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Physiological studies to optimize algal biomass production in phytoremediation processes

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Introduction

## 1. Microalgae for mass cultures

Algae are primarily oxygen-releasing photosynthetic organisms with simple body plans no roots, stems, or leaves, and usually are aquatic organisms. They do not form a single monophyletic group and consequently cannot be easily defined. Although algae are an ubiquitous group, individual species occupy specific habitats. Some algae are attached to a substrate like plants, some are motile like animals, some are simply suspended in water, some grow loosely on soil, trees, and animals, and some form symbiotic relationships with other organisms (e.g., hexacoralli, fungi). The internal cell structure of algae varies greatly. The complex multicellular structures found in seaweeds, lack in microalgae (Lee 2008; Graham et al. 2009).

Microalgae show a huge variety of shapes and forms, and there are several adjectives to define the common forms: amoeboid, palmelloid, flagellate, sarcinoid, coccoid, filamentous (Fig. 1). Moreover, the structure of microalgae is more diverse than that found among animal and plant cells. There are two different cell structure models: prokaryotic and eukaryotic cells. The cyanobacteria or blue-green algae have relatively simple cells, a prokaryotic cell structure. This reflects the broad phylogenetic diversity of algae, their adaptation to many environments, and 3.5 billion years of evolutionary change. A cyanobacterial cell contains many sheet-like thylakoids, and these thylakoids appear as parallel lines in thin sections viewed in the transmission electron microscope. The cells divide by fission, or pinching, that converts one larger cell into two smaller cells. The ultrastructure of eukaryotic cells is much more complex, their evolutionary history spans about 1.5–2.0 billion years, and the structures vary significantly within and among algal classes. Eukaryotes possess a number of organelles and these are important metabolic compartments that allow specialization; they have mitochondria, Golgi bodies, endoplasmic reticulum, and other typical eukaryotic organelles (Martin, 2010).
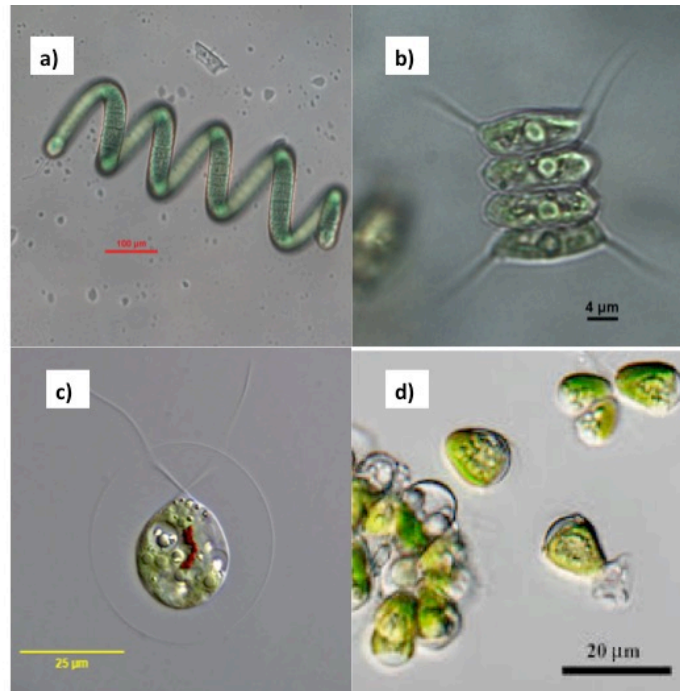
Figure 1 Microalgal species: (a) *Arthrospira platensis* **(40x); (b)**
Desmodesmus communis **(100x); (c)** *Haematococcus pluvialis* **(100x);**
Botryococcus braunii **(100x)**

1.1 The cell structure

The dominant organelle of eukaryotic algae is the chloroplast, which contains pigments to capture light for the photosynthesis processes. Plastids present different arrangements that vary among algal groups. Many plastids have a pyrenoid, that is an accumulation of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). RuBisCO is the dominant protein involved in the Calvin cycle of photosynthesis. For instance green algae store starch grains, carbohydrate or lipid storage within the chloroplast, while all other algae photosynthates storage is outside the plastid (Ball et al., 2011).

The Golgi body and endoplasmic reticulum are generally similar in structure to those of other eukaryotes. Algae use these organelles to produce organic, silicate, or calcium carbonate scales as well as flagellar hairs and other structures.

