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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
microalgal or cyanobacterial growth, while others, such as NO$_2$ or NH$_3$ have detrimental effects when present in high concentrations. Nutrients in the gaseous form, such as CO$_2$ and NO face a major limitation which is related mainly to their mass transfer from the gaseous to the liquid state. Since the cultivation of microalgae and cyanobacteria consumes considerable quantities of nutrients, strategies to improve the nutrient application efficiency are needed. Additionally, a promising strategy to improve microalgal and cyanobacterial production sustainability is the utilization of waste streams by recycling and waste nutrients. However, major constraints of using waste streams are the reduction of the range of the biomass applications due to production of contaminated biomass and the possible low bio-availability of some nutrients.

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

### 1. Introduction

Microalgae and cyanobacteria are photoautotrophic microorganisms, which are presently cultivated to produce numerous high value products, such as vitamins, pigments, proteins, fatty acids, polysaccharides etc. In the near future it is expected that the dedicated market for microalgal high-value compounds will significantly expand (Borowitzka 2013, Spolaore et al. 2006). Moreover, microalgae are considered as a potential biomass feedstock for the production of biofuels and it is believed that they will play a significant role in the sector of renewable energy
(Gouveia 2011, Schenk et al. 2008). However, to fulfill only the global needs for transportation fuels using microalgal biomass as feedstock, the cultivation of microalgae rises several practical questions and has some significant constraints, such as high land areas use and high consumption of energy, water and nutrients (Borowitzka and Moheimani 2013, Chisti 2013). The mass production of microalgae for biofuels production presupposes the application of massive quantities of nutrients (fertilizers) (Borowitzka and Moheimani 2013). Since microalgae can be cultivated in non-arable land it is believed that microalgal biomass production for biofuels will not compete with food production. However, the competition between biomass for biofuels and food production might be transmitted to the competition for nutrient (fertilizers) availability. Because microalgal biomass has low content of cellulose compared to terrestrial crops, it contains three times the amount of nutrients compared to biomass of terrestrial plants. As a result, the nutrient demand for microalgal biomass production is much higher than that for agricultural crops (Elser et al. 2000).

Microalgae during photosynthesis utilize solar energy and along with several essential nutrients (C, N, P, S, K, Fe etc.) to synthesize their biomass compounds and to multiply their cells. Microalgae need specific quantities of those essential elements in order to be capable to produce biomass. Possible deficiency of one of the elements will cause growth reduction (Liebig’s law of the minimum). Considering the universal Redfield C:N:P ratio of 106:16:1 of phytoplankton elemental composition, all of the essential elements have to be present in appropriate ratios, in adequate quantities and in bio-available chemical form in the
cultivation medium so that the growth of microalgae will not be limited (Spaargaren 1996). Consequently, in the literature the recipes of the cultivation media are frequently considered as fixed. Nevertheless, the experience of cultivating microalgae in various types of wastewater with diverse nutrient compositions and the evidence that phytoplankton stoichiometry diverges from the canonical Redfield ratio under specific conditions (Arrigo 2005) suggest that the cultivation media could be flexible and could be adapted to the microalgal metabolic needs.

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

2. Carbon

Photosynthesis is a complex process through which light energy and inorganic carbon is converted into organic matter. Carbon contributes to all organic
compounds, and is the main microalgal (including cyanobacterial) biomass element, amounting up to 65% of dry weight. The carbon content though, varies significant among the species and culture conditions and can range between 17.5 and 65% by dry weight. However, the majority of the species contain about 50% carbon (Grobbelaar 2004). Limitation by other nutrients than carbon (e.g. nitrogen or phosphorus) generally results in an increase in the carbon content of the biomass.

2.1. Buffer system

Carbon is mainly taken up by photosynthetic microorganisms in its inorganic form of CO$_2$. However, since the majority of microalgae are aquatic microorganisms which thrive in liquid habitats, CO$_2$ is dissolved in the aquatic environment. When CO$_2$ is dissolved in water it reacts with the water molecules (H$_2$O) and forms a weak acid-base buffer system, having the following equilibrium (Figure 1):

\[
\text{CO}_2(\text{aq}) + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3^* \rightleftharpoons \text{HCO}_3^- + \text{H}^+ \approx \text{CO}_3^{2-} + 2\text{H}^+
\]

where, H$_2$CO$_3^*$ refers to CO$_2$(aq) + H$_2$CO$_3$

The above equilibrium depends on the pH of the solution, which means that the relative amount of the dissolved inorganic carbon (DIC) species is strictly related to the pH of the solution. Based on the equilibrium of the carbon species (Figure 1), in the range in which the majority of the microalgae thrive, i.e. between pH 6.5 and 10, the dominant carbon form is bicarbonate (HCO$_3^-$). Lowering pH
values, more $H_2CO_3^+$ is gradually formed and when pH reaches that of $pK_2$ the concentration of $H_2CO_3^+$ is equal to $HCO_3^-$. At pH lower than $pK_2$ the $H_2CO_3^+$ is predominant. In contrast, increasing pH values more $CO_2^-$ is gradually formed and when pH reaches that of $pK_3$ the concentration of $CO_3^{2-}$ is equal to $HCO_3^-$. At pH higher than $pK_3$ the $CO_3^{2-}$ is predominant.

2.2 Carbon fixation and up-take

Inorganic carbon is fixed inside the microalgal cells and is converted to organic form through the Calvin cycle. The first step of the Calvin cycle is the assimilation of $CO_2$ and its incorporation into a three-carbon compound. Microalgae and cyanobacteria possess fundamentally the C$_3$ pathway photosynthesis, however in some species the evidence for a C$_3$-C$_4$ intermediate photosynthesis exist (Roberts et al. 2007, Xu et al. 2012). The assimilation of $CO_2$ is catalyzed by Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco). Rubisco can utilize only $CO_2$ and therefore $CO_2$ is the ultimate substrate for carbon fixation (Price et al. 2008).

