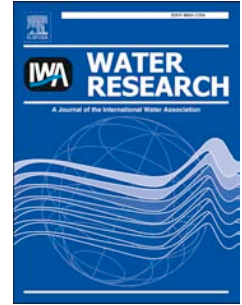*(article begins on next page)*

# Accepted Manuscript

Microalgal and cyanobacterial cultivation: The supply of nutrients

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10

11    **Abstract**

12

13    Microalgae and cyanobacteria are a promising new source of biomass that may

14    complement agricultural crops to meet the increasing global demand for food, feed,

15    biofuels and chemical production. Microalgae and cyanobacteria cultivation does

16    not interfere directly with food production, but care should be taken to avoid

17    indirect competition for nutrient (fertilizer) supply. Microalgae and cyanobacteria

18    production requires high concentrations of essential nutrients (C,N,P,S,K,Fe, etc.). In

19    the present paper the application of nutrients and their uptake by microalgae and

20    cyanobacteria is reviewed. The main focus is on the three most significant nutrients,

21    i.e. carbon, nitrogen and phosphorus; however other nutrients are also reviewed.

22    Nutrients are generally taken up in the inorganic form, but several organic forms of

23    them are also assimilable. Some nutrients do not display any inhibition effect on

24    microalgal or cyanobacterial growth, while others, such as $NO_2$ or $NH_3$ have

25    detrimental effects when present in high concentrations. Nutrients in the gaseous

26    form, such as $CO_2$ 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

38

### 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
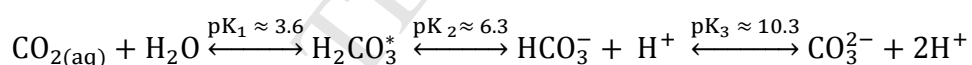
93    compounds, and is the main microalgal (including cyanobacterial) biomass element,

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 $CO_2$. However, since the majority of microalgae are aquatic microorganisms

103   which thrive in liquid habitats, $CO_2$ is dissolved in the aquatic environment. When

104   $CO_2$ is dissolved in water it reacts with the water molecules ($H_2O$) and forms a weak

105   acid-base buffer system, having the following equilibrium (**Figure 1**):

106

$$CO_{2(aq)} + H_2O \xrightleftharpoons{pK_1 \approx 3.6} H_2CO_3^* \xrightleftharpoons{pK_2 \approx 6.3} HCO_3^- + H^+ \xrightleftharpoons{pK_3 \approx 10.3} CO_3^{2-} + 2H^+$$

107

108   where, $H_2CO_3^*$ refers to $CO_{2(aq)} + H_2CO_3$

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 ($HCO_3^-$). 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. $CO_2$

139    can be taken up either passively through (1) membrane diffusion i.e. crossing and

140    inserting the cell as free $CO_2$, or (2) actively through membrane transport

141    mechanisms (pumps). $HCO_3^-$ is taken up only actively through transport

142    mechanisms; however, bicarbonate can be converted by the metalloenzyme

143    carbonic anhydrase (CA) to $CO_2$ ($HCO_3^- \xrightarrow{CA} CO_2 + OH^-$), 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    $CO_2$ concentration than with high $CO_2$ 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 $CO_2$ is preferred over $HCO_3^-$

150    (Aizawa and Miyachi 1986). It was shown that the active uptake of $CO_2$ is

151    significantly faster than that of $HCO_3^-$ (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 $CO_2$ 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 $CO_2$-

164 concentrating mechanism (CCM). The CCM involves various processes by which the

165 intracellular concentration of $CO_2$ 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 $CO_2$ concentration is low and $O_2$ concentration high, Rubisco will react

170 with $O_2$ rather than $CO_2$ and will produce $CO_2$ rather than assimilate $CO_2$

171 (photorespiration). By increasing the ratio $CO_2/O_2$ 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 $HCO_3^-$ to $CO_2$, 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 $HCO_3^-$ or $CO_2$ than of diffusion of $CO_2$ (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 $HCO_3^-$ to give $CO_2$, which can subsequently be taken up by diffusion

182 (Price et al. 2008, Van Den Hende et al. 2012).

8

183    Another strategy developed by microalgae to obtain $CO_2$ from aquatic

184    environments with relative high pH is the process of calcification, in which $CaCO_3$ is

185    precipitated following the reactions (Jansson and Northen 2010, Moheimani et al.

186    2012):

187

$$Ca^{2+} + 2HCO_3^- \rightleftharpoons CaCO_3 + CO_2 + H_2O$$

188

189    However, in marine environments the principal path of calcification is by reacting of

190    calcium cations with carbonate:

191

$$Ca^{2+} + CO_3^{2-} \rightleftharpoons CaCO_3$$
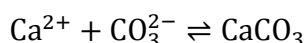
192

193    Although no $CO_2$ is produced in this reaction, removal of $CO_3^{2-}$ 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 $Ca^{2+}$ 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 ($HCO_3^- \xrightarrow{CA} CO_2 + OH^-$),

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).

