 transporters involved in photosynthesis and respiration, some enzymes, *etc.* When metals are present in excess,  
451 they induce an oxidative stress [159-160] and antioxidant defense mechanisms already cited above. To cope with  
452 metals in excess, many microalgae excrete exopolysaccharides that adsorb metals in the medium and prevent  
453 them to enter inside the cells [161-162]. Polysaccharide depolymerization by different procedures allows the  
454 **obtention** a variety of oligomers with potential applications in therapeutics and in biotechnology [10]. However,  
455 in the presence of Cd, the xanthophyll cycle in *Phaeodactylum tricornutum* is inhibited [163]. The impact of  
456 metals depends on their speciation and the growth medium pH [164].

457

**458 Temperature**

459 Microalgae can synthesize VLCPUFA as major fatty acid components [165]. Experiments in controlled  
460 conditions are necessary in order to select species producing those PUFAs, in what conditions, at what stage of  
461 their growth, and in what lipid class. Tonon *et al.* [158] have shown that fatty acids accumulate during the  
462 stationary phase of growth when nitrate concentration in the growth medium was low. EPA production is higher  
463 at 8°C than at 25°C in the red microalga *P. purpureum* [166]. In the marine diatom *Nitzschia leavis* cultivated at  
464 15, 19 and 23°C, growth is enhanced at the highest temperature but the lowest temperature favours the  
465 distribution of PUFAs in phospholipids and increases EPA content in TAGs [167]. As in terrestrial plants, the  
466 increase of PUFAs in membrane was suggested to be a strategy to maintain membrane fluidity under low  
467 temperature.

468

**469 LARGE-SCALE CULTURE AND BIOMOLECULE PRODUCTION**

470 Microalgae are a source for a variety of bioactive compounds. However, they remain largely unexplored and,  
471 until now, very few commercial achievements of microalgal biotechnology have emerged [168]. During the last  
472 decades, researchers and engineers have developed several cultivation technologies that are still in use today.  
473 Seen very often as obvious, the subsequent culture of a given microalgae can be more difficult than expected in  
474 the attempt to up-scaling. Numerous drawbacks and difficulties await the entrepreneur wishing to set up a  
475 commercial production. The choice of photobioreactors, light systems, control for pH, CO<sub>2</sub> etc.. batch or  
476 continuous cultures, management of nutrients, water supply, water treatment onward and outward as well as the  
477 energy needed will constitute a strategic debate. Concerning the biological aspects, once the proper selected  
478 strain is chosen, the type of culture system, the feeding strategies (photoautotrophy, heterotrophy, mixotrophy  
479 reviewed hereafter), the confrontation with pathogens, contaminants and predators will enter in the game.

480

**481 Photoautotrophic production**

482 Photoautotrophic productions use light as the source of energy thank to photosynthesis and CO<sub>2</sub> as the source of  
483 carbon. They are currently processed with either open ponds or closed systems, that can use sun light and  
484 artificial light. However, the major constraint that phototrophic production must address is how efficiently light  
485 is used. Indeed, productivity is tightly related to the surface to volume ratio of the cultivation system **and** many  
486 recent technological developments tended to **improve this ratio**.

487 Originally, open-ponds and raceways were used for microalgae production, but the quest for increased  
488 productivity and better control led to the development of closed photobioreactors. The latter are usually  
489 recognized as achieving higher biomass productivity than open systems [60,169-170]. Nevertheless, the  
490 maximum biomass productivity does not necessarily **match** the maximum productivity for a particular molecule,  
491 neither the maximal **economic** efficiency [171]. It is beyond the scope of this article to enter into the  
492 argumentation of the pro and contra open ponds *versus* **photobioreactors**. The solution might lie in between when  
493 the two technologies will be integrated in the same production line. Controlled production system like  
494 photobioreactors renders easier to explore the metabolic versatility of microalgae with different production  
495 strategies. Despite their high initial investment, photobioreactors provide a variety of attractive benefits for  
496 bioactive molecule production, when compared to open systems. First, they make possible monospecific **and**  
497 axenic cultures **as well**. They also are characterized by reproducible cultivation conditions and accurate control  
498 for abiotic factors such as temperature, pH, irradiance, evaporation and hydrodynamics. The production of a  
499 particular molecule can take advantage of these controls since abiotic factors can substantially impact the  
500 biochemical composition of microalgae, as discussed above.