Since the form and the amount of dissolved inorganic carbon depends on pH, salinity, pressure and temperature (Figure 2) microalgae have developed various mechanisms, by which, in each specific environment they can utilize the available dissolved inorganic carbon (DIC). Consequently, the utilization of the preferred form of carbon is species dependent. Many species of microalgae and cyanobacteria can take up both $CO_2$ and $HCO_3^-$, some may take up only $CO_2$ or $HCO_3^-$ (Camiro-Vargas et al. 2005) and some extremely alkaliphilic cyanobacteria can utilize $CO_3^{2-}$ (Mikhodyuk et al. 2008).
The carbon up-take (Figure 1) is performed either passively or actively. CO$_2$ can be taken up either passively through (1) membrane diffusion i.e. crossing and inserting the cell as free CO$_2$, or (2) actively through membrane transport mechanisms (pumps). HCO$_3^-$ is taken up only actively through transport mechanisms; however, bicarbonate can be converted by the metalloenzyme carbonic anhydrase (CA) to CO$_2$ ($\text{HCO}_3^- \rightarrow \text{CO}_2 + \text{OH}^-$), which can subsequently be taken up either passively or actively, as mentioned before (Badger and Spalding 2004). CA's activity is found to be influenced by the DIC concentration and it appears to be higher in microalgae and cyanobacteria cultivated in media with low CO$_2$ concentration than with high CO$_2$ concentrations (Aizawa and Miyachi 1986).

The form of inorganic carbon utilization depends also on its concentration in the medium; in high DIC concentration it seems that CO$_2$ is preferred over HCO$_3^-$ (Aizawa and Miyachi 1986). It was shown that the active uptake of CO$_2$ is significantly faster than that of HCO$_3^-$ (Matsuda et al. 1999) and is energetically favourable (Moazami-Goudarzi and Colman 2012). The pH of the cultivation medium affects the type of carbon uptake; for example at low pH, two marine microalgae species of Stichococcus, proved to take up only CO$_2$ by diffusion, while the active uptake appeared to be occur at pH higher than 6 (Moazami-Goudarzi and Colman 2012).

Since the active transportation of inorganic carbon is an energy consuming process and because the preferred form for inorganic carbon up-take depends on the microalgal species the quantitative evaluation of the contribution of each species of dissolved inorganic carbon to the carbon fixation is very significant (Matsuda et
al. 1999) and more research is needed at least for the potentially economical significant species.

CA in microalgae and cyanobacteria is thought to be a part of the CO$_2$-concentrating mechanism (CCM). The CCM involves various processes by which the intracellular concentration of CO$_2$ around the Rubisco is elevated compared to its concentration in the extracellular surrounding environment. In other words, the main aim of the CCM is to actively transport and concentrate the inorganic carbon, in order to be used as substrate for its fixation (Azov 1982, Price et al. 2008). When intracellular CO$_2$ concentration is low and O$_2$ concentration high, Rubisco will react with O$_2$ rather than CO$_2$ and will produce CO$_2$ rather than assimilate CO$_2$ (photorespiration). By increasing the ratio CO$_2$/O$_2$ due to CCM this reduces the rate of photorespiration (Roberts et al. 2007).

CA in the majority of freshwater and marine microalgae and cyanobacteria is located intracellularly; but some species excrete it in the extracellular surrounding space, converting the HCO$_3^-$ to CO$_2$, which is then diffused or actively transported inside the cells. However, in alkaline environments the extracellular CA activity is low, and therefore in marine environments the uptake of carbon occur mainly by actively transportation of HCO$_3^-$ or CO$_2$ than of diffusion of CO$_2$ (Amoroso et al. 1998, Huertas et al. 2000). Moreover, microalgae and cyanobacteria are found to excrete H$^+$ ions to regulate their intracellular pH by homeostasis. The excreted H$^+$ might react with HCO$_3^-$ to give CO$_2$, which can subsequently be taken up by diffusion (Price et al. 2008, Van Den Hende et al. 2012).
Another strategy developed by microalgae to obtain CO$_2$ from aquatic environments with relative high pH is the process of calcification, in which CaCO$_3$ is precipitated following the reactions (Jansson and Northen 2010, Moheimani et al. 2012):

$$\text{Ca}^{2+} + 2\text{HCO}_3^- \rightleftharpoons \text{CaCO}_3 + \text{CO}_2 + \text{H}_2\text{O}$$

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

$$\text{Ca}^{2+} + \text{CO}_3^{2-} \rightleftharpoons \text{CaCO}_3$$

Although no CO$_2$ is produced in this reaction, removal of CO$_3^{2-}$ from solution serves as a buffer against pH rise, especially in alkaline environments (Borowitzka and Larkum 1987, Jansson and Northen 2010). Extracellular calcification is also thought to serve as a light shield, protecting the cell against excessive irradiance, as a way for excretion of Ca$^{2+}$ to prevent intracellular toxicity and as a way to enhance nutrient uptake (Van Den Hende et al. 2012).

Photosynthetic activity, and in particular the extracellular or/and intracellular conversion of bicarbonate to carbon dioxide ($\text{HCO}_3^- \xrightarrow{CA} \text{CO}_2 + \text{OH}^-$), produces OH$^-$, which gradually causes an increase of the pH of the medium, which can reach even a value of 11. In the case of intracellular production of OH$^-$ the microorganisms take up H$^+$ from the surrounding in order to neutralize the
produced intracellular OH\(^{-}\). In contrast, it seems that the passive uptake of CO\(_2\) itself does not alkalize the medium because it leaves no hydroxyl ion during its utilization and fixation (Chi et al. 2011, Shiraiwa et al. 1993). The gradually rise of the medium pH can affect negatively the growth of the microorganisms, either by the alkaline environment itself or due to low useful carbon availability, as CO\(_3^{2-}\) gradually becomes the dominant DIC form available (Shiraiwa et al. 1993). Therefore, the control of the pH is a significant issue to maintain microalgal growth. To control the rising pH, a strategy is to acidify the medium, either by applying CO\(_2\) or by using inorganic or organic acids (Grobbelaar 2004). However, the consumption of other nutrients and the degradation of various metabolites (Molina Grima et al. 1999) or the excretion of several organic acids by the microorganisms might also influence the pH values of the cultivation medium.