11

250    The most plain model given the mass transfer rate of $CO_2$ ($M_{CO_2}$) 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, $\alpha$ is the specific area

254    available for mass transfer and, $C^*_{CO_2}$ is the $CO_2$ concentration in the liquid equal to

255    partial pressure in gas phase and $C_{CO_2}$ is the $CO_2$ concentration in the liquid (Van

256    Den Hende et al. 2012). The enhancement of the mass transfer rate of $CO_2$ 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    $CO_2$ from the air into the water is too slow to replace the assimilated $CO_2$ (Suh and

262    Lee 2003). In these systems $CO_2$ has to be provided actively in the one or the

263    another form. The three main ways of applying $CO_2$ to the cultures are (1) pumping

264    air, (2) pumping concentrated $CO_2$ and (3) bicarbonate salts.

265

266    **2.4 Sources for providing inorganic carbon**

267    As was mentioned before, the application of $CO_2$ 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 $CO_2$ is provided by the aeration of the culture with air ($\approx$400 ppmv

271    $CO_2$) 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 $CO_2$ seems also to be costly. Flue gas is a lower-cost alternative.

274  It has a $CO_2$ 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 $CO_2$ in concentrations ranging from 10 up

280  to 25% and can be used as $CO_2$ 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 NOx, SOx, 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 $CO_2$ sources which could be used are biogas derived from the anaerobic

285  digestion process (with a $CO_2$ content of 20-40%) (Travieso et al. 1993) or the $CO_2$

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 $CO_2$ (Gouveia 2011). However, as it was pointed out from Van Den

290  Hende et al. (2012) the critical issue of applying $CO_2$ is not its concentration in the

291  applied gas but the combination of $CO_2$ concentration and its flow rate, consequently

292  the mass of the dissolved inorganic carbon. Because the dissolution of $CO_2$ in water

293  results to its acidification, a high mass flux of $CO_2$ 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 $CO_2$ concentration affects the growth, making this

296 distinction challenging (Yang and Gao 2003). The degree of acidification of the

297 medium due to $CO_2$ 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 $CO_2$ should match the rate of

300 $CO_2$ 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% $CO_2$ gas (Olaizola 2003). That means that the addition of $CO_2$ on

304 demand is independent of the $CO_2$ 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 $CO_2$ 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, $Na_2CO_3 + CO_2 + H_2O \leftrightarrow NaHCO_3$, (Nelson

311 et al. 2009) and ammonium bicarbonate, $NH_4HCO_3$, (Aaron and Tsouris 2005) or

312 urea, $(NH_2)_2CO$, (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 $CO_2$ (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 $CO_2$,

14

318    (for example NaHCO$_3$ solubility is >90g/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 $CO_2$, 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

$$NH_4^+ + OH^- \xrightarrow{\text{pK=9.25}} NH_3 + H_2O$$
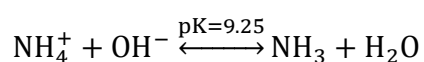
437

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 ($NH_3$). 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 $CO_2$ to form ammonium bicarbonate, $CO_2 + NH_3 + H_2O \leftrightarrow NH_4HCO_3$,

491 (Aaron and Tsouris 2005) or urea, $CO_2 + 2NH_3 \leftrightarrow H_2NCOONH_4 \leftrightarrow (NH_2)_2CO +$

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 $N_2$**

496     Some cyanobacteria (such as *Oscilatoria* sp., *Nostoc* sp., *Anabaena* sp. etc.) and

497 some diatoms (such as *Rhizosolenia* and *Hemiaulusare* which have cyanobacterial

498 symbionts) are diazotrophic microorganisms, which means that they can assimilate

499 dinitrogen ($N_2$) as their sole nitrogen source by the reduction of $N_2$ to $NH_4^+$ using

22

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 $NH_3$ according to the

503   following equation (Großkopf and LaRoche 2012):

504

505   $$N_2 + 8H^+ + 8F_{red} + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 8F_{dox} + 16P_i$$

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

23

$$(NH_2)_2CO + H_2O \xrightarrow{\text{Enzyme}} NH_3 + H_2CO_3$$
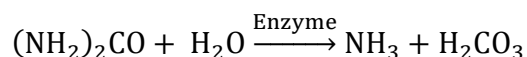
523

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, Vílchez 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 $NH_4^+>NO>NO_2^->NO_3^->$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 comparison 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-availibility (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 Tromballa 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**

29

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 (Grobbelaar 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 $(NH_4)_2SO_4\text{-}MgSO_4$, $MgCl_2$, $MgHPO_4$, $Mg(NO_3)_2$ 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 $MgSO_4$, $(NH_4)_2SO_4$, $K_2SO_4$, $(NH_4)_2SO_4$-$MgSO_4$ 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 ans 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 in 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 $(Ca_3(PO_4)_2, Ca(NO)_3$ 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:

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

750  where, $\mu$ is the current specific growth rate, $\mu_{max}$ 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

34

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 disserve further

867 exploration.