501 Most of the commercial productions use photoautotrophic cultivation processes, with pigments, health food and  
502 aquaculture being the main markets. Several commercial companies produce Asta with *Haematococcus* : Mera  
503 Pharmaceuticals (Hawaii) reports a biomass production of about 6.6 T/year using closed tubular  
504 photobioreactors. Similar culture systems have been used by Algatechnologies (Israel) and Fuji Health Science  
505 (Hawaii). However, the production cost of Asta with *Haematococcus* is still high because of physiological (slow  
506 growth rate) and technical (two-stage production process) constraints. Thus **from the economic point of view,**  
507 **Asta produced with *Haematococcus* can hardly compete with the synthetic pigment [92].**

508 *Dunaliella* natural  $\beta$ -carotene is another widely distributed pigment from microalgae. Its global production  
509 through autotrophic cultivation is estimated at about 1.2 T/year [12]. Two cultivation processes are currently  
510 used for  $\beta$ -carotene production. Betaten (Adelaide Australia) or Aquacaroten (Subiaco, Australia) grow this  
511 microalgae in unmixed open ponds and Betaten reported a  $\beta$ -carotene production of about 13 T/year (about 400  
512 ha of culture area). The associated production costs appear relatively low considering **the optimal climate and,**  
513 **unlike other systems,** no pumping is required [172]. Raceway ponds (intensive mode) are operated by the Nature  
514 Beta Technologies company (Eilat, Israel), reporting a  $\beta$ -carotene production of 3 tonnes per year. Several  
515 studies have been attempted to grow *Dunaliella* in closed photobioreactors, although up to date, none of these  
516 trials led to any significant production even at the pilot scale [173]. Several other little companies commercialize

517 a variety of microalgae grown photoautotrophically for their high amount in EPA and DHA. For example,  
518 *Isochrysis sp.* is produced by Innovative Aquaculture Products Ltd (Lasqueti Island, Canada) and the diatom *O.*  
519 *aurita* is produced by BlueBiotech InT (Kollmar Germany) and Innovalg (Bouin, France). In the latter, *O.*  
520 *aurita* is grown photoautotrophically in open air 1,000 m<sup>2</sup> raceways and co-cultured with the macroalgae  
521 *Chondrus crispus*, for increased productivity [65].

522

### 523 **Heterotrophic production**

524 Studies on microalgae heterotrophy were initiated in the 60s and demonstrated that some species could grow on  
525 organic carbon sources, such as sugars or organic acids, replacing the traditional support of light energy. The  
526 number of studies further increased in the 2000s with the growing interest for biofuel from microalgae. Among  
527 the microalgae species currently cultivated, only a few (e.g., *Chlorella protothecoides*, *Cryptocodinium cohnii*,  
528 *Schizochytrium limacinum*, *Haematococcus pluvialis*) have been successfully grown heterotrophically [174].  
529 Conversely to photoautotrophy where productivity is related to the illuminated area of the culture, productivity  
530 for heterotrophic cultures relies on organic carbon concentration in the bulk volume of the culture. This  
531 facilitates the up-scaling for commercial production and usually results in higher productivity, with biomass  
532 production being one order of magnitude higher than for photoautotrophically grown cultures [175] and in  
533 reduced production, harvest and maintenance costs. For instance, high biomass concentration (45 g L<sup>-1</sup>) and  
534 volumetric productivity (20 g L<sup>-1</sup> d<sup>-1</sup>) were achieved in heterotrophic cultures of *Nitzschia alba* [176].

535 Heterotrophic culture requires axenic conditions, a major drawback when compared to photoautotrophy. As  
536 pointed out by **Bumback et al.** [177], any, even minor, contamination introduced with the inoculum could easily  
537 outcompete the microalgal species for the organic carbon source. The prerequisite for axenicity and the  
538 additional care for its maintenance necessarily impact the production costs. Additionally, heterotrophic culture  
539 might not bring the same diversity and the same **biochemical composition** as reached with photoautotrophy. Yet,  
540 **Perez-Garcia et al.** [174] reported the possibility to produce lutein with *Dunaliella sp.* and Asta with *Chlorella*  
541 *zofingiensis* grown heterotrophically. **Wang & Peng** [178] reported the first growth-associated biosynthesis of  
542 Asta with *Chlorella zofingiensis* heterotrophic cultures using glucose as organic carbon source. This study  
543 suggested that maximal biomass and Asta production could be obtained simultaneously by a one stage culturing  
544 rather than the two stage process that was proposed for *Haematococcus*. Although commercial production of  
545 Asta with heterotrophic *Chlorella zofingiensis* culture has not yet been reported, this species may be a promising  
546 alternative to *Haematococcus* for the mass production of Asta. Besides, commercial production of

547 heterotrophically grown *Chlorella* in fermentor is common in Japan and Korea, mainly for aquaculture and  
548 health food applications [179]. Martek (USA) also successfully produces DHA health food with heterotrophic  
549 *Cryptocodinium cohnii* cultivation [180].