2.3 Factors affecting solubility and dissolution of CO\(_2\)

As mentioned before, microalgae thrive in an aquatic environment and therefore the CO\(_2\) is in dissolved form. When gas CO\(_2\) is supplied to the medium, the main influencing parameters are its solubility and its mass transfer rate. CO\(_2\) solubility in water at 25°C and 1 atm is about 1.5 g/L. However, the solubility of CO\(_2\) in aquatic environments varies significant and depends on pH, salinity, pressure and temperature. As shown in Figure 2, solubility of CO\(_2\) in water decreases with increasing salinity and temperature and increases with increasing pressure. pH therefore not only affects the DIC species equilibrium, but also affects the total amount of DIC that is available in solution. In alkaline solutions, the excess OH\(^{-}\) ions
react with CO$_2$ to form HCO$_3^-$, resulting to a higher bicarbonate-carbonate alkalinity and consequently a higher total carbon availability (Münkel et al. 2013).

In addition to the solubility of CO$_2$, which refers to the amount of CO$_2$ that can be dissolved in water under specific conditions, the mass transfer rate also influences the total carbon availability. The transfer of CO$_2$ from the gaseous to the liquid phase is affected by various design parameters of the cultivation system. Factors that influence the CO$_2$ mass transfer to the cultivation medium is the specific contact area between the gas and the liquid phase and the concentration gradient between the gas and the liquid phase. In open pond cultivation systems, CO$_2$ is often transferred passively from the atmosphere to the cultivation medium through the water surface of the pond. Because the specific contact area is low and the concentration gradient is also low (due to the low concentration of CO$_2$ in the atmosphere), the mass transfer rate of CO$_2$ into the solution is often too low to compensate for microalgal uptake. To increase the transfer rate, the culture medium is often sparged with a CO$_2$-rich gas. The contact time of the bubble with the medium (determined by the rising speed of the bubbles) and size of the gas bubbles will mainly determine the gas transfer rate, in addition to the pH and alkalinity of the medium, the degree of contamination with suspended matter and the general CO$_2$ saturation state of the medium (Rubio et al. 1999, Suh and Lee 2003, Takemura and Yabe 1999). In high pH values the mass transfer of CO$_2$ is faster than in low pH values, because the dissolution is mainly due to the chemical reaction of CO$_2$ and OH$^-$ which is faster than the hydration of CO$_2$ to H$_2$CO$_3^-$ (Takemura and Matsumoto 2000, Van Den Hende et al. 2012).
The most plain model given the mass transfer rate of CO$_2$ ($M_{\text{CO}_2}$) is (Van Den Hende et al. 2012):

$$M_{\text{CO}_2} = k_L \alpha (C^*_{\text{CO}_2} - C_{\text{CO}_2})$$

where, $k_L$ is the liquid-phase mass transfer coefficient, $\alpha$ is the specific area available for mass transfer and, $C^*_{\text{CO}_2}$ is the CO$_2$ concentration in the liquid equal to partial pressure in gas phase and $C_{\text{CO}_2}$ is the CO$_2$ concentration in the liquid (Van Den Hende et al. 2012). The enhancement of the mass transfer rate of CO$_2$ can be achieved by increasing on the one hand the parameter of $k_L \alpha$ (Van Den Hende et al. 2012) or on the other hand by increasing the pH values of the cultures (Chi et al. 2011).

In fast growing microalgal systems, the simple diffusion and dissolution of CO$_2$ from the air into the water is too slow to replace the assimilated CO$_2$ (Suh and Lee 2003). In these systems CO$_2$ has to be provided actively in the one or the another form. The three main ways of applying CO$_2$ to the cultures are (1) pumping air, (2) pumping concentrated CO$_2$ and (3) bicarbonate salts.

### 2.4 Sources for providing inorganic carbon

As was mentioned before, the application of CO$_2$ in microalgal cultivation plays a major role and contributes significantly to the total production costs (about the 50% of the cost of producing the biomass) (Chisti 2013, Kadam 1997, Rubio et al. 1999). When CO$_2$ is provided by the aeration of the culture with air ($\approx$400 ppmv CO$_2$) the air volume which should be passing through the culture has to be
enormous, resulting to high energy consumption for its pumping. However, the use of commercial pure CO₂ seems also to be costly. Flue gas is a lower-cost alternative. It has a CO₂ content of 10-20%. When using flue gases, important costs are associated with the recovery, compression and/or transportation of flue gas, unless the cultivation plant is near the flue gas source, allowing simple transportation and direct application of the flue gases (Kadam 1997, Lam et al. 2012).

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

There is a vast number of published studies that deal with the tolerance of various microalgal strains in cultures that are cultivated with various degrees of concentrated CO₂ (Gouveia 2011). However, as it was pointed out from Van Den Hende et al. (2012) the critical issue of applying CO₂ is not its concentration in the applied gas but the combination of CO₂ concentration and its flow rate, consequently the mass of the dissolved inorganic carbon. Because the dissolution of CO₂ in water results to its acidification, a high mass flux of CO₂ to the liquid phase could lead to a significant decrease in medium's pH. However, it is not entirely clear whether the
acidification or the free CO₂ concentration affects the growth, making this
distinction challenging (Yang and Gao 2003). The degree of acidification of the
medium due to CO₂ addition is an outcome of the equilibrium between the CO₂
concentration in the gas and liquid phase, and is affected by the partial pressure and
the alkalinity (Goldman et al. 1982). Ideally, the rate of CO₂ should match the rate of
CO₂ assimilation by the microalgae (Rubio et al. 1999) with the simultaneously
regulation and adjustment of pH to optimal values for each microalgal strain. This
strategy is proven successful in cultures with various microalgal species, even when
applying 100% CO₂ gas (Olaizola 2003). That means that the addition of CO₂ on
demand is independent of the CO₂ concentration in the gas and depends mainly on
the operational pH.