868

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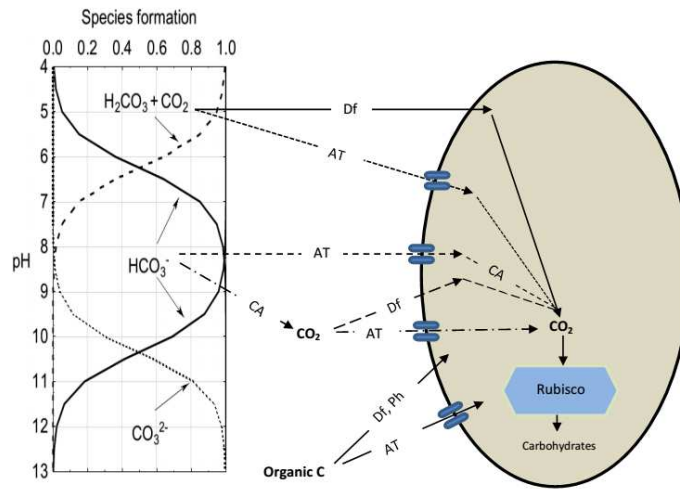
**Figure.1** Inorganic carbon uptake of Df:Diffusion, AT: Active Transport, CA: Carbonic Anhydrase, Ph: phosphorylation
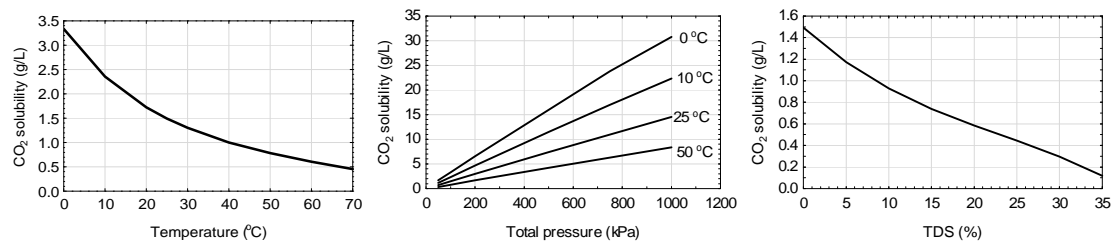
**Figure 2** a) total dissolved solids (TDS) for temperature 25°C and pressure 101 kPa. Figures a and b are based on Carroll et al. (1991) and Figure c on Enick and Klara (1990).
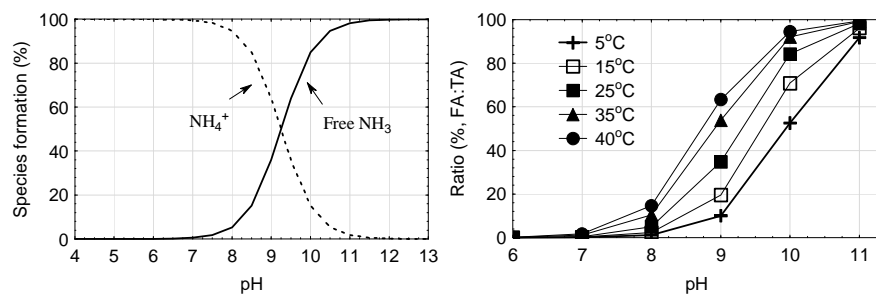
**Figure 3.** Effect of pH and temperature on the concentration of free ammonia and the ratio of free ammonia (FA) to total ammonia (TA).
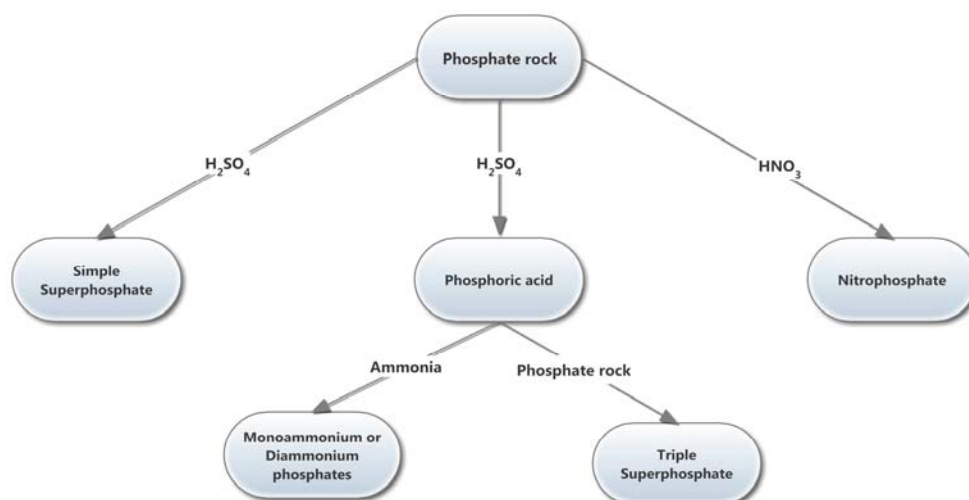
**Figure 4**. Flowchart of phosphorus fertilizers production

- 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 disserve further exploration