550

### 551 **Mixotrophic production**

552 If mixotrophy is defined so as to include osmotrophy, most of microalgae can be considered as mixotrophic.  
553 Many microalgae can grow on dissolved organic carbon [181] and, under inorganic nitrogen stress, use organic  
554 sources of nitrogen [182].

555 When microalgae are grown with CO<sub>2</sub> as the sole carbon source, light provides the energy necessary for biomass  
556 production. However, under photoautotrophic conditions, growth is often limited by light availability and, during  
557 the night, the productivity is further reduced by respiration. Mixotrophic microalgae can concurrently drive  
558 phototrophy and heterotrophy to utilize organic energy and both inorganic and organic carbon substrates, thus  
559 leading to a synergetic effect of the two processes that enhances the **culture** productivity. *Yang et al.* [183]  
560 demonstrated that biomass yield on the supplied energy was four folds higher for true mixotrophically grown  
561 *Chlorella pyrenoidosa* than for the photoautotrophic culture. They also highlighted that cyclic autotrophic/  
562 heterotrophic cultivations, could lead to even more efficient utilization of energy for biomass production than the  
563 true mixotrophy. Moreover, mixotrophy can overcome light limitation occurring at high densities. This  
564 mechanism has been demonstrated to be important for *Scenedesmus obliquus* [184] and is suggested to be widely  
565 spread among mixotrophic microalgae in general.

566 Hence, high productivity is one of the major benefits associated with mixotrophy. For some microalgae, the  
567 growth performance under mixotrophic conditions can even exceed that achieved with heterotrophic cultures.  
568 Indeed, *Pulz & Gross* [12] pointed out that the maximum specific growth rate of *Chlorella vulgaris* and  
569 *Haematococcus pluvialis* growing mixotrophically was the sum of the photosynthetic and heterotrophic specific  
570 growth rates. Besides, *Stadnichuck et al.* [185] reported higher Chl *a*, carotenoids, phycocyanin and  
571 allophycocyanin content in *Galdieria partita* grown mixotrophically than in heterotrophically cultures.  
572 Mixotrophy can therefore overcome some of the drawbacks experienced with heterotrophic cultures [186] and  
573 might be an efficient means for enhanced production of light-induced pigments in microalgae. However, as for  
574 heterotrophic cultures, mixotrophic cultures require axenic conditions to prevent bacteria from outcompeting  
575 microalgae for organic substrates. Research will be needed to cope with the risk of favouring the prokaryotic part  
576 in the culture. To date, the processing of mixotrophic cultivation implies the availability and maintenance of



577 axenic strains, the investment for sterilizable photobioreactors and higher operation costs. However, the higher  
578 productivity achieved with mixotrophy cultures could balance these drawbacks.

579 It is well documented that some economically important microalgae can be grown mixotrophically  
580 (*Haematococcus pluvialis*, *Scenedesmus acutus*, *Chlorella vulgaris*, *Nannochloropsis* sp.). However, despite the  
581 indisputable assets of mixotrophy, only one company reported the use of mixotrophic processes for industrial  
582 Asta production. Indeed, BioReal (Sweden) was the first company in the world to produce and commercialize  
583 from 15 to 30 T/year of Asta-rich biomass using mixotrophy culture in indoor closed photobioreactors [172].

584

## 585 CONCLUSIONS AND FUTURE DIRECTIONS

586 Microalgae represent a subset of single cell microorganisms that generally grow autotrophically using carbon  
587 dioxide as the sole carbon source and light as energy. They are ubiquitous in nature, occupying every type of  
588 ecological niche. Microalgae represent a major untapped resource of genetic potential for valuable bioactive  
589 agents and fine biochemicals. Screening studies should reveal the existence of new molecules potentially  
590 interesting for their biological activities. From the basic point of view, the mechanisms of action of the already  
591 marketed products should be better established. For instance, it has been reported that, beyond  $\omega$ -3 and  
592 antioxidants, fish oil also contain peptides having bioactive activity. Many of them have an interest for health  
593 and pharmaceutical industries. In their natural environment, algae are subjected simultaneously to different  
594 abiotic factors with daily and seasonal variations that may be stressful, such as tidal movements, temperature,  
595 light levels or UV radiations. To cope with stress, the synthesis of molecules of interest such as antioxidants,  
596 PUFAs and glycerol is increased in tolerant microalgae. More basic research on this point should be performed  
597 to elucidate the metabolic and regulation circuits involved in these productions. This will help to discover what  
598 are the interactions between several abiotic factors and mechanisms involved in the biochemical responses. *In*  
599 *silico* research, biochemical characterization of microalgal products and in the same way the research of  
600 biological activities of algal extracts seem promising for biotechnology applications. Many molecules produced  
601 by microalgae show a high structural diversity and should be considered as potent bioactive molecules able to  
602 significantly modulate human cell functions, in a physiological or pathological context, at very low  
603 concentrations. Additional studies of their biological activity *in vivo* are required to precise their absorption,  
604 metabolism and interest as potential natural anticancer or cardioprotective agents. The development of efficient  
605 purification processes will stimulate their study and pharmaceutical development.



606 The cultivation means to produce bioactive compounds are various. Important are the source of energy and the  
607 biomass yield. The selection for high producing strains, the optimization of culture modes and harvesting and the  
608 management of molecule expression in cultures are crucial steps for the future. Whatever the species and  
609 molecules produced, the harvesting system is an expensive and limiting step that has to be adapted to preserve  
610 together the algae integrity but also the one of the molecule. Ideally, microalgae producers look for strains with a  
611 high valuable-product productivity. However, until now, the main commercial productions rely on a few wild-  
612 type strains and the selection for original strains with a high potential for biotechnology remains a challenge for  
613 the industry. Pioneer studies for strain selection were initiated in the 90s. The combination of mutagenesis to a  
614 selection procedure resulted in substantially increased production for pigments [187], PUFAs [188] or  
615 triacylglycerides [189]. These techniques offer an appealing alternative to GMOs.

616 Transgenic microalgae can be also used as bioreactor for production of therapeutic and industrial recombinant  
617 proteins [190-191]. To date, a variety of recombinant proteins have been expressed from nucleus and chloroplast  
618 of *Chlamydomonas reinhardtii*. These include pharmaceutical proteins, antibodies, vaccines, and others that  
619 showed a biological activity comparable to the same proteins produced by traditional commercial techniques  
620 [192]. Our groups were quickly intrigued by the potential of microalgae as a means to produce therapeutic  
621 proteins [193]. A private company was born from this research: Algenics, which is, to date, the first European  
622 privately-held biotechnology company focusing on innovative uses of microalgae to produce recombinant  
623 biotherapeutics (<http://www.algenics.com>). Concerning the use of microalgae as a platform of recombinant  
624 proteins, the recent success led to several patents [194-197] with the successful production of erythropoietin in  
625 *Phaeodactylum tricorutum* (unpublished work). The production costs for microalgal therapeutic proteins are  
626 very attractive (*i.e.*, the cost for recombinant antibody is estimated to 0.002 US\$ and 150 US\$ per gram from  
627 microalgae and mammalian cell culture respectively [198]). Moreover, this cost could fall **provided that**  
628 recombinant protein production is coupled with recovery of valuable natural product. However, to the best of our  
629 knowledge, no microalgal therapeutic proteins have been yet commercially used.

630 Microalgae can also be used in biotransformation experiments. In such experiments, immobilized microalgae are  
631 incubated with particular substrates to use the in situ enzymes to produce products. Such a method has been used  
632 to study the potential of green microalgae such as *Chlamydomonas sp.* and *Oocystis sp.* to produce new  
633 monoterpenes. The molecular engineering described above combined with biotransformation principle opens  
634 many new avenues for algal biotechnology.

635

636 **ABBREVIATIONS:** Asta: astaxanthin, Car: carotenoids, Chl: chlorophyll, DHA: docosahexaenoic acid, EPA:  
637 all-Z-eicosa-5,8,11,14,17-pentaenoic acid, Fuco: fucoxanthin, MAAs: mycosporine-like amino acids, **P-gp: P-**  
638 **glycoprotein**, PUFA: polyunsaturated fatty acids, MDR : multi-drug resistance, TAGS: triacylglycerols, Viola:  
639 violaxanthin, VLC: very long chain, Zea: zeaxanthin

640

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646

#### 647 **SUPPLEMENTARY MATERIAL**

648 **Fig. S1. Algae represent less than 10% of the total number of identified species.**

649 The original data [S1] were not including the unicellular species. In order to take into account these organisms,  
650 we have substituted the number of original species by the number of species found in the AlgaeBase database  
651 (<http://www.algaebase.org/>) although this number is probably largely underestimated.

652 [S1] The World Conservation Union. IUCN Red List of Threatened Species. Summary Statistics for Globally  
653 Threatened Species (1996–2010).  
654 [http://www.iucnredlist.org/documents/summarystatistics/2010\\_1RL\\_Strats\\_Table\\_1.pdf](http://www.iucnredlist.org/documents/summarystatistics/2010_1RL_Strats_Table_1.pdf). Accessed 31/01/2012.

655

656 **Fig S2. Number of publications describing a compound from algae having a biological activity.**

657 The numbers of publications were taken from the Web of knowledge database  
658 (<http://www.webofknowledge.com/>). Search performed in December 2011.

659

660 **Fig. S3.** Exemple of mycosporine-like amino acids.

661

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1108 **Fig. 1. Main plasma biochemical parameters in rats fed with different diets.**

1109 Glucose, triacylglycerol and cholesterol levels were determined using colorimetric kits (glucose RTU,  
1110 cholesterol RTU, triglycerides enzymatique PAP 150, respectively, from bioMerieux, Marcy-l'Etoile, France).  
1111 Results are expressed ( $\text{mmol L}^{-1}$ ) as mean  $\pm$  SEM for  $n = 4$  animals. After analysis of variance, the means were  
1112 compared by Fisher's least significant difference test. Means assigned different superscript letters were  
1113 significantly different ( $p < 0.05$ ).

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1116 **Fig. 2. Effects of  $\omega$ 3 fatty acid marine sources on total lipid  $\omega$ 3 fatty acid composition in plasma, liver,**  
1117 **heart and kidneys in rats fed with different diets.**

1118 After extraction of lipids, fatty acid methyl esters were obtained according to the method of Slover and Lanza  
1119 [81]. Fatty acid composition was performed on a GC-Focus apparatus as previously described [82]. Results are  
1120 expressed (% molar) as mean  $\pm$  SEM for n = 4 animals. After analysis of variance, the means were compared by  
1121 Fisher's least significant difference test. Means assigned different superscript letters were significantly different  
1122 ( $p < 0.05$ ).

1123

1124 Table 1. Main chlorophyll and carotenoid types in the various taxons of photosynthetic organisms.  
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Pigment type	Red algae	Brown algae	Green algae
Phycocerythrin, phycocyanin, allophycocyanin	+	-	-
Chl a	+	+	+
Chl b	-	-	+
Chl c	-	+	-
$\beta$ -carotene	Unicellular	+	+
Fucoxanthin	-	+	-
Violaxanthin	+	+	+
Lutein	Pluricellular	-	+
Zeaxanthin	+	+	+
Canthaxanthin	-	-	-
Xanthophyll cycle	-	+	+

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1127 Table 2. Total lipid content (% of dry weight) and EPA and DHA content (molar percentage) of some  
1128 species of microalgae [59-69].  
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Classes	Species	Lipid content	EPA	DHA
<i>Chlorophyceae</i>	<i>Tetraselmis suecica</i>	15-23	1-5	<1
	<i>Chlorella sp.</i>	28-32	1-5	<1
	<i>Dunaliella primolecta</i>	23	<1	<1
<i>Prymnesiophyceae</i>	<i>Isochrysis sp.</i>	25-33	<1	10-20
	<i>Pavlova lutheri</i>	20-25	>20	10-20
<i>Bacillariophyceae</i>	<i>Skeletonema costatum</i>	13	10-20	1-5
	<i>Thalassiosira pseudonana</i>	24	15	1
	<i>Odontella aurita</i>	7-13	>25	1-2
	<i>Phaeodactylum tricorutum</i>	20-30	26	2
	<i>Nitzschia sp.</i>	45-47	25-30	<1
<i>Dinophyceae</i>	<i>Cryptocodinium cohnii</i>	20	45	<1
<i>Rhodophyceae</i>	<i>Porphyridium cruentum</i>	10-15	21	<1

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