To avoid problems associated with the direct application of untreated flue
gas, the CO₂ could be recovered from the flue gas and purified. Several methods exist
for this purpose (Aaron and Tsouris 2005), however the methods who might be of
particular interest for microalgal production are those by which bicarbonate salts
are formed, such as sodium bicarbonate, Na₂CO₃ + CO₂ + H₂O ↔ NaHCO₃, (Nelson
et al. 2009) and ammonium bicarbonate, NH₄HCO₃, (Aaron and Tsouris 2005) or
urea, (NH₂)₂CO, (Meessen 2000). Ammonium bicarbonate and urea can be used
also as nitrogen source (see nitrogen section).

There are two main concerns about using bicarbonates: they cost more than
three times as much as gaseous CO₂ (Suh and Lee 2003) and they can only be
applied on microalgal species which tolerate high pH and high ionic strength (Chi et
al. 2011). However, bicarbonate salts have higher solubility when compared to CO₂,
(for example NaHCO$_3$ solubility is $>90$g/L at 25°C) and it is expected that their application efficiency would be higher than CO$_2$ application. It is a challenge and a necessity to screen and find strains that can thrive with high growth rates under high values of pH, alkalinity and ion strength (Chi et al. 2011). Recently, Chi et al. (2013) studied the NaHCO$_3$ tolerance of some cyanobacteria and microalgae, and found that the highest NaHCO$_3$ concentrations were 0.3 M for *Synechocystis* sp. PCC6803, 0.6 M for *Cyanothece* sp., 0.1 M for *Chlorella sorokiniana*, 0.6 M for *Dunaliella salina*, and 0.3 M for *Dunaliella viridis* and *Dunaliella primolecta*. Beside these species, *A. platensis* is also known to be very tolerant in high alkalinity (Kebede 1997). Nevertheless, the use of bicarbonate salts could be have some advantages over CO$_2$. The use of bicarbonate results in accumulation and in a higher lipid content in, which is attractive for biodiesel production (Gardner et al. 2012).

Another source for inorganic carbon are wastewaters streams that are rich in bicarbonate-carbonate alkalinity, especially the anaerobically digested livestock wastes. During the anaerobic digestion, the organic fraction of the wastes is converted to CH$_4$ and CO$_2$. A portion of the latter is dissolved to the liquor and generates the bicarbonate-carbonate buffer of the anaerobic liquor (Markou and Georgakakis 2011).

### 2.5 Applying of organic carbon

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

3. Nitrogen

Nitrogen is the second most abundant element in microalgal biomass, and its content ranges from 1% up to 14% (typically around 5-10%) of dry weight (Grobbelaar 2004). Nitrogen participates in essential biomass biochemical compounds, such as nucleic acids (DNA, RNA), amino acids (proteins) and pigments such as chlorophylls and phycocyanin. It can be taken up in inorganic form of $\text{NO}_3^-$, $\text{NO}_2^-$, $\text{NO}$, $\text{NH}_4^+$ and in some cases $\text{N}_2$, but also in organic form, like urea or amino acids (Flores and Herrero 2005, Perez-Garcia et al. 2011). The most important path for nitrogen assimilation is through the glutamine synthetase enzyme system, by which glutamate reacts with ammonium (driven energetically by ATP) to form the amino acid glutamine.

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

3.1 Applying nitrogen

3.1.1 Inorganic nitrogen
3.1.2 Nitrogen oxides

Nitrate ($\text{NO}_3^-$) is the most commonly used mineral nitrogen form for microalgae and cyanobacteria cultivation on synthetic media. The most frequently used nitrate salts are NaNO$_3$ and, less frequently, KNO$_3$ (Grobbelaar 2004). Nitrate is taken up by active mechanisms and therefore consumes energy (Graham and Wilcox 2000). Nitrate does not display toxic effects to cells, and microalgae can tolerate concentrations of up to 100 mM of nitrate (Jeanfils et al. 1993). However, it was observed that the growth is negatively affected when the concentration of nitrate is increased (Jeanfils et al. 1993). Possibly, the activity of nitrate reductase is enhanced when nitrate concentrations are high and these results in a high intracellular concentration of nitrite and ammonium, both of which are toxic to the cells (Chen et al. 2009, Jeanfils et al. 1993, Kim et al. 2013).

Nitric oxide (NO) could be considered as interesting nitrogen form mainly when flue gases are used. Nitric oxide has a very low solubility in the cultivation medium, and this low solubility is considered to be the rate-limiting factor to supply NO to microalgal cultures (Jin et al. 2008, Nagase et al. 2001). To improve the solubility of NO, ferrous-complexed EDTA can be added to the cultivation medium (Jin et al. 2008, Santiago et al. 2010) or the NO bubble retention time can be increased and/or bubble size decreased (Nagase et al. 1998, Nagase et al. 1997). Dissolved nitric oxide is oxidized to nitrite or nitrate in the presence of dissolved oxygen, which both can be taken up by the microalgae (Nagase et al. 1997). However, due to the fact that nitric oxide is a small and non-polar molecule, it diffuses directly into the cells and is oxidized intracellularly to nitrite/nitrate. Nitric oxide, however, is a free radical and
high intracellular concentrations will have detrimental effects (Nagase et al. 2001, Yoshihara et al. 1996). The degree of the tolerance to NO is species-dependent (Brown 1996).