Another interesting organelle is the vacuole, and eukaryotic cells may posses one or more types of vacuoles. The function of the vacuole is to maintain a positive osmotic pressure for the cell rigidity. Some algae use vacuoles for storage products or remodelling of subcellular compartments, particularly under stress. These algae produce low-molecular-weight carbohydrates (laminarin, chrysolaminarin) in the cytosol, and because the molecules are small (e.g., 20–40 glucose residues) they affect the osmolarity of the cell. To avoid a surge in osmotic pressure, these small carbohydrates are kept within specialized vacuoles. In freshwater algae, the contractile vacuole is an osmoregulatory organelle to remove osmotic water from cells.

1.2 Biochemical characteristics

Algae provide an exceptional diversity of biomolecules that recently has interested many applicative studies and commercial companies. The potential of the algal biochemistry is in the possibility to manipulate the biomolecules to some degree by altering the growth conditions.

Proteins

Algal proteins are exceptionally diverse and can be manipulated by environmental changes (Grossman et al., 1995). Protein-rich cells are often actively growing/dividing cells. Furthermore, some organisms sequester nitrogen when it is available in the environment and they store the excess nitrogen in proteins; when nitrogen becomes limited, they digest these storage proteins to release the nitrogen. For instance Cyanobacteria as *Spirulina* and *Synechococcus* have peptidoglycan cell walls and, therefore, are an excellent source of proteins, that is, 40–60% of the dry weight is protein (Becker, 2007). Green algae are also good sources (e.g., *Chlorella*, *Scenedesmus*); *Euglena gracilis* as well as *Porphyridium* produce up to 30–60% protein by dry weight.

Lipids

The cellular lipid composition and diversity can be manipulated (Hu et al., 2008; Wang et al., 2009). For instance under low nitrogen conditions (e.g., in the stationary phase), cells carry out photosynthesis and produce lipids from photosynthetically fixed carbon (e.g., 3-phosphoglycerate). Fatty acid and sterol diversity are found in cellular membranes among algal groups. A wide range of algae produce lipids as storage products (i.e., oleaginous algae), and frequently the lipids can be observed as oil droplets in cells. These lipids are largely polyunsaturated fatty acids (PUFAs), including the omega-3 PUFAs arachidonic acid (AA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA). The heterokont algae (e.g., *Chaetoceros*, *Nannochloropsis*) and the haptophytes typically use oil droplets as a storage product, especially when their carbohydrate storage is chrysolaminarin like. The *Pinguiophyceae* store EPA in large quantities, and DHA is stored in many haptophyte algae (Guschina and Harwood, 2006; Kawachi et al., 2002; Khozin-Goldberg et al., 2011). Moreover a considerable number of chlorophyte green algae (e.g., *Scenedesmus*, *Chlorella*) store oils under stress (Guschina and Harwood, 2006).

Carbohydrates

The carbohydrate storage product in many algae is starch or a starch-like product (e.g., green and red algae, cryptophytes, dinoflagellates). These starches have a primary α-1,4-linked glucan molecular backbone, and typically the backbone chain has α-1,6-linked side chains (Ball et al., 2011). The starches are large molecules (i.e., colloidal particles or larger particles). Another group of algae utilize a β-1,3-linked glucan backbone (e.g., heterokont algae, haptophytes, euglenoids). The degree of

polymerization varies significantly for these laminarin and paramylon products. The smaller molecules, such as chrysolaminarin, consist of fewer than 30 glucose residues, and therefore to avoid osmotic problems, the molecules are maintained in vacuoles. At the other end of the spectrum, large paramylon grains, such as those found in euglenoids, are easily visible in a light microscope.

1.3 Industrial potentials

Because of their great biodiversity, microalgae can produce an enormous variety of high-value compounds for human nutrition, medical applications, cosmetics, and agrochemical industry. In the industry, microalgae have been used as source for a wide variety of practical and potential metabolic products, such as food supplements, pharmacological substances, lipids, polymers, toxins, pigments, enzymes, biomass, wastewater treatment, and ''green energy''. Microalgae are also important in aquaculture as a source of nutrients, in production of oxygen, in consumption of carbon dioxide, and in consumption of nitrogen-based compounds. The main driving force to grow microalgae commercially is harvesting metabolic products, feed for marine and terrestrial organisms, food supplements for humans, or to use the microalgae for environmental processes, such as wastewater treatment, fertilization of soils, biofuels, and phytoremediation of toxic wastes.

Recently many reviews (Apt and Behrens, 1999; Chisti, 2007; Pulz et al., 2001) have been focused on the status of microalgal applications in aquaculture, food, pharmaceutical and environmental applications .

First of all Wijffels, (2008) observed that microalgae have potentially an areal productivity superior to traditional agricultural crops. Realistic estimates for areal productivity are in the order of magnitude of 40-80 tonnes of dry matter per year depending on the technology used and the location of production. Microalgae are not a well-studied group from a biotechnological point of view: of the tens of thousands of microalgal species believed to exist, only a few thousand strains are kept in collections around the world, only a few hundred have been investigated for chemical content and only a handful have been cultivated in industrial quantities (tons per year quantities). Because they are largely unexplored, the microalgae represent a rich opportunity for discovery; the expected rate of rediscovery (finding metabolites already described) is expected to be far lower than for other groups of better-studied organisms (Hunter et al., 1996).

Nowadays microalgae are produced for high-value products in niche markets; however, if the cost price of production goes down, it is expected that new markets will open with every step in reduction. Initially, most probably the production of edible oils for food and fish feed will become feasible, but after some time production of bulk chemicals, biomaterials, and biodiesel may also become feasible. For that the technology needs to develop from a small- sized activity to an industrial scale technology with a multidisciplinary approach (Wijffels et al., 2010).

Microalgae have been also proposed as an appropriate source for hydrocarbons due to their potentially high yield of lipid- and carbohydrate-rich biomass per acre. While algal biofuels may prove to be a renewable and sustainable alternative to petroleum energy, they have yet to be viably produced on a commercial scale. However, the incentives to make these technologies successful are significant and have thus resulted in considerable investment (Singh et al., 2011).

The most common species of microalgae cultivated on commercial large scale are: *Chlorella* and *Spirulina* for health food, *Dunaliella salina* for β-carotene, *Haematococcus pluvialis* for astaxanthin, many marine microalage for aquaculture feeds (e.g. *Skeletonema*, *Chaetoceros*, *Thalassiorira*, *Tetraselmis* and *Isochrysis*) (Borowitzka, 1999) and *Crypthecodinium* for DHA (Olaizola, 2003).

2. PHYSIOLOGY

2.1 Autotrophy and photosynthesis

Photosynthesis is a unique process of sunlight energy conversion in which inorganic compounds and light energy are converted into organic matter. Today, the most common procedure for cultivation of microalgae is autotrophic growth. Because all microalgae are photosynthetic, and many microalgae are especially efficient solar energy convertors, microalgae are cultivated in illuminated environments naturally or artificially. Under autotrophic cultivation, the cells harvest light energy and use CO_2 as a carbon source. The introduction of sufficient natural or artificial light to allow massive growth and dense populations is the main objective and a limiting factor of the cultivation.

Oxygenic photosynthesis can be expressed as a redox reaction driven by light energy (harvested by chlorophyll molecules) in which carbon dioxide and water are converted into carbohydrates and oxygen. This process is traditionally divided into two stages, the so-called light reactions and dark reactions (Fig.2). In the light reactions, which are bound on photosynthetic membranes, the light energy is converted to chemical energy providing a biochemical reductant $NADPH_2$ and a high-energy compound ATP. In the dark reactions, which take place in the stroma, $NADPH_2$ and ATP are utilised in the sequential biochemical reduction of carbon dioxide to carbohydrates.
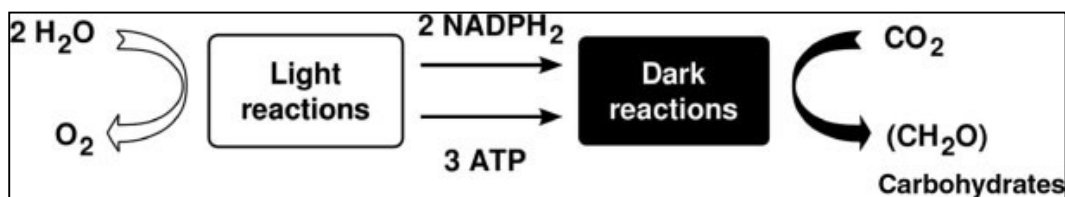

Figure 2 Major products of the light and dark reaction of photosynthesis.

The classical description of photosynthetic activity is based on measurements of oxygen evolution in proportion to light intensity, the so-called light–response (P/I) curve (Fig.3). The initial slope $\alpha = P_{max}/I_k$, where I_k represents the saturation irradiance and P_{max} is the maximum rate of photosynthesis. In the dark, there is a net consumption of oxygen as a consequence of respiration (the negative part of the curve in Fig. 2). Thus, gross photosynthesis is considered as the sum of net photosynthesis (O_2 evolution) and respiration (O_2 uptake). At low irradiance (light-limited region), the rate of photosynthesis depends linearly on light intensity. With increasing light intensity, photosynthesis becomes less and less efficient as the dark enzymatic reactions utilising fixed energy become rate limiting. Finally, it reaches a plateau the maximum (light-saturated) rate of photosynthesis P_{max}. Under prolonged supra-optimal irradiance, photosynthetic rates usually decline from the light-saturated value. This phenomenon is commonly referred to as photoinhibition of photosynthesis.
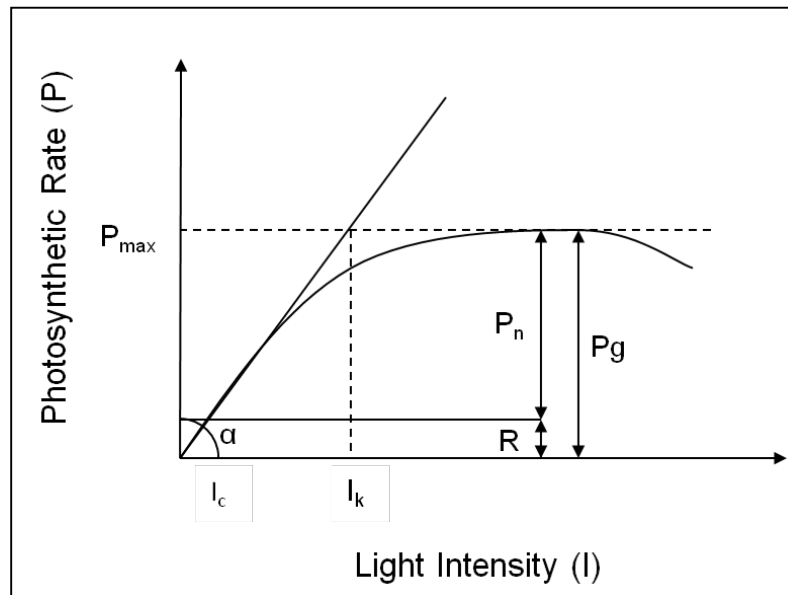
Figure 3 Light saturation curve of photosynthesis. P_{max} is the maximum rate of photosynthesis, I_c is the compensation light intensity, I_k is the light intensity at which saturations occurs, R is the respiration, P_n is the net photosynthesis and P_g is the gross photosynthesis.

Pigments

The absorption of light energy and its conversion into chemical energy occurs in multi-protein complexes, called photosystems, located in the thylakoid membrane. A photosystem has two closely linked components, an antenna containing light-absorbing pigments and a reaction center comprising a complex of proteins and two chlorophyll a molecules. Each antenna (named by analogy with radio antennas) contains one or more light-harvesting complexes (LHCs). The energy of the light captured by LHCs is funneled to the two chlorophylls in the reaction center, where the primary events of photosynthesis occur. Found in both eukaryotic and prokaryotic, chlorophyll *a* is the principal pigment involved in photosynthesis, being present in both antennas and reaction centers. There are three major classes of pigments: chlorophylls, carotenoids, and phycobilins. The presence of various antenna pigments, which absorb light at different wavelengths, greatly extends the range of light that can be absorbed and used for photosynthesis.

Measure the photosynthesis

There are several indirect and direct methods to measure the photosynthetic efficiency of microalgae, in order to evaluate the stress conditions, due to changes in several environmental conditions. Routine measurements of photosynthetic oxygen production in algal cultures are usually carried out with an oxygen electrode (Walker, 1993).

A Clark-type oxygen electrode, which is the most widely used, consists of a platinum cathode (but gold or other metals can also be used) and a silver/silver chloride anode. Oxygen production is usually expressed in μmol or mg $O2$ per mg^{-1}(Chl) h^{-1}, or per cell h^{-1}.

An indirect method to measure the photosynthetic carbon fixation in cell suspension is using the

infrared gas analysis, with special electrodes to measure the partial pressure of carbon dioxide (pCO_2) in solutions. The principle is based on the relationship between pH and the concentration of CO_2 and bicarbonate in the solution.

Another way to measure the carbon allocation is the method of ^{14}C radiolabelling. It also provides a measure of the photosynthetic assimilation rate. The population (or culture) of microalgae is exposed to ^{14}C for a fixed period of time. The reaction is then stopped by the addition of concentrated HCl and the amount of ^{14}C incorporated is determined by a scintillation counter.

Finally one of the most common and useful techniques in photosynthesis research is the measure of the chlorophyll fluorescence (Masojídek et al., 2010). Chlorophyll fluorescence directly reflects the performance of photochemical processes in photosystem II (PS II). Upon illumination, the PS II chlorophyll molecules are excited to a singlet excited state (Chl a). The energy of the excited state is transferred to the reