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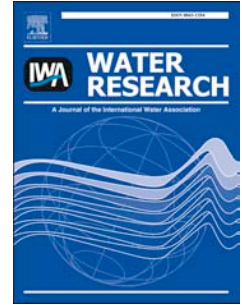
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Microalgal and cyanobacterial cultivation: The supply of nutrients

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Abstract

Microalgae and cyanobacteria are a promising new source of biomass that may complement agricultural crops to meet the increasing global demand for food, feed, biofuels and chemical production. Microalgae and cyanobacteria cultivation does not interfere directly with food production, but care should be taken to avoid indirect competition for nutrient (fertilizer) supply. Microalgae and cyanobacteria production requires high concentrations of essential nutrients (C,N,P,S,K,Fe, etc.). In the present paper the application of nutrients and their uptake by microalgae and cyanobacteria is reviewed. The main focus is on the three most significant nutrients, i.e. carbon, nitrogen and phosphorus; however other nutrients are also reviewed. Nutrients are generally taken up in the inorganic form, but several organic forms of them are also assimilable. Some nutrients do not display any inhibition effect on

24 microalgal or cyanobacterial growth, while others, such as NO₂ or NH₃ have
25 detrimental effects when present in high concentrations. Nutrients in the gaseous
26 form, such as CO₂ and NO face a major limitation which is related mainly to their
27 mass transfer from the gaseous to the liquid state. Since the cultivation of
28 microalgae and cyanobacteria consumes considerable quantities of nutrients,
29 strategies to improve the nutrient application efficiency are needed. Additionally, a
30 promising strategy to improve microalgal and cyanobacterial production
31 sustainability is the utilization of waste streams by recycling and waste nutrients.
32 However, major constraints of using waste streams are the reduction of the range of
33 the biomass applications due to production of contaminated biomass and the
34 possible low bio-availability of some nutrients.

35

36 **Keywords:** biofuels; biomass; cyanobacteria; high-value compounds; microalgae;
37 nutrients

38

39 **1. Introduction**

40 Microalgae and cyanobacteria are photoautotrophic microorganisms, which are
41 presently cultivated to produce numerous high value products, such as vitamins,
42 pigments, proteins, fatty acids, polysaccharides etc. In the near future it is expected
43 that the dedicated market for microalgal high-value compounds will significantly
44 expand (Borowitzka 2013, Spolaore et al. 2006). Moreover, microalgae are
45 considered as a potential biomass feedstock for the production of biofuels and it is
46 believed that they will play a significant role in the sector of renewable energy

47 (Gouveia 2011, Schenk et al. 2008). However, to fulfill only the global needs for
48 transportation fuels using microalgal biomass as feedstock, the cultivation of
49 microalgae rises several practical questions and has some significant constraints,
50 such as high land areas use and high consumption of energy, water and nutrients
51 (Borowitzka and Moheimani 2013, Chisti 2013). The mass production of microalgae
52 for biofuels production presupposes the application of massive quantities of
53 nutrients (fertilizers) (Borowitzka and Moheimani 2013). Since microalgae can be
54 cultivated in non-arable land it is believed that microalgal biomass production for
55 biofuels will not compete with food production. However, the competition between
56 biomass for biofuels and food production might be transmitted to the competition
57 for nutrient (fertilizers) availability. Because microalgal biomass has low content of
58 cellulose compared to terrestrial crops, it contains three times the amount of
59 nutrients compared to biomass of terrestrial plants. As a result, the nutrient demand
60 for microalgal biomass production is much higher than that for agricultural crops
61 (Elser et al. 2000).

62 Microalgae during photosynthesis utilize solar energy and along with several
63 essential nutrients (C, N, P, S, K, Fe etc.) to synthesize their biomass compounds and
64 to multiply their cells. Microalgae need specific quantities of those essential
65 elements in order to be capable to produce biomass. Possible deficiency of one of
66 the elements will cause growth reduction (Liebig's law of the minimum).
67 Considering the universal Redfield C:N:P ratio of 106:16:1 of phytoplankton
68 elemental composition, all of the essential elements have to be present in
69 appropriate ratios, in adequate quantities and in bio-available chemical form in the

70 cultivation medium so that the growth of microalgae will not be limited (Spaargaren
71 1996). Consequently, in the literature the recipes of the cultivation media are
72 frequently considered as fixed. Nevertheless, the experience of cultivating
73 microalgae in various types of wastewater with diverse nutrient compositions and
74 the evidence that phytoplankton stoichiometry diverges from the canonical Redfield
75 ratio under specific conditions (Arrigo 2005) suggest that the cultivation media
76 could be flexible and could be adapted to the microalgal metabolic needs.

77 It is very significant in practice and in large-scale cultivation systems to
78 adapt the cultivation medium to the needs of microalgal growth under the specific
79 environmental conditions, in order to achieve high yields per mass of applied
80 nutrient. The knowledge about the nutrient application and the microalgal uptake of
81 the nutrients is of particular significance. The present article aims to review and to
82 present the most important issues about the application of the most essential
83 nutrients for the production of microalgae using either synthetic fertilizers or
84 wastewater streams. The review will focus and discuss not only issues related to the
85 physiology of microalgae/cyanobacteria but also will discuss technical concerns
86 about the application of nutrients for biomass production. The main focus will be on
87 the nutrients carbon, nitrogen and phosphorus; however the minor nutrients
88 potassium, magnesium, sulfur and calcium will also be reviewed.

89

90 **2. Carbon**

91 Photosynthesis is a complex process through which light energy and inorganic
92 carbon is converted into organic matter. Carbon contributes to all organic

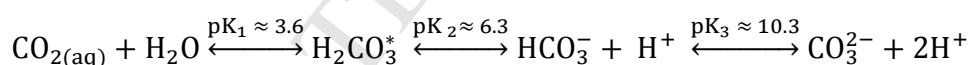
93 compounds, and is the main microalgal (including cyanobacterial) biomass element,
 94 amounting up to 65% of dry weight. The carbon content though, varies significant
 95 among the species and culture conditions and can range between 17.5 and 65% by
 96 dry weight. However, the majority of the species contain about 50% carbon
 97 (Grobbelaar 2004). Limitation by other nutrients than carbon (e.g. nitrogen or
 98 phosphorus) generally results in an increase in the carbon content of the biomass.

99

100 2.1. Buffer system

101 Carbon is mainly taken up by photosynthetic microorganisms in its inorganic
 102 form of CO₂. However, since the majority of microalgae are aquatic microorganisms
 103 which thrive in liquid habitats, CO₂ is dissolved in the aquatic environment. When
 104 CO₂ is dissolved in water it reacts with the water molecules (H₂O) and forms a weak
 105 acid-base buffer system, having the following equilibrium (**Figure 1**):

106



107

108 where, H₂CO₃^{*} refers to CO_{2(aq)} + H₂CO₃

109

110 The above equilibrium depends on the pH of the solution, which means that
 111 the relative amount of the dissolved inorganic carbon (DIC) species is strictly
 112 related to the pH of the solution. Based on the equilibrium of the carbon species
 113 (**Figure 1**), in the range in which the majority of the microalgae thrive, i.e. between
 114 pH 6.5 and 10, the dominant carbon form is bicarbonate (HCO₃⁻). Lowering pH

115 values, more H_2CO_3^* is gradually formed and when pH reaches that of pK_2 the
116 concentration of H_2CO_3^* is equal to HCO_3^- . At pH lower than pK_2 the H_2CO_3^* is
117 predominant. In contrast, increasing pH values more CO_3^{2-} is gradually formed and
118 when pH reaches that of pK_3 the concentration of CO_3^{2-} is equal to HCO_3^- . At pH
119 higher than pK_3 the CO_3^{2-} is predominant.

120

121 2.2 Carbon fixation and up-take

122 Inorganic carbon is fixed inside the microalgal cells and is converted to
123 organic form through the Calvin cycle. The first step of the Calvin cycle is the
124 assimilation of CO_2 and its incorporation into a three-carbon compound. Microalgae
125 and cyanobacteria possess fundamentally the C_3 pathway photosynthesis, however
126 in some species the evidence for a C_3 - C_4 intermediate photosynthesis exist (Roberts
127 et al. 2007, Xu et al. 2012). The assimilation of CO_2 is catalyzed by Ribulose-1,5-
128 bisphosphate carboxylase oxygenase (Rubisco). Rubisco can utilize only CO_2 and
129 therefore CO_2 is the ultimate substrate for carbon fixation (Price et al. 2008).