Nitrite ($\text{NO}_2^-$) is frequently found in natural environments as an intermediate product of the nitrification process, that is, the oxidation of ammonia to nitrate. However, nitrite is also an intracellular intermediate of the nitrogen metabolism, that is the product of reduction of nitrate to nitrite by the nitrate reductase, which then is reduced further to ammonium through the action of the nitrite reductase. The main way of nitrite uptake is through active transportation, but diffusion has been also reported for green microalgae and cyanobacteria (Flores et al. 1987, Fuggi 1993). Although nitrite can be taken up and used as a nitrogen source, at high concentrations it has toxic effects (Chen et al. 2012). Yang et al. (2004) observed that a nitrite concentration of 4 mM extended the lag phase in cultures of *Botryococcus braunii* to 10 days, while an increase of nitrite to 8 mM causes a total inhibition of microalgal growth. Also in the study of Chen et al. (2011), in which *Microcystis aeruginosa* was cultivated in a medium with 50 mg/l nitrate, the addition of nitrite from 0 to 15 mg/l had a gradual negative effect on cell growth. The uptake of nitrite seems to be reduced under low CO$_2$ concentration, suggesting that CO$_2$ is required for nitrite uptake (Flores et al. 1987, Vilchez and Vega 1994), while high CO$_2$ concentration seems to affect positively the nitrite reductase activity, enhancing the nitrite assimilation (Hu and Zhang 2008).

3.1.3 Ammonia/ammonium
Ammonia is a volatile molecule, but, unlike CO$_2$, its solubility is very high (about 35% (w/w) at 25°C) and it is found frequently as a liquid solution. When ammonia is dissolved in water it reacts with water to form a buffer system of ammonia/ammonium:

\[ \text{NH}_4^+ + \text{OH}^- \xrightarrow{pK=9.25} \text{NH}_3 + \text{H}_2\text{O} \]

The equilibrium between the forms of ammonium (ionized form) and free ammonia (the unionized gaseous form present in the solution) depends mainly on the pH (Figure 3). At pH values higher than 9.25 (=pK at 25°C) the dominant species is the free ammonia (NH$_3$). Temperature has also a significant effect on the ammonia/ammonium species equilibrium; the pK value decreases as temperatures increase, which means that free ammonia start to be dominant at lower pH values when temperatures are high.

Ammonia/ammonium is the preferred nitrogen source for microalgae/cyanobacteria because its uptake and assimilation consumes less energy compared to the other nitrogen sources (Boussiba and Gibson 1991, Graham and Wilcox 2000, Perez-Garcia et al. 2011). However, the microalgal biomass production or the growth rate using ammonia/ammonium as nitrogen source is similar as when nitrate is used as nitrogen source (Boussiba 1989, Park et al. 2010) or even lower (Kim et al. 2013, Lin et al. 2007). A serious constraint when using ammonia/ammonium is the potential toxicity. Free ammonia has detrimental effect on microalgae in relatively low concentrations (2 mM) (Abeliovich and Azov 1976,
Azov and Goldman 1982). The main factor affecting the toxicity is the pH of the cultivation medium, which determines whether the toxic form of free ammonia is dominant or the non-toxic ammonium ion. Microalgae and cyanobacteria take up ammonium actively by transportation mechanisms and can thus control intracellular concentrations. But free ammonia diffuses passively into cells, which have therefore little control over intracellular concentrations, which can sometimes become toxic. Free ammonia affects the photosynthetic system and in particular induces photo damage of photosystem II (Azov and Goldman 1982, Drath et al. 2008). The toxicity of ammonium ions is considerably less than that of free ammonia (Källqvist and Svenson 2003), and therefore free ammonia should be considered as the main toxic factor affecting the viability of the cells. However, the degree of the toxicity is species dependent. For instance *Arthrospira*, which thrives in media with very high pH (even more than 11) (Boussiba 1989), is found to be more resistant to ammonia toxicity than other cyanobacteria or microalgae. The degree of toxicity seems to be related to the difference between the intracellular and extracellular (medium) pH values. At low pH differences, as it is in case of *Arthrospira*, the resistance to ammonia toxicity is higher (Belkin and Boussiba 1991a, b).

Ammonia toxicity should be taken into consideration when wastewaters rich in ammonia are used as the cultivation medium. To avoid the negative effect of free ammonia, one strategy is to regulate the pH values and keep them well below the pK of ammonia/ammonium in order to limit the concentration of free ammonia (Azov and Goldman 1982) or to dilute the wastewater to avoid an inhibitory ammonia
concentration (Olguín et al. 2003) or to use a fed-batch cultivation mode, in which ammonia is added gradually to the culture medium (Rodrigues et al. 2010). It is worth mentioning that the assimilation of different nitrogen forms influences the pH of the culture medium. If ammonium is applied as the nitrogen source the pH may drop due to the release of $H^+$ during assimilation, while the pH will increase due to the release of $OH^-$ when nitrate is applied (Perez-Garcia et al. 2011). An additional constraint of using ammonia for nitrogen application for microalgae growth is that it can be lost from the cultivation media due to volatilization, especially at higher pH values (Markou et al. 2014a).

For the production of a series of nitrogen fertilizers (such as urea, ammonium nitrate, urea-ammonium-nitrate (UAN), ammonium bicarbonate etc.), ammonia is the main feedstock. Thus ammonia as fertilizer represents the lowest direct energy input for the cultivation of microalgae (Johnson et al. 2013). Ammonia is used to react with $CO_2$ to form ammonium bicarbonate, $CO_2 + NH_3 + H_2O \leftrightarrow NH_4HCO_3$, (Aaron and Tsouris 2005) or urea, $CO_2 + 2NH_3 \leftrightarrow H_2NCOONH_4 \leftrightarrow (NH_2)_2CO + H_2O$, (Meessen 2000), which both could be used as nitrogen and carbon sources for the cultivation of microalgae.