130 Since the form and the amount of dissolved inorganic carbon depends on pH,
131 salinity, pressure and temperature (**Figure 2**) microalgae have developed various
132 mechanisms, by which, in each specific environment they can utilize the available
133 dissolved inorganic carbon (DIC). Consequently, the utilization of the preferred
134 form of carbon is species dependent. Many species of microalgae and cyanobacteria
135 can take up both CO_2 and HCO_3^- , some may take up only CO_2 or HCO_3^- (Camiro-
136 Vargas et al. 2005) and some extremely alkaliphilic cyanobacteria can utilize CO_3^{2-}
137 (Mikhodyuk et al. 2008).

138 The carbon up-take (**Figure 1**) is performed either passively or actively. CO₂
139 can be taken up either passively through (1) membrane diffusion i.e. crossing and
140 inserting the cell as free CO₂, or (2) actively through membrane transport
141 mechanisms (pumps). HCO₃⁻ is taken up only actively through transport
142 mechanisms; however, bicarbonate can be converted by the metalloenzyme
143 carbonic anhydrase (CA) to CO₂ ($\text{HCO}_3^- \xrightarrow{\text{CA}} \text{CO}_2 + \text{OH}^-$), which can subsequently be
144 taken up either passively or actively, as mentioned before (Badger and Spalding
145 2004). CA's activity is found to be influenced by the DIC concentration and it
146 appears to be higher in microalgae and cyanobacteria cultivated in media with low
147 CO₂ concentration than with high CO₂ concentrations (Aizawa and Miyachi 1986).
148 The form of inorganic carbon utilization depends also on its concentration in the
149 medium; in high DIC concentration it seems that CO₂ is preferred over HCO₃⁻
150 (Aizawa and Miyachi 1986). It was shown that the active uptake of CO₂ is
151 significantly faster than that of HCO₃⁻ (Matsuda et al. 1999) and is energetically
152 favourable (Moazami-Goudarzi and Colman 2012). The pH of the cultivation
153 medium affects the type of carbon uptake; for example at low pH, two marine
154 microalgae species of *Stichococcus*, proved to take up only CO₂ by diffusion, while
155 the active uptake appeared to be occur at pH higher than 6 (Moazami-Goudarzi and
156 Colman 2012).

157 Since the active transportation of inorganic carbon is an energy consuming
158 process and because the preferred form for inorganic carbon up-take depends on
159 the microalgal species the quantitative evaluation of the contribution of each species
160 of dissolved inorganic carbon to the carbon fixation is very significant (Matsuda et

161 al. 1999) and more research is needed at least for the potentially economical
162 significant species.

163 CA in microalgae and cyanobacteria is thought to be a part of the CO₂-
164 concentrating mechanism (CCM). The CCM involves various processes by which the
165 intracellular concentration of CO₂ around the Rubisco is elevated compared to its
166 concentration in the extracellular surrounding environment. In other words, the
167 main aim of the CCM is to actively transport and concentrate the inorganic carbon,
168 in order to be used as substrate for its fixation (Azov 1982, Price et al. 2008). When
169 intracellular CO₂ concentration is low and O₂ concentration high, Rubisco will react
170 with O₂ rather than CO₂ and will produce CO₂ rather than assimilate CO₂
171 (photorespiration). By increasing the ratio CO₂/O₂ due to CCM this reduces the rate
172 of photorespiration (Roberts et al. 2007).

173 CA in the majority of freshwater and marine microalgae and cyanobacteria is
174 located intracellularly; but some species excrete it in the extracellular surrounding
175 space, converting the HCO₃⁻ to CO₂, which is then diffused or actively transported
176 inside the cells. However, in alkaline environments the extracellular CA activity is
177 low, and therefore in marine environments the uptake of carbon occur mainly by
178 actively transportation of HCO₃⁻ or CO₂ than of diffusion of CO₂ (Amoroso et al.
179 1998, Huertas et al. 2000). Moreover, microalgae and cyanobacteria are found to
180 excrete H⁺ ions to regulate their intracellular pH by homeostasis. The excreted H⁺
181 might react with HCO₃⁻ to give CO₂, which can subsequently be taken up by diffusion
182 (Price et al. 2008, Van Den Hende et al. 2012).

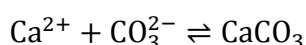
183 Another strategy developed by microalgae to obtain CO₂ from aquatic
184 environments with relative high pH is the process of calcification, in which CaCO₃ is
185 precipitated following the reactions (Jansson and Northen 2010, Moheimani et al.
186 2012):



188

189 However, in marine environments the principal path of calcification is by reacting of
190 calcium cations with carbonate:

191



192

193 Although no CO₂ is produced in this reaction, removal of CO₃²⁻ from solution serves
194 as a buffer against pH rise, especially in alkaline environments (Borowitzka and
195 Larkum 1987, Jansson and Northen 2010). Extracellular calcification is also thought
196 to serve as a light shield, protecting the cell against excessive irradiance, as a way
197 for excretion of Ca²⁺ to prevent intracellular toxicity and as a way to enhance
198 nutrient uptake (Van Den Hende et al. 2012).

199 Photosynthetic activity, and in particular the extracellular or/and
200 intracellular conversion of bicarbonate to carbon dioxide ($\text{HCO}_3^- \xrightarrow{\text{CA}} \text{CO}_2 + \text{OH}^-$),
201 produces OH⁻, which gradually causes an increase of the pH of the medium, which
202 can reach even a value of 11. In the case of intracellular production of OH⁻ the
203 microorganisms take up H⁺ from the surrounding in order to neutralize the

204 produced intracellular OH^- . In contrast, it seems that the passive uptake of CO_2 itself
205 does not alkalize the medium because it leaves no hydroxyl ion during its utilization
206 and fixation (Chi et al. 2011, Shiraiwa et al. 1993). The gradually rise of the medium
207 pH can affect negatively the growth of the microorganisms, either by the alkaline
208 environment itself or due to low useful carbon availability, as CO_3^{2-} gradually
209 becomes the dominant DIC form available (Shiraiwa et al. 1993). Therefore, the
210 control of the pH is a significant issue to maintain microalgal growth. To control the
211 rising pH, a strategy is to acidify the medium, either by applying CO_2 or by using
212 inorganic or organic acids (Grobbelaar 2004). However, the consumption of other
213 nutrients and the degradation of various metabolites (Molina Grima et al. 1999) or
214 the excretion of several organic acids by the microorganisms might also influence
215 the pH values of the cultivation medium.

216

217 **2.3 Factors affecting solubility and dissolution of CO_2**

218 As mentioned before, microalgae thrive in an aquatic environment and
219 therefore the CO_2 is in dissolved form. When gas CO_2 is supplied to the medium, the
220 main influencing parameters are its solubility and its mass transfer rate. CO_2
221 solubility in water at 25°C and 1 atm is about 1.5 g/L. However, the solubility of CO_2
222 in aquatic environments varies significant and depends on pH, salinity, pressure and
223 temperature. As shown in **Figure 2**, solubility of CO_2 in water decreases with
224 increasing salinity and temperature and increases with increasing pressure. pH
225 therefore not only affects the DIC species equilibrium, but also affects the total
226 amount of DIC that is available in solution. In alkaline solutions, the excess OH^- ions

227 react with CO_2 to form HCO_3^- , resulting to a higher bicarbonate-carbonate alkalinity
228 and consequently a higher total carbon availability (Münkel et al. 2013).

229 In addition to the solubility of CO_2 , which refers to the amount of CO_2 that can
230 be dissolved in water under specific conditions, the mass transfer rate also
231 influences the total carbon availability. The transfer of CO_2 from the gaseous to the
232 liquid phase is affected by various design parameters of the cultivation system.
233 Factors that influence the CO_2 mass transfer to the cultivation medium is the specific
234 contact area between the gas and the liquid phase and the concentration gradient
235 between the gas and the liquid phase. In open pond cultivation systems, CO_2 is often
236 transferred passively from the atmosphere to the cultivation medium through the
237 water surface of the pond. Because the specific contact area is low and the
238 concentration gradient is also low (due to the low concentration of CO_2 in the
239 atmosphere), the mass transfer rate of CO_2 into the solution is often too low to
240 compensate for microalgal uptake. To increase the transfer rate, the culture medium
241 is often sparged with a CO_2 -rich gas. The contact time of the bubble with the
242 medium (determined by the rising speed of the bubbles) and size of the gas bubbles
243 will mainly determine the gas transfer rate, in addition to the pH and alkalinity of
244 the medium, the degree of contamination with suspended matter and the general
245 CO_2 saturation state of the medium (Rubio et al. 1999, Suh and Lee 2003, Takemura
246 and Yabe 1999). In high pH values the mass transfer of CO_2 is faster than in low pH
247 values, because the dissolution is mainly due to the chemical reaction of CO_2 and OH^-
248 which is faster than the hydration of CO_2 to H_2CO_3 (Takemura and Matsumoto 2000,
249 Van Den Hende et al. 2012).

250 The most plain model given the mass transfer rate of CO₂ (M_{CO₂}) is (Van Den
251 Hende et al. 2012):

$$M_{CO_2} = k_L \alpha (C_{CO_2}^* - C_{CO_2})$$

252

253 where, k_L is the liquid-phase mass transfer coefficient, α is the specific area
254 available for mass transfer and, $C_{CO_2}^*$ is the CO₂ concentration in the liquid equal to
255 partial pressure in gas phase and C_{CO_2} is the CO₂ concentration in the liquid (Van
256 Den Hende et al. 2012). The enhancement of the mass transfer rate of CO₂ can be
257 achieved by increasing on the one hand the parameter of $k_L \alpha$ (Van Den Hende et al.
258 2012) or on the other hand by increasing the pH values of the cultures (Chi et al.
259 2011).

260 In fast growing microalgal systems, the simple diffusion and dissolution of
261 CO₂ from the air into the water is too slow to replace the assimilated CO₂ (Suh and
262 Lee 2003). In these systems CO₂ has to be provided actively in the one or the
263 another form. The three main ways of applying CO₂ to the cultures are (1) pumping
264 air, (2) pumping concentrated CO₂ and (3) bicarbonate salts.

265

266 **2.4 Sources for providing inorganic carbon**

267 As was mentioned before, the application of CO₂ in microalgal cultivation
268 plays a major role and contributes significantly to the total production costs (about
269 the 50% of the cost of producing the biomass) (Chisti 2013, Kadam 1997, Rubio et
270 al. 1999). When CO₂ is provided by the aeration of the culture with air (≈ 400 ppmv
271 CO₂) the air volume which should be passing through the culture has to be

272 enormous, resulting to high energy consumption for its pumping. However, the use
273 of commercial pure CO₂ seems also to be costly. Flue gas is a lower-cost alternative.
274 It has a CO₂ content of 10-20%. When using flue gases, important costs are
275 associated with the recovery, compression and/or transportation of flue gas, unless
276 the cultivation plant is near the flue gas source, allowing simple transportation and
277 direct application of the flue gases (Kadam 1997, Lam et al. 2012).

278 Flue gases such as those derived from coal-fired plants, cement production
279 plants, or natural gas combustion contain CO₂ in concentrations ranging from 10 up
280 to 25% and can be used as CO₂ source. The use of untreated flue gases as a carbon
281 source in microalgal cultivation is well studied and several constraints have been
282 pointed out, such as the inhibiting effect of NO_x, SO_x, pH and temperature (see the
283 reviews of (Farrelly et al. 2013, Lam et al. 2012, Van Den Hende et al. 2012). Other
284 potential CO₂ sources which could be used are biogas derived from the anaerobic
285 digestion process (with a CO₂ content of 20-40%) (Travieso et al. 1993) or the CO₂
286 that is a by-product of alcoholic fermentation (Bezerra et al. 2013).

287 There is a vast number of published studies that deal with the tolerance of
288 various microalgal strains in cultures that are cultivated with various degrees of
289 concentrated CO₂ (Gouveia 2011). However, as it was pointed out from Van Den
290 Hende et al. (2012) the critical issue of applying CO₂ is not its concentration in the
291 applied gas but the combination of CO₂ concentration and its flow rate, consequently
292 the mass of the dissolved inorganic carbon. Because the dissolution of CO₂ in water
293 results to its acidification, a high mass flux of CO₂ to the liquid phase could lead to a
294 significant decrease in medium's pH. However, it is not entirely clear whether the

295 acidification or the free CO₂ concentration affects the growth, making this
296 distinction challenging (Yang and Gao 2003). The degree of acidification of the
297 medium due to CO₂ addition is an outcome of the equilibrium between the CO₂
298 concentration in the gas and liquid phase, and is affected by the partial pressure and
299 the alkalinity (Goldman et al. 1982). Ideally, the rate of CO₂ should match the rate of
300 CO₂ assimilation by the microalgae (Rubio et al. 1999) with the simultaneously
301 regulation and adjustment of pH to optimal values for each microalgal strain. This
302 strategy is proven successful in cultures with various microalgal species, even when
303 applying 100% CO₂ gas (Olaizola 2003). That means that the addition of CO₂ on
304 demand is independent of the CO₂ concentration in the gas and depends mainly on
305 the operational pH.

306 To avoid problems associated with the direct application of untreated flue
307 gas, the CO₂ could be recovered from the flue gas and purified. Several methods exist
308 for this purpose (Aaron and Tsouris 2005), however the methods who might be of
309 particular interest for microalgal production are those by which bicarbonate salts
310 are formed, such as sodium bicarbonate, $\text{Na}_2\text{CO}_3 + \text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{NaHCO}_3$, (Nelson
311 et al. 2009) and ammonium bicarbonate, NH_4HCO_3 , (Aaron and Tsouris 2005) or
312 urea, $(\text{NH}_2)_2\text{CO}$, (Meessen 2000). Ammonium bicarbonate and urea can be used
313 also as nitrogen source (see nitrogen section).

314 There are two main concerns about using bicarbonates: they cost more than
315 three times as much as gaseous CO₂ (Suh and Lee 2003) and they can only be
316 applied on microalgal species which tolerate high pH and high ionic strength (Chi et
317 al. 2011). However, bicarbonate salts have higher solubility when compared to CO₂,

318 (for example NaHCO_3 solubility is $>90\text{g/L}$ at 25°C) and it is expected that their
319 application efficiency would be higher than CO_2 application. It is a challenge and a
320 necessity to screen and find strains that can thrive with high growth rates under
321 high values of pH, alkalinity and ion strength (Chi et al. 2011). Recently, Chi et al.
322 (2013) studied the NaHCO_3 tolerance of some cyanobacteria and microalgae, and
323 found that the highest NaHCO_3 concentrations were 0.3 M for *Synechocystis* sp.
324 PCC6803, 0.6 M for *Cyanothece* sp., 0.1 M for *Chlorella sorokiniana*, 0.6 M for
325 *Dunaliella salina*, and 0.3 M for *Dunaliella viridis* and *Dunaliella primolecta*. Beside
326 these species, *A. platensis* is also known to be very tolerant in high alkalinity
327 (Kebede 1997). Nevertheless, the use of bicarbonate salts could be have some
328 advantages over CO_2 . The use of bicarbonate results in accumulation and in a higher
329 lipid content in, which is attractive for biodiesel production (Gardner et al. 2012).

330 Another source for inorganic carbon are wastewaters streams that are rich in
331 bicarbonate-carbonate alkalinity, especially the anaerobically digested livestock
332 wastes. During the anaerobic digestion, the organic fraction of the wastes is
333 converted to CH_4 and CO_2 . A portion of the latter is dissolved to the liquor and
334 generates the bicarbonate-carbonate buffer of the anaerobic liquor (Markou and
335 Georgakakis 2011).

336

337 **2.5 Applying of organic carbon**

338 Microalgae are autotrophic microorganisms, however some species are
339 capable to grow on organic molecules either heterotrophically or mixotrophically.
340 The organic molecules are used by the microalgae as a source of carbon and/or

341 energy (Chojnacka and Marquez-Rocha 2004, Perez-Garcia et al. 2011). However,
342 the utilization of organic molecules is species and strain dependent, which means
343 that some species can assimilate a specific organic molecule, while other species do
344 not (Mühling et al. 2005, Sun et al. 2008). The main ways of organic carbon uptake
345 into the cells is diffusion, active transportation and phosphorylation (Perez-Garcia
346 et al. 2011). The best known utilized organic molecules are: monosaccharides (such
347 as glucose, fructose etc.), volatile fatty acids (such as acetic acid), glycerol and urea
348 (Chen and Zhang 1997, Heredia-Arroyo et al. 2011, Hsieh and Wu 2009). The
349 capability of some microalgae to utilize organic molecules has a lot of potential in
350 the field of wastewater treatment. The use of organic molecules as an energy and/or
351 carbon source by the microalgae has as an added benefit that the organic load of the
352 wastewater is reduced. Heterotrophic and/or mixotrophic growth have some
353 advantages over autotrophic growth, such as the higher growth rates and higher
354 biomass concentrations, but they have also disadvantages, mainly the high cost of
355 organic substrates and the high potentiality of contamination by other heterotrophs
356 (Chojnacka and Zielińska 2012). Organic molecules for heterotrophic and/or
357 mixotrophic grow can be originated directly from wastewaters rich in bioavailable
358 organic matter (Bhatnagar et al. 2011) or after their pre-treatment by an hydrolytic
359 phase to convert organic matter to bioavailable volatile fatty acids (mainly acetate)
360 (Hu et al. 2013). The latter is a very interesting approach, since theoretically all the
361 organic matter (such as carbohydrates, lipids and proteins) can be converted by
362 anaerobic bacteria to acetate rendering all types of wastewaters suitable for

363 microalgal grow. However, for the application of acetate as carbon source the
364 screening of capable microalgal species is needed.

365

366 **3. Nitrogen**

367 Nitrogen is the second most abundant element in microalgal biomass, and its
368 content ranges from 1% up to 14% (typically around 5-10%) of dry weight
369 (Grobbelaar 2004). Nitrogen participates in essential biomass biochemical
370 compounds, such as nucleic acids (DNA, RNA), amino acids (proteins) and pigments
371 such as chlorophylls and phycocyanin. It can be taken up in inorganic form of NO_3^- ,
372 NO_2^- , NO , NH_4^+ and in some cases N_2 , but also in organic form, like urea or amino
373 acids (Flores and Herrero 2005, Perez-Garcia et al. 2011). The most important path
374 for nitrogen assimilation is through the glutamine synthetase enzyme system, by
375 which glutamate reacts with ammonium (driven energetically by ATP) to form the
376 amino acid glutamine.

377 Microalgae have a very high protein content when compared to terrestrial
378 plants (30-60%)(Becker 1994), and therefore the nitrogen requirements are high.
379 The nitrogen supply along with carbon supply for microalgal production is one of
380 the main nutrient expenses and is an indirect energy input to the cultivation of
381 microalgae (Borowitzka and Moheimani 2013).

382

383 **3.1 Applying nitrogen**

384 **3.1.1 Inorganic nitrogen**

385

386 3.1.2 Nitrogen oxides

387 Nitrate (NO_3^-) is the most commonly used mineral nitrogen form for microalgae
388 and cyanobacteria cultivation on synthetic media. The most frequently used nitrate
389 salts are NaNO_3 and, less frequently, KNO_3 (Grobbelaar 2004). Nitrate is taken up by
390 active mechanisms and therefore consumes energy (Graham and Wilcox 2000).
391 Nitrate does not display toxic effects to cells, and microalgae can tolerate
392 concentrations of up to 100 mM of nitrate (Jeanfils et al. 1993). However, it was
393 observed that the growth is negatively affected when the concentration of nitrate is
394 increased (Jeanfils et al. 1993). Possibly, the activity of nitrate reductase is
395 enhanced when nitrate concentrations are high and these results in a high
396 intracellular concentration of nitrite and ammonium, both of which are toxic to the
397 cells (Chen et al. 2009, Jeanfils et al. 1993, Kim et al. 2013).

398 Nitric oxide (NO) could be considered as interesting nitrogen form mainly when
399 flue gases are used. Nitric oxide has a very low solubility in the cultivation medium,
400 and this low solubility is considered to be the rate-limiting factor to supply NO to
401 microalgal cultures (Jin et al. 2008, Nagase et al. 2001). To improve the solubility of
402 NO, ferrous-complexed EDTA can be added to the cultivation medium (Jin et al.
403 2008, Santiago et al. 2010) or the NO bubble retention time can be increased and/or
404 bubble size decreased (Nagase et al. 1998, Nagase et al. 1997). Dissolved nitric oxide
405 is oxidized to nitrite or nitrate in the presence of dissolved oxygen, which both can
406 be taken up by the microalgae (Nagase et al. 1997). However, due to the fact that
407 nitric oxide is a small and non-polar molecule, it diffuses directly into the cells and is
408 oxidized intracellularly to nitrite/nitrate. Nitric oxide, however, is a free radical and

409 high intracellular concentrations will have detrimental effects (Nagase et al. 2001,
410 Yoshihara et al. 1996). The degree of the tolerance to NO is species-dependent
411 (Brown 1996).

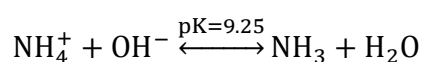
412 Nitrite (NO_2^-) is frequently found in natural environments as an intermediate
413 product of the nitrification process, that is, the oxidation of ammonia to nitrate.
414 However, nitrite is also an intracellular intermediate of the nitrogen metabolism,
415 that is the product of reduction of nitrate to nitrite by the nitrate reductase, which
416 then is reduced further to ammonium through the action of the nitrite reductase.
417 The main way of nitrite uptake is through active transportation, but diffusion has
418 been also reported for green microalgae and cyanobacteria (Flores et al. 1987, Fuggi
419 1993). Although nitrite can be taken up and used as a nitrogen source, at high
420 concentrations it has toxic effects (Chen et al. 2012). Yang et al. (2004) observed
421 that a nitrite concentration of 4 mM extended the lag phase in cultures of
422 *Botryococcus braunii* to 10 days, while an increase of nitrite to 8 mM causes a total
423 inhibition of microalgal growth. Also in the study of Chen et al. (2011), in which
424 *Microcystis aeruginosa* was cultivated in a medium with 50 mg/l nitrate, the
425 addition of nitrite from 0 to 15 mg/l had a gradual negative effect on cell growth.
426 The uptake of nitrite seems to be reduced under low CO_2 concentration, suggesting
427 that CO_2 is required for nitrite uptake (Flores et al. 1987, Vílchez and Vega 1994),
428 while high CO_2 concentration seems to affect positively the nitrite reductase activity,
429 enhancing the nitrite assimilation (Hu and Zhang 2008).

430

431 **3.1.3 Ammonia/ammonium**

432 Ammonia is a volatile molecule, but, unlike CO₂, its solubility is very high (about
433 35% (w/w) at 25°C) and it is found frequently as a liquid solution. When ammonia
434 is dissolved in water it reacts with water to form a buffer system of
435 ammonia/ammonium:

436



437

438 The equilibrium between the forms of ammonium (ionized form) and free
439 ammonia (the unionized gaseous form present in the solution) depends mainly on
440 the pH (**Figure 3**). At pH values higher than 9.25 (=pK at 25°C) the dominant species
441 is the free ammonia (NH₃). Temperature has also a significant effect on the
442 ammonia/ammonium species equilibrium; the pK value decreases as temperatures
443 increase, which means that free ammonia start to be dominant at lower pH values
444 when temperatures are high.

445 Ammonia/ammonium is the preferred nitrogen source for
446 microalgae/cyanobacteria because its uptake and assimilation consumes less
447 energy compared to the other nitrogen sources (Boussiba and Gibson 1991, Graham
448 and Wilcox 2000, Perez-Garcia et al. 2011). However, the microalgal biomass
449 production or the growth rate using ammonia/ammonium as nitrogen source is
450 similar as when nitrate is used as nitrogen source (Boussiba 1989, Park et al. 2010)
451 or even lower (Kim et al. 2013, Lin et al. 2007). A serious constraint when using
452 ammonia/ammonium is the potential toxicity. Free ammonia has detrimental effect
453 on microalgae in relatively low concentrations (2 mM) (Abeliovich and Azov 1976,

454 Azov and Goldman 1982). The main factor affecting the toxicity is the pH of the
455 cultivation medium, which determines whether the toxic form of free ammonia is
456 dominant or the non-toxic ammonium ion. Microalgae and cyanobacteria take up
457 ammonium actively by transportation mechanisms and can thus control
458 intracellular concentrations. But free ammonia diffuses passively into cells, which
459 have therefore little control over intracellular concentrations, which can sometimes
460 become toxic. Free ammonia affects the photosynthetic system and in particular
461 induces photo damage of photosystem II (Azov and Goldman 1982, Drath et al.
462 2008). The toxicity of ammonium ions is considerable less than that of free
463 ammonia (Källqvist and Svenson 2003), and therefore free ammonia should be
464 considered as the main toxic factor affecting the viability of the cells. However, the
465 degree of the toxicity is species dependent. For instance *Arthrospira*, which thrives
466 in media with very high pH (even more than 11) (Boussiba 1989), is found to be
467 more resistant to ammonia toxicity than other cyanobacteria or microalgae. The
468 degree of toxicity seems to be related to the difference between the intracellular and
469 extracellular (medium) pH values. At low pH differences, as it is in case of
470 *Arthrospira*, the resistance to ammonia toxicity is higher (Belkin and Boussiba
471 1991a, b).

472 Ammonia toxicity should be taken into consideration when wastewaters rich in
473 ammonia are used as the cultivation medium. To avoid the negative effect of free
474 ammonia, one strategy is to regulate the pH values and keep them well below the pK
475 of ammonia/ammonium in order to limit the concentration of free ammonia (Azov
476 and Goldman 1982) or to dilute the wastewater to avoid an inhibitory ammonia

477 concentration (Olguín et al. 2003) or to use a fed-batch cultivation mode, in which
478 ammonia is added gradually to the culture medium (Rodrigues et al. 2010). It is
479 worth mentioning that the assimilation of different nitrogen forms influences the pH
480 of the culture medium. If ammonium is applied as the nitrogen source the pH may
481 drop due to the release of H⁺ during assimilation, while the pH will increase due to
482 the release of OH⁻ when nitrate is applied (Perez-Garcia et al. 2011). An additional
483 constraint of using ammonia for nitrogen application for microalgae growth is that it
484 can be lost from the cultivation media due to volatilization, especially at higher pH
485 values (Markou et al. 2014a).

486 For the production of a series of nitrogen fertilizers (such as urea, ammonium
487 nitrate, urea-ammonium-nitrate (UAN), ammonium bicarbonate etc.), ammonia is
488 the main feedstock. Thus ammonia as fertilizer represents the lowest direct energy
489 input for the cultivation of microalgae (Johnson et al. 2013). Ammonia is used to
490 react with CO₂ to form ammonium bicarbonate, $\text{CO}_2 + \text{NH}_3 + \text{H}_2\text{O} \leftrightarrow \text{NH}_4\text{HCO}_3$,
491 (Aaron and Tsouris 2005) or urea, $\text{CO}_2 + 2\text{NH}_3 \leftrightarrow \text{H}_2\text{NCOONH}_4 \leftrightarrow (\text{NH}_2)_2\text{CO} +$
492 H_2O , (Meessen 2000), which both could be used as nitrogen and carbon sources for
493 the cultivation of microalgae.

494

495 **3.1.4 Molecular N₂**

496 Some cyanobacteria (such as *Oscillatoria* sp., *Nostoc* sp., *Anabaena* sp. etc.) and
497 some diatoms (such as *Rhizosolenia* and *Hemiaulus* which have cyanobacterial
498 symbionts) are diazotrophic microorganisms, which means that they can assimilate
499 dinitrogen (N₂) as their sole nitrogen source by the reduction of N₂ to NH₄⁺ using

500 the nitrogenase enzyme complex (Benemann 1979, Gallon 2001, Peccia et al. 2013,
501 Stal 2000, Zehr 2011). However, the nitrogen-fixing process is a very energy-costly
502 process, and it consumes sixteen ATP for the generation of two NH₃ according to the
503 following equation (Großkopf and LaRoche 2012):

504



506

507 where F_{red} and F_{dox} are the reduced and oxidized form of ferredoxin,
508 respectively, and P_i is inorganic phosphate.

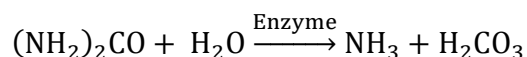
509 Probably due to the energetically costly nature of nitrogen fixation, it is a
510 process with a very low reaction rate and therefore it is considered to be unsuitable
511 for high-rate production of cyanobacteria (Grobbelaar 2004). However, nitrogen
512 fixing cyanobacteria have been suggested to be used for the production of nitrogen
513 fertilizers (Benemann 1979, Razon 2012).

514

515 **3.2.1 Organic nitrogen**

516 Microalgae can utilize nitrogen from organic forms such as urea and some
517 amino acids. Urea and amino acids are transported actively into the cells and are
518 metabolized intracellularly (Flores and Herrero 2005, Perez-Garcia et al. 2011). The
519 most significant organic nitrogen form that could be used as nitrogen source for
520 microalgae cultivation is urea. Generally, urea is hydrolyzed to ammonia and
521 carbonic acid which both can be utilized by microalgae and cyanobacteria.

522



523

524 Many researchers reported that urea has a positive influence in the growth of
525 some species, such as in *A. platensis* (Danesi et al. 2002), *Chlorella* sp. (Hsieh and
526 Wu 2009) or *Coccomyxa acidophila* (Casal et al. 2011) and their growth rates are
527 equal or even higher compared to cultures using other nitrogen sources.

528 Beside urea, microalgae are also capable to utilize nitrogen from amino acids, in
529 autotrophic as well as heterotrophic cultivation mode. However the capability of
530 microalgae to grow on amino acids as nitrogen source is species dependent and
531 growth rates vary significant between the microalgal species and the amino acid
532 used (Flores and Herrero 2005, Neilson and Larsson 1980). Various wastewaters
533 derived from the livestock or food processing sector could be used as organic
534 nitrogen source, however the presence of bacteria in the culture which convert the
535 organic nitrogen to inorganic one seems to be necessary (Li et al. 2011,
536 Pehlivanoglu and Sedlak 2004).

537

538 3.3 Counter-repression

539 The simultaneous presence of more than one nitrogen form in the cultivation
540 medium affects the uptake of nitrogen. It has been shown that there is a repression
541 of the uptake of some nitrogen forms when other forms are simultaneous present in
542 the medium. When ammonia/ammonium is present the uptake of nitrite and nitrate
543 is repressed (Boussiba and Gibson 1991, Fernandez and Galvan 2007, Vilchez and
544 Vega 1994) and microalgae will first completely remove ammonia/ammonium and

545 only then utilize the other forms (Boussiba and Gibson 1991). Ammonium represses
546 specific permeases for the active transport of nitrite or nitrate into the cells and also
547 represses the synthesis of nitrate and nitrite reductase (Darley 1982, Florencio and
548 Vega 1983, Garbayo et al. 2002, Ohmori et al. 1977). On the other hand, a high
549 nitrate concentration also inhibits ammonium/ammonia uptake (Florencio and
550 Vega 1983). Ammonium/ammonia also represses urea uptake, but the
551 concentration at which repression occurs seems to differ between species (Molloy
552 and Syrett 1988).

553 Likewise, nitrate and nitrite mutually inhibit each other. In the cyanobacterium
554 *Anacystis nidulans* nitrate hindered the active uptake of nitrite. However in cultures
555 of *C. reinhardtii* nitrite inhibited nitrate uptake only in immobilized cells, while in
556 the free cells nitrite did not block nitrate uptake (Garbayo et al. 2002).

557 As has been mentioned above, microalgae prefer to take up the most reduced
558 form of nitrogen. It seems that the order of preference for nitrogen utilization is
559 $\text{NH}_4^+ > \text{NO} > \text{NO}_2^- > \text{NO}_3^- > \text{urea}$ (Garbayo et al. 2002, Nagase et al. 2001, Perez-Garcia et
560 al. 2011).

561

562 **4. Phosphorus**

563 Phosphorous is one of the most important nutrients for microalgal growth and
564 its biomass content varies from 0.05% up to 3.3% (Grobbelaar 2004). Phosphorus is
565 an component of several organic molecules that are essential to the metabolism,
566 such as nucleic acids (RNA and DNA), membrane phospholipids and ATP (Geider

567 and La Roche 2002). Phosphorus is frequently a limiting nutrient for microalgae,
568 especially in natural environments (Oliver and Ganf 2000).

569 Unlike carbon and nitrogen nutrients, which are renewable although their
570 production is an energy intensive process, phosphorus is derived from fossil
571 phosphate-rocks (calcium phosphates), which are non-renewable and their reserves
572 are considered to be depleted in the future (Elser 2012). There are various types of
573 phosphorus fertilizers that could be used as phosphorus source for microalgae
574 cultivation, such as potassium-, sodium- and ammonium-phosphates or
575 superphosphates, but all are produced using phosphate-rock as feedstock (**Figure**
576 **4**).

577 In natural environments as well as in wastewaters, phosphorus is present in
578 various forms such as orthophosphate, polyphosphate, pyrophosphate,
579 metaphosphate and their organic forms (Cembella et al. 1982, Yeoman et al. 1988).
580 It is well known that phosphorus is taken up by the cells in the orthophosphate
581 form, however other inorganic and organic phosphorus forms (mainly dissolved
582 organic phosphorus but also insoluble phosphorus compounds) can also be utilized
583 by microalgae (Huang and Hong 1999, Whitton 1991). In case of dissolved organic
584 phosphorus (DOP), there are two ways of utilization: either by actively up-take into
585 the cells or by extracellular mineralization by phosphatase enzymes (Dyhrman and
586 Ruttenberg 2006, Hua-sheng et al. 1995). The capability of DOP to be taken-up
587 depends on its chemical composition. However, most of the DOP compounds cannot
588 be directly assimilated by microalgae and have first to be mineralized (Dyhrman
589 and Ruttenberg 2006). In case of inorganic phosphorus forms other than

590 orthophosphate, in order to be rendered suitable for uptake by microalgae they
591 have first to be converted to orthophosphate. This is accomplished also by the
592 action of various phosphatase enzymes (Kuenzler and Perras 1965, Lin 1977). The
593 enzymes for the conversion of the various forms into orthophosphate are
594 intracellular, extracellular or attached to the cell wall of algae. The activity of these
595 enzymes is affected from various environmental factors, such as pH, temperature,
596 light and the presence of metals or other inhibitors (Kuenzler and Perras 1965, Lin
597 1977, Whitton et al. 2005). Orthophosphate is taken up actively, while passive
598 diffusion corresponds to a small fraction of total inorganic phosphorus influx
599 (Cembella et al. 1982). The uptake rate of phosphorus is affected by the cell
600 condition and by several environmental factors, such as available energy (light), pH,
601 temperature, salinity/ionic-strength of the cultivation medium and available ions
602 such as K^+ , Na^+ and Mg^{2+} (Cembella et al. 1982, Corell 1998, Falkner et al. 1980,
603 Rigby et al. 1980, Seale et al. 1987). The cellular production of these enzymes is
604 significantly enhanced when available phosphorus is decreased and when cells are
605 phosphorus limited or starved (Dyhrman and Ruttenberg 2006, Hua-sheng et al.
606 1995).

607 Contradictory data are reported regarding the uptake of phosphorus as
608 organic compounds. In some studies the phosphorus uptake rate of organic
609 compounds was lower when compared to the uptake rate of orthophosphate, a
610 fact that resulted in phosphorus limitation and reduction of the biomass production
611 (Ellwood et al. 2012, Kuenzler and Perras 1965). In other studies growth rates were
612 equal or almost equal when phosphorus was supplied as organic phosphates or

613 inorganic phosphates (Whitton 1991, Whitton et al. 2005). In the presence of
614 metals, phosphate may also form complexes with humic substances. Such
615 phosphate-metal-humic complexes may have a low bio-availability (Li and Brett
616 2013).

617 Microalgae and cyanobacteria may accumulate intracellular phosphorus
618 reserves as polyphosphate granules. This phosphorus reserve can be used as a
619 phosphorus source when phosphate becomes depleted in the surrounding medium.
620 This behavior is known as luxury uptake and is observed in microalgae as well as
621 cyanobacteria (Bolsunovskii and Kosinenko 2000, Powell et al. 2009, Shively 1988).
622 This capability to store excess phosphorus can be exploited for removal of
623 phosphorus from wastewaters (Powell et al. 2011). However, in cultures in which
624 synthetic fertilizers are used, luxury uptake should be avoided in order to maximize
625 the biomass yield per mass of added nutrient.

626 At increased pH due to photosynthesis and the alkalization of the cultivation
627 medium, polyvalent cations, such as calcium and magnesium may precipitate with
628 phosphates (Hartley et al. 1997, Hoffmann 1998) and this may reduce the
629 availability of phosphorus (Cembella et al. 1982), especially in wastewaters in which
630 the content of divalent cations is high.

631

632 **5. Potassium**

633 Potassium, along with nitrogen and phosphorus is one of the three primary
634 macronutrients for biomass production. Potassium content in some microalgae
635 ranges from 1.2% to 1.5% (Tokuşoglu and Ünal 2003), however it could be as high

636 as 7.5% (Grobbelaar 2004). Potassium plays a significant biological role, because it
637 is an activator for a number of enzymes involved in photosynthesis and respiration,
638 it affects protein and carbohydrate synthesis and regulates the osmotic potential of
639 cells (Checchetto et al. 2013, Hopkins and Hüner 2009, Malhotra and Glass 1995).
640 Potassium is taken up actively by the cells when concentrations are low, but it can
641 be also taken up passively at high concentrations (Malhotra and Glass 1995,
642 Trombala 1978). Potassium can be applied to microalgal cultures as various salts,
643 such as K_2HPO_4 , KH_2PO_4 , KNO_3 , KSO_4 , KCl etc. or using wastewater streams. Wastes
644 and wastewaters from the agro-industrial sector seem to be rich in potassium, for
645 example swine slurry content ranges from 3 to 7.5 g l^{-1} (as K_2O) and poultry manure
646 contain potassium up to 32.5 mg g^{-1} (Markou and Georgakakis 2011). However, it is
647 not known whether microalgae can utilize only inorganic potassium or are capable
648 to utilize organic-bounded potassium by performing hydrolysis.

649

650 **6. Others nutrients**

651 For an unhindered microalgal growth, the cultivation medium has to contain several
652 other nutrients (micro-nutrients) besides carbon, nitrogen, phosphorus and
653 potassium (macro-nutrients). Essential micro-nutrients are Mg, S, Ca, Na, Cl, Fe, Zn,
654 Cu, Mo, Mn, B and Co. Wastewaters and seawater are a good source for most of these
655 nutrients (Markou and Georgakakis 2011, Pohl et al. 1987).

656

657 **6.1 Magnesium**

658 Magnesium is an essential element for microalgal biomass production. Magnesium
659 content in microalgae ranges between 0.35% and 0.7% (Tokuşoglu and Ünal 2003),
660 however a content as high as 7.5% can be found in some species (Grobbeelaar 2004).
661 Magnesium participates in vital cell processes such as ATP reactions for carbon
662 fixation and is an activator for several major enzymes. Also it is a constituent of the
663 photosynthetic apparatus and in particular of the chlorophylls (Hopkins and Hüner
664 2009). In the microalga *Chlorella vulgaris*, it was observed that cell multiplication
665 and synthesis of cell material are independently affected by magnesium availability.
666 It seems that the process of multiplication requires larger amounts of magnesium
667 than does the production of dry matter (Finkle and Appleman 1953). Magnesium in
668 aqueous solutions is mainly presented as the Mg^{2+} cation. However, when pH is high,
669 Mg^{2+} may precipitate as magnesium phosphate or magnesium hydroxide. These
670 mineral precipitates may induce flocculation of microalgal biomass, which may not
671 be desirable. However, the algal-mineral complexes are formed only at very high pH
672 values, higher than 11 (Hoffmann 1998, Vandamme et al. 2012). At those high pH
673 values, only a few alkalophilic species can thrive. Magnesium is provided to the
674 cultures mainly as $MgSO_4$, however several other magnesium salts are available and
675 could be used, such as $(NH_4)_2SO_4$ - $MgSO_4$, $MgCl_2$, $MgHPO_4$, $Mg(NO_3)_2$ etc.

676 On the other hand, wastewaters are often deficient in magnesium (Hoffmann
677 1998) and it was reported that supplementation of magnesium is necessary when
678 hydrolyzed human urine (Tuantet et al. 2014), anaerobic digestion effluents of
679 piggery wastes (Park et al. 2010) or the aqueous phase from the hydrothermal

680 liquefaction of the same microalgae are used as a substrate for microalgae
681 cultivation (Garcia Alba et al. 2013).

682

683 **6.2 Sulfur**

684 Sulfur is also a significant macronutrient for the growth of microalgae and its
685 biomass content ranges from 0.15% to 1.6% (Grobbelaar 2004). It is a component of
686 the amino acids cysteine and methionine and of sulfolipids that are part of the lipid
687 bilayer of the cell membranes. Moreover it is a constituent of vitamins, regulatory
688 compounds, and a number of sulfur-containing secondary metabolites (e.g. dimethyl
689 sulphoxide, which is common in marine microalgae) (Melis and Chen 2005). Sulfur
690 can be found in many different forms, however, the sulfur demands of microalgae
691 are fulfilled mainly by the uptake in form of sulfate (SO_4^{2-}), while other forms such
692 as sulfide are toxic (Oren et al. 1979). Sulfate is taken up actively involving transport
693 mechanisms. In marine environments sulfate concentration is relative high (29 mM)
694 and it is believed that it is unlikely to be a growth limiting factor for marine
695 microalgae (Giordano et al. 2008, Hawkesford 2008). When sulfate is depleted from
696 growth media it is documented that the uptake rate of sulfate in various microalgae
697 is induced (Weiss et al. 2001). Sulfate is available in various forms of fertilizers and
698 can be provided as MgSO_4 , $(\text{NH}_4)_2\text{SO}_4$, K_2SO_4 , $(\text{NH}_4)_2\text{SO}_4\text{-MgSO}_4$ etc. Wastewaters are
699 also a source for sulfates, and especially some industrial wastewaters, such as those
700 derived from paper milling, food processing and distillery, are very rich in sulfates
701 (Hulshoff Pol et al. 1998, Lens et al. 1998, Sarti et al. 2010, Silva et al. 2002).

702

703 **6.3 Calcium**

704 Calcium is a significant element for microalgal growth, since it is an important
705 constituent of cell walls. It also affects the cell division and is a secondary messenger
706 that affects the overall morphogenesis (Kylin and Das 1967, Plieth et al. 1997).
707 Calcium participates also in the process of calcification (see carbon section). Calcium
708 content in microalgal biomass varies from 0.2% to 1.4 % (Kay and Barton 1991,
709 Tokuşoglu and Ünal 2003) but it can reach 8% (Grobbelaar 2004). Calcium in
710 aquatic environments is mainly in form of Ca^{2+} and it is taken-up actively as well as
711 passively by diffusion. However, relatively little is known about the transport of Ca^{2+}
712 into the cell (Moheimani et al. 2012). Increased intracellular Ca^{2+} concentration for
713 prolonged periods has a negative effect on growth (Karimova et al. 2000).

714 High calcium concentrations in the cultivation medium along with high pH
715 values results to the formation of CaCO_3 and various other calcium salts which
716 precipitate, decreasing the alkalinity of the medium and the concentration of some
717 minerals such as iron and phosphorus (Shimamatsu 2004). Calcium is frequently
718 added to the cultures as CaCl_2 , however some other forms could be also used
719 ($\text{Ca}_3(\text{PO}_4)_2$, $\text{Ca}(\text{NO}_3)_2$ etc.).

720

721 **6.4 Iron**

722 Among trace elements, iron is one of the most essential elements required by
723 microalgae. Iron, as a transition metal, is associated with enzymes through complex
724 formation with S or N groups of various amino acids. Iron is involved in fundamental
725 enzymatic processes such as oxygen metabolism, electron transfer, nitrogen

726 assimilation, and DNA, RNA and chlorophyll synthesis (Naito et al. 2005, Straus
727 2004). However, iron has some physico-chemical characteristics that reduce its bio-
728 availability. In aqueous oxic environments because Fe^{2+} is quickly oxidized iron is
729 mainly in the Fe^{3+} form. Fe^{3+} forms oxides and hydroxides, which are insoluble.
730 Moreover iron is easily adsorbed onto particle surfaces, resulting to a low bio-
731 availability. In natural environments, and in domestic wastewaters, iron is
732 frequently bio-unavailable and this can limit the growth capability of microalgae
733 and cyanobacteria (Mostafa and Mahmoud 2012, Sunda and Huntsman 1995).
734 Therefore, iron is frequently supplied in cultures as chelated complexes, which
735 render it bio-available. Iron deficient cultures display low growth rates (Sandmann
736 1985), while high iron concentration in some microalgal species induce lipid
737 synthesis and increasing its content in the biomass (Liu et al. 2008, Yeesang and
738 Cheirsilp 2011).

739

740 **7. Effect of nutrient limitation/starvation in growth and biomass composition**

741 Microalgae and cyanobacteria adjust their nutrient uptake and requirements
742 according to the nutrient availability in the surroundings. They can store excess
743 quantities of a nutrient (luxury uptake) but also can grow with lower quantities of a
744 nutrient. However, concentrations lower than a specific threshold of a nutrient
745 affects the general growth rates of microalgae and cyanobacteria. The effect of the
746 limitation of a nutrient on algal growth is best described by the Droop model (Droop
747 1968, Lemesle and Mailleret 2008, Sommer 1991), according to which, growth rates

748 are dependent on the intracellular concentration of a nutrient. This relationship is
749 described with the following model:

$$\mu = \mu_{max} \left(1 - \frac{k_q}{Q}\right)$$

750 where, μ is the current specific growth rate, μ_{max} is the maximum specific growth
751 rate for a given cultivation system, Q is the current intracellular concentration of the
752 nutrient and k_q is the minimum intracellular concentration of the nutrient
753 (subsistence quota). The subsistence quota is a threshold under which microalgae
754 and cyanobacteria do not grow (Droop 1968).

755 When a nutrient becomes limiting, microalgae often adjust their biomass
756 composition, either triggering the accumulation of carbohydrates or lipids or by
757 altering the content of other compounds such as proteins and pigments (Hu 2004,
758 Pirson et al. 1951, Turpin 1991). In the last years there is an intensification of the
759 research on the topic of nutrient starvation for the accumulation of lipids for the
760 production of bio-diesel (Breuer et al. 2012, Li et al. 2012, Mujtaba et al. 2012,
761 Rodolfi et al. 2009), and, to a lesser extent, for the accumulation of carbohydrates
762 for bio-ethanol production (Ho et al. 2013, Markou et al. 2013, Miranda et al. 2012).
763 The cultivation under nutrient limitation could be a strategy to significantly reduce
764 the nutrient demand for microalgae cultivation while at the same time to achieve a
765 very high biomass yield per mass of nutrient added. However, the decreased
766 nutrient demand should be carefully balanced against a decreased growth rate. This
767 could be moderated by using optimized cultivation media or by using a multi-stage

768 cultivation system (Dragone et al. 2011, Markou et al. 2012a). However, more
769 research is needed in this field.

770 Recently, it was shown that there is a specified relationship between
771 intracellular phosphorus limitation and biomass composition (carbohydrates,
772 proteins and lipids) of the cyanobacterium *Arthrospira* (Markou 2012). This
773 relationship was expanded by the study of Adams et al. (2013), dealing with
774 nitrogen limitation and lipid content of several eukaryotic microalgae. In both
775 studies there was shown that a minimum intracellular nutrient concentration exists
776 in which the desired biomass component is in its maximum concentration, and
777 which is gradually reduced when the intracellular nutrient increases. However, the
778 effect of nutrient limitation on growth of the microalgae seems to be depended on
779 the microalgal species (Adams et al. 2013) and the kind of nutrient. It is in general
780 known that the effect of a nutrient on the growth depends on how much low can the
781 subsistence quota of the particular nutrient be. Lower subsistence quota of a
782 nutrient means that the microalga or the cyanobacterium is more flexible and
783 offsets the limitation of the nutrient on the growth (Grobbelaar 2004).

784

785 **8. Wastewater as nutrient source**

786 As was mentioned before, wastewater streams are good source for specific nutrients
787 and the use of wastewater could reduce the cost of synthetic fertilizer.
788 Microalgae/cyanobacteria cultivation in wastewater has a double advantage; on the
789 one hand valuable biomass is produced while treatment of the wastewater occurs
790 (Markou and Georgakakis 2011, Rawat et al. 2011). However, the major constrain of

791 using wastewater as nutrient source is that it reduces the range of the biomass
792 applications because the produced biomass is possibly contaminated by various
793 pollutants that are present in the wastewater. Therefore, microalgae produced on
794 wastewater should mainly be used for the production of biofuels rather than food or
795 feed applications. However, to overcome this issue, it has been suggested that the
796 nutrients could be separated from the wastewater and subsequently added to the
797 cultivation medium, using a physic-chemical technology for nutrient recovery from
798 wastewater. Potential technologies for this purpose are adsorption (for instance on
799 zeolite), precipitation (such as struvite), air stripping (such as ammonia stripping)
800 etc. (De-Bashan and Bashan 2004, Markou et al. 2014b, Renou et al. 2008). An
801 additional constrain is that several wastewaters, especially of the industrial and
802 agro-industrial sector, contain various inhibitors in concentrations that could be
803 have detrimental effects on microalgal cells. Inhibitors, such as ammonia, nitrite,
804 heavy metals, polyphenolic compounds, organic acids etc. might render wastewater
805 inappropriate for microalgal or cyanobacterial growth (Cai et al. 2013, Markou et al.
806 2012b, Olguín 2012). Also the deficiency of some wastewater on essential nutrients
807 and their low bio-availability could be limit their application (Mostafa and
808 Mahmoud 2012). However, in many cases the growth of microalgae on wastewater
809 was similar compared to the growth on synthetic media (Arbib et al. 2014, Martínez
810 et al. 2000).

811 Wastewater can also contain several microorganisms (mainly bacteria) that
812 could compete with microalgae. The presence of these competitors might have
813 negative effect on the microalgal growth and even if the competitors have higher

814 growth rates than microalgae and if the conditions are favorable for the growth of
815 the first, the microalgal culture might be fail (Wang et al. 2013). In addition, in some
816 wastewater types, the content of suspended solids or dissolved colored organic
817 compounds might have negative effect on growth of microalgae due to the decrease
818 of light penetration inside the cultures (Depraetere et al. 2013), however this issue
819 might not be very important if the wastewater contain organic carbon and the
820 microalgal species can be grow in hetero- or mixo-trophic mode.