### 3.1.4 Molecular N$_2$

Some cyanobacteria (such as *Oscillatoria* sp., *Nostoc* sp., *Anabaena* sp. etc.) and some diatoms (such as *Rhizosolenia* and *Hemiaulusare* which have cyanobacterial symbionts) are diazotrophic microorganisms, which means that they can assimilate dinitrogen (N$_2$) as their sole nitrogen source by the reduction of N$_2$ to NH$_4^+$ using
the nitrogenase enzyme complex (Benemann 1979, Gallon 2001, Peccia et al. 2013, Stal 2000, Zehr 2011). However, the nitrogen-fixing process is a very energy-costly process, and it consumes sixteen ATP for the generation of two NH$_3$ according to the following equation (Großkopf and LaRoche 2012):

$$N_2 + 8H^+ + 8F_{\text{red}} + 16\text{ATP} \rightarrow 2\text{NH}_3 + \text{H}_2 + 16\text{ADP} + 8F_{\text{dox}} + 16\text{P}_i$$

where $F_{\text{red}}$ and $F_{\text{dox}}$ are the reduced and oxidized form of ferredoxin, respectively, and $\text{P}_i$ is inorganic phosphate.

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

### 3.2.1 Organic nitrogen

Microalgae can utilize nitrogen from organic forms such as urea and some amino acids. Urea and amino acids are transported actively into the cells and are metabolized intracellularly (Flores and Herrero 2005, Perez-Garcia et al. 2011). The most significant organic nitrogen form that could be used as nitrogen source for microalgae cultivation is urea. Generally, urea is hydrolyzed to ammonia and carbonic acid which both can be utilized by microalgae and cyanobacteria.
\[(\text{NH}_2)_2\text{CO} + \text{H}_2\text{O} \xrightarrow{\text{Enzyme}} \text{NH}_3 + \text{H}_2\text{CO}_3\]

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

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

### 3.3 Counter-repression

The simultaneous presence of more than one nitrogen form in the cultivation medium affects the uptake of nitrogen. It has been shown that there is a repression of the uptake of some nitrogen forms when other forms are simultaneous present in the medium. When ammonia/ammonium is present the uptake of nitrite and nitrate is repressed (Boussiba and Gibson 1991, Fernandez and Galvan 2007, Vílchez and Vega 1994) and microalgae will first completely remove ammonia/ammonium and
only then utilize the other forms (Boussiba and Gibson 1991). Ammonium represses specific permeases for the active transport of nitrite or nitrate into the cells and also represses the synthesis of nitrate and nitrite reductase (Darley 1982, Florencio and Vega 1983, Garbayo et al. 2002, Ohmori et al. 1977). On the other hand, a high nitrate concentration also inhibits ammonium/ammonia uptake (Florencio and Vega 1983). Ammonium/ammonia also represses urea uptake, but the concentration at which repression occurs seems to differ between species (Molloy and Syrett 1988).

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

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

4. Phosphorus

Phosphorous is one of the most important nutrients for microalgal growth and its biomass content varies from 0.05% up to 3.3% (Grobbelaar 2004). Phosphorus is an component of several organic molecules that are essential to the metabolism, such as nucleic acids (RNA and DNA), membrane phospholipids and ATP (Geider
Phosphorus is frequently a limiting nutrient for microalgae, especially in natural environments (Oliver and Ganf 2000).

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

In natural environments as well as in wastewaters, phosphorus is present in various forms such as orthophosphate, polyphosphate, pyrophosphate, metaphosphate and their organic forms (Cembella et al. 1982, Yeoman et al. 1988). It is well known that phosphorus is taken up by the cells in the orthophosphate form, however other inorganic and organic phosphorus forms (mainly dissolved organic phosphorus but also insoluble phosphorus compounds) can also be utilized by microalgae (Huang and Hong 1999, Whitton 1991). In case of dissolved organic phosphorus (DOP), there are two ways of utilization: either by actively up-take into the cells or by extracellular mineralization by phosphatase enzymes (Dyhrman and Ruttenberg 2006, Hua-sheng et al. 1995). The capability of DOP to be taken-up depends on its chemical composition. However, most of the DOP compounds cannot be directly assimilated by microalgae and have first to be mineralized (Dyhrman and Ruttenberg 2006). In case of inorganic phosphorus forms other than
orthophosphate, in order to be rendered suitable for uptake by microalgae they have first to be converted to orthophosphate. This is accomplished also by the action of various phosphatase enzymes (Kuenzler and Perras 1965, Lin 1977). The enzymes for the conversion of the various forms into orthophosphate are intracellular, extracellular or attached to the cell wall of algae. The activity of these enzymes is affected from various environmental factors, such as pH, temperature, light and the presence of metals or other inhibitors (Kuenzler and Perras 1965, Lin 1977, Whitton et al. 2005). Orthophosphate is taken up actively, while passive diffusion corresponds to a small fraction of total inorganic phosphorus influx (Cembella et al. 1982). The uptake rate of phosphorus is affected by the cell condition and by several environmental factors, such as available energy (light), pH, temperature, salinity/ionic-strength of the cultivation medium and available ions such as K⁺, Na⁺ and Mg²⁺ (Cembella et al. 1982, Corell 1998, Falkner et al. 1980, Rigby et al. 1980, Seale et al. 1987). The cellular production of these enzymes is significantly enhanced when available phosphorus is decreased and when cells are phosphorus limited or starved (Dyhrman and Ruttenberg 2006, Hua-sheng et al. 1995).

Contradictory data are reported regarding the uptake of phosphorus as organic compounds. In some studies the phosphorus uptake rate of organic compounds was lower when comparison to the uptake rate of orthophosphate, a fact that resulted in phosphorus limitation and reduction of the biomass production (Ellwood et al. 2012, Kuenzler and Perras 1965). In other studies growth rates were equal or almost equal when phosphorus was supplied as organic phosphates or
inorganic phosphates (Whitton 1991, Whitton et al. 2005). In the presence of metals, phosphate may also form complexes with humic substances. Such phosphate-metal-humic complexes may have a low bio-availability (Li and Brett 2013).

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

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

5. Potassium

Potassium, along with nitrogen and phosphorus is one of the three primary macronutrients for biomass production. Potassium content in some microalgae ranges from 1.2% to 1.5% (Tokuşoğlu and Ünal 2003), however it could be as high
as 7.5% (Grobbelaar 2004). Potassium plays a significant biological role, because it is an activator for a number of enzymes involved in photosynthesis and respiration, it affects protein and carbohydrate synthesis and regulates the osmotic potential of cells (Checchetto et al. 2013, Hopkins and Hüner 2009, Malhotra and Glass 1995). Potassium is taken up actively by the cells when concentrations are low, but it can be also taken up passively at high concentrations (Malhotra and Glass 1995, Tromballa 1978). Potassium can be applied to microalgal cultures as various salts, such as K₂HPO₄, KH₂PO₄, KNO₃, KSO₄, KCl etc. or using wastewater streams. Wastes and wastewaters from the agro-industrial sector seem to be rich in potassium, for example swine slurry content ranges from 3 to 7.5 g l⁻¹ (as K₂O) and poultry manure contain potassium up to 32.5 mg g⁻¹ (Markou and Georgakakis 2011). However, it is not known whether microalgae can utilize only inorganic potassium or are capable to utilize organic-bounded potassium by performing hydrolysis.

6. Others nutrients

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

6.1 Magnesium
Magnesium is an essential element for microalgal biomass production. Magnesium content in microalgae ranges between 0.35% and 0.7% (Tokuşoglu and Ünal 2003), however a content as high as 7.5% can be found in some species (Grobbelaar 2004). Magnesium participates in vital cell processes such as ATP reactions for carbon fixation and is an activator for several major enzymes. Also it is a constituent of the photosynthetic apparatus and in particular of the chlorophylls (Hopkins and Hüner 2009). In the microalga *Chlorella vulgaris*, it was observed that cell multiplication and synthesis of cell material are independently affected by magnesium availability. It seems that the process of multiplication requires larger amounts of magnesium than does the production of dry matter (Finkle and Appleman 1953). Magnesium in aqueous solutions is mainly presented as the Mg$^{2+}$ cation. However, when pH is high, Mg$^{2+}$ may precipitate as magnesium phosphate or magnesium hydroxide. These mineral precipitates may induce flocculation of microalgal biomass, which may not be desirable. However, the algal-mineral complexes are formed only at very high pH values, higher than 11 (Hoffmann 1998, Vandamme et al. 2012). At those high pH values, only a few alkalophilic species can thrive. Magnesium is provided to the cultures mainly as MgSO$_4$, however several other magnesium salts are available and could be used, such as (NH$_4$)$_2$SO$_4$-MgSO$_4$, MgCl$_2$, MgHPO$_4$, Mg(NO$_3$)$_2$ etc.

On the other hand, wastewaters are often deficient in magnesium (Hoffmann 1998) and it was reported that supplementation of magnesium is necessary when hydrolyzed human urine (Tuantet et al. 2014), anaerobic digestion effluents of piggery wastes (Park et al. 2010) or the aqueous phase from the hydrothermal
liquefaction of the same microalgae are used as a substrate for microalgae cultivation (Garcia Alba et al. 2013).

6.2 Sulfur

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

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

High calcium concentrations in the cultivation medium along with high pH values result to the formation of CaCO$_3$ and various other calcium salts which precipitate, decreasing the alkalinity of the medium and the concentration of some minerals such as iron and phosphorus (Shimamatsu 2004). Calcium is frequently added to the cultures as CaCl$_2$, however some other forms could be also used (Ca$_3$(PO$_4$)$_2$, Ca(NO)$_3$ etc.).

6.4 Iron

Among trace elements, iron is one of the most essential elements required by microalgae. Iron, as a transition metal, is associated with enzymes through complex formation with S or N groups of various amino acids. Iron is involved in fundamental enzymatic processes such as oxygen metabolism, electron transfer, nitrogen
assimilation, and DNA, RNA and chlorophyll synthesis (Naito et al. 2005, Straus 2004). However, iron has some physico-chemical characteristics that reduce its bioavailability. In aqueous oxic environments because Fe$^{2+}$ is quickly oxidized iron is mainly in the Fe$^{3+}$ form. Fe$^{3+}$ forms oxides and hydroxides, which are insoluble. Moreover iron is easily adsorbed onto particle surfaces, resulting to a low bioavailability. In natural environments, and in domestic wastewaters, iron is frequently bio-unavailable and this can limit the growth capability of microalgae and cyanobacteria (Mostafa and Mahmoud 2012, Sunda and Huntsman 1995). Therefore, iron is frequently supplied in cultures as chelated complexes, which render it bio-available. Iron deficient cultures display low growth rates (Sandmann 1985), while high iron concentration in some microalgal species induce lipid synthesis and increasing its content in the biomass (Liu et al. 2008, Yeesang and Cheirsilp 2011).