821

822 **9. Recycling of nutrients**

823 To reduce the amounts of input of fertilizers required for microalgal and
824 cyanobacterial biomass production, several nutrient recycling strategies are
825 suggested. Two main approaches for nutrient recycling can be considered: one
826 source is the spent medium after the biomass has been harvested and the second
827 source is the biomass residues that remains after extraction of the desirable
828 compounds. Concerning the nutrients left on the cultivation medium after
829 harvesting of biomass, a strategy is to recycle the medium and to supplement only
830 the exhausted nutrients. However, as was shown this strategy has a main constraint
831 related to the presence of several autoinhibitory organic compounds that
832 microalgae might excrete to the medium and/or the presence and the growth of
833 unwanted bacteria (González-López et al. 2013, Rodolfi et al. 2003). Sterilization
834 and degradation of these compounds using various methods could be an approach
835 to allow medium recycling. González-López et al. (2013) investigated various
836 sterilization methods and found that ozonation gave the best results for medium

837 recycling. Nevertheless, the study of Hadj-Romdhane et al. (2013) showed that
838 growth of *Chlorella vulgaris* was almost unaffected after 63 days of medium
839 recycling, a fact that might indicate that the capability of medium reuse is species
840 dependent. Nevertheless, more research is needed in this field.

841 Concerning the nutrients left in biomass residues after the extraction of the
842 desired compounds (i.e. lipids, proteins pigments etc.) the leftover biomass could be
843 treated by several technologies such as anaerobic digestion (Ehimen et al. 2011, Ras
844 et al. 2011) or by hydrothermal liquefaction (Garcia Alba et al. 2013, Shuping et al.
845 2010) so that on the one hand valuable biofuels could be produced (biomethane,
846 bio-oil etc.) and on the other hand the mineralization of the organic fraction could
847 be occurred. Consequently nutrients contained in biomass could be recycled.
848 However, not all of the organic fraction of the biomass can be mineralized but only a
849 portion, that it is determined by the parameters used in each technology. The
850 recycling of the aqueous phase of hydrothermal liquefaction could be reduce the
851 application of nitrogen at half (Garcia Alba et al. 2013), while in the anaerobic
852 digestion under moderate hydraulic retention time (HRT) the mineralization is
853 about 19 and 68% at 16 and 30 days of HRT (Collet et al. 2011).

854

855 **10. Conclusions**

856 It seems that in the future the cultivation of microalgal and cyanobacterial biomass
857 will play a significant role in the sector of biotechnology for the production of
858 valuable organic compounds and of biofuels. However, their cultivation will
859 consume considerable amounts of nutrients, raising questions about the

860 sustainability of such biomass production systems. Optimization of nutrient supply
861 is extremely important to increase sustainability and to avoid shifts in global
862 nutrient supplies. Therefore the understanding of microalgal and cyanobacterial
863 biology combined with the understanding of bio-availability of nutrients is essential
864 to meet previous mentioned goals. Most of the research and even commercial
865 production is done using synthetic nutrient resources; however the usage of
866 wastewater and nutrient recycling are interesting routes that deserve further
867 exploration.

868

869 **References**

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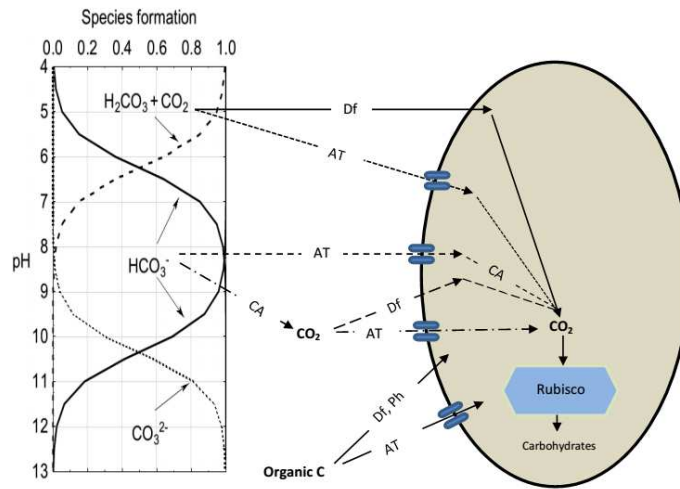


Figure.1 Inorganic carbon uptake of Df:Diffusion, AT: Active Transport, CA: Carbonic Anhydrase, Ph: phosphorylation

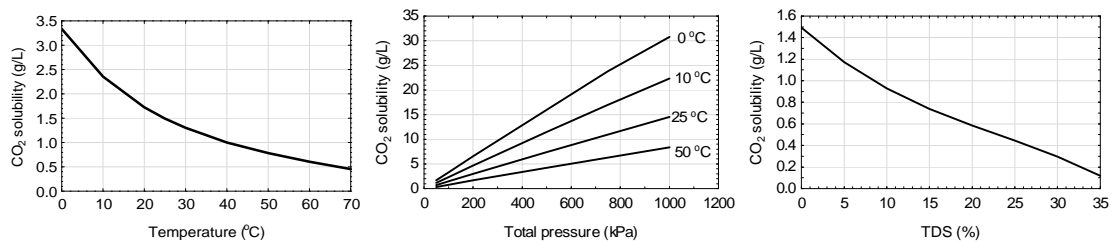


Figure 2 a) total dissolved solids (TDS) for temperature 25°C and pressure 101 kPa. Figures a and b are based on Carroll et al. (1991) and Figure c on Enick and Klara (1990).

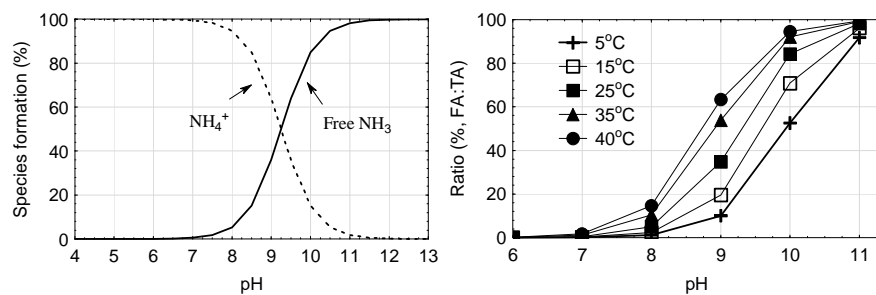


Figure 3. Effect of pH and temperature on the concentration of free ammonia and the ratio of free ammonia (FA) to total ammonia (TA).

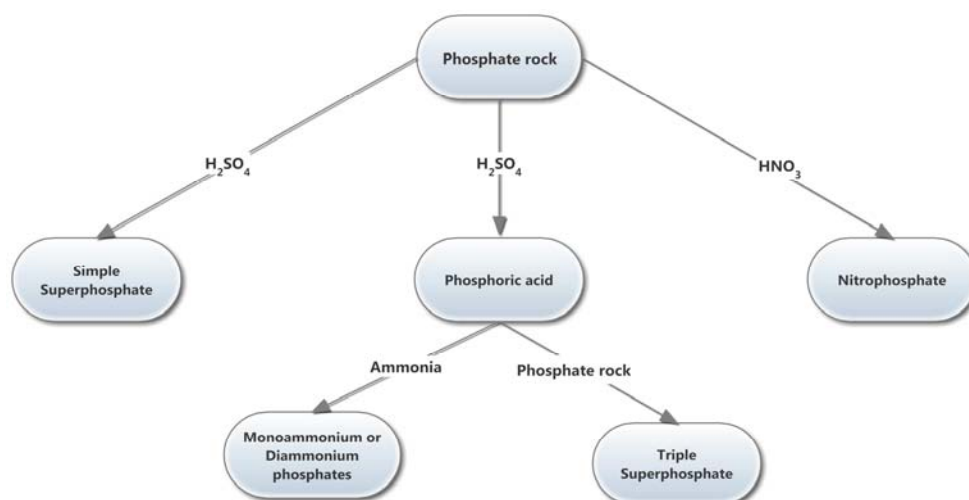


Figure 4. Flowchart of phosphorus fertilizers production

- We review the application of nutrients and their uptake by microalgae/cyanobacteria
- We focus mainly on inorganic and organic forms of C, N and P
- However, the nutrients K, Mg, S, Ca and Fe are also reviewed
- Nutrient supply optimization is very important for sustainable biomass production
- Usage of wastewater and nutrient recycling deserve further exploration