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

Microalgae and cyanobacteria adjust their nutrient uptake and requirements according to the nutrient availability in the surroundings. They can store excess quantities of a nutrient (luxury uptake) but also can grow with lower quantities of a nutrient. However, concentrations lower than a specific threshold of a nutrient affects the general growth rates of microalgae and cyanobacteria. The effect of the limitation of a nutrient on algal growth is best described by the Droop model (Droop 1968, Lemesle and Mailleret 2008, Sommer 1991), according to which, growth rates
are dependent on the intracellular concentration of a nutrient. This relationship is described with the following model:

\[ \mu = \mu_{\text{max}} (1 - \frac{k_q}{Q}) \]

where, \( \mu \) is the current specific growth rate, \( \mu_{\text{max}} \) is the maximum specific growth rate for a given cultivation system, \( Q \) is the current intracellular concentration of the nutrient and \( k_q \) is the minimum intracellular concentration of the nutrient (subsistence quota). The subsistence quota is a threshold under which microalgae and cyanobacteria do not grow (Droop 1968).

When a nutrient becomes limiting, microalgae often adjust their biomass composition, either triggering the accumulation of carbohydrates or lipids or by altering the content of other compounds such as proteins and pigments (Hu 2004, Pirson et al. 1951, Turpin 1991). In the last years there is an intensification of the research on the topic of nutrient starvation for the accumulation of lipids for the production of bio-diesel (Breuer et al. 2012, Li et al. 2012, Mujtaba et al. 2012, Rodolfi et al. 2009), and, to a lesser extent, for the accumulation of carbohydrates for bio-ethanol production (Ho et al. 2013, Markou et al. 2013, Miranda et al. 2012).

The cultivation under nutrient limitation could be a strategy to significantly reduce the nutrient demand for microalgae cultivation while at the same time to achieve a very high biomass yield per mass of nutrient added. However, the decreased nutrient demand should be carefully balanced against a decreased growth rate. This could be moderated by using optimized cultivation media or by using a multi-stage
cultivation system (Dragone et al. 2011, Markou et al. 2012a). However, more research is needed in this field.

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

8. Wastewater as nutrient source

As was mentioned before, wastewater streams are good source for specific nutrients and the use of wastewater could reduce the cost of synthetic fertilizer. Microalgae/cyanobacteria cultivation in wastewater has a double advantage; on the one hand valuable biomass is produced while treatment of the wastewater occurs (Markou and Georgakakis 2011, Rawat et al. 2011). However, the major constrain of
using wastewater as nutrient source is that it reduces the range of the biomass applications because the produced biomass is possibly contaminated by various pollutants that are present in the wastewater. Therefore, microalgae produced on wastewater should mainly be used for the production of biofuels rather than food or feed applications. However, to overcome this issue, it has been suggested that the nutrients could be separated from the wastewater and subsequently added to the cultivation medium, using a physic-chemical technology for nutrient recovery from wastewater. Potential technologies for this purpose are adsorption (for instance on zeolite), precipitation (such as struvite), air stripping (such as ammonia stripping) etc. (De-Bashan and Bashan 2004, Markou et al. 2014b, Renou et al. 2008). An additional constrain is that several wastewaters, especially of the industrial and agro-industrial sector, contain various inhibitors in concentrations that could be have detrimental effects on microalgal cells. Inhibitors, such as ammonia, nitrite, heavy metals, polyphenolic compounds, organic acids etc. might render wastewater inappropriate for microalgal or cyanobacterial growth (Cai et al. 2013, Markou et al. 2012b, Olguín 2012). Also the deficiency of some wastewater on essential nutrients and their low bio-availability could be limit their application (Mostafa and Mahmoud 2012). However, in many cases the growth of microalgae on wastewater was similar compared to the growth on synthetic media (Arbib et al. 2014, Martínez et al. 2000).

Wastewater can also contain several microorganisms (mainly bacteria) that could compete with microalgae. The presence of these competitors might have negative effect on the microalgal growth and even if the competitors have higher
growth rates than microalgae and if the conditions are favorable for the growth of
the first, the microalgal culture might be fail (Wang et al. 2013). In addition, in some
wastewater types, the content of suspended solids or dissolved colored organic
compounds might have negative effect on growth of microalgae due to the decrease
of light penetration inside the cultures (Depraetere et al. 2013), however this issue
might not be very important if the wastewater contain organic carbon and the
microalgal species can be grow in hetero- or mixo-trophic mode.

9. Recycling of nutrients

To reduce the amounts of input of fertilizers required for microalgal and
cyanobacterial biomass production, several nutrient recycling strategies are
suggested. Two main approaches for nutrient recycling can be considered: one
source is the spent medium after the biomass has been harvested and the second
source is the biomass residues that remains after extraction of the desirable
compounds. Concerning the nutrients left on the cultivation medium after
harvesting of biomass, a strategy is to recycle the medium and to supplement only
the exhausted nutrients. However, as was shown this strategy has a main constraint
related to the presence of several autoinhibitory organic compounds that
microalgae might excrete to the medium and/or the presence and the growth of
unwanted bacteria (González-López et al. 2013, Rodolfi et al. 2003). Sterilization
and degradation of these compounds using various methods could be an approach
to allow medium recycling. González-López et al. (2013) investigated various
sterilization methods and found that ozonation gave the best results for medium
recycling. Nevertheless, the study of Hadj-Romdhane et al. (2013) showed that growth of *Chlorella vulgaris* was almost unaffected after 63 days of medium recycling, a fact that might indicate that the capability of medium reuse is species dependent. Nevertheless, more research is needed in this field.

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

**10. Conclusions**

It seems that in the future the cultivation of microalgal and cyanobacterial biomass will play a significant role in the sector of biotechnology for the production of valuable organic compounds and of biofuels. However, their cultivation will consume considerable amounts of nutrients, raising questions about the
sustainability of such biomass production systems. Optimization of nutrient supply is extremely important to increase sustainability and to avoid shifts in global nutrient supplies. Therefore the understanding of microalgal and cyanobacterial biology combined with the understanding of bio-availability of nutrients is essential to meet previous mentioned goals. Most of the research and even commercial production is done using synthetic nutrient resources; however the usage of wastewater and nutrient recycling are interesting routes that deserve further exploration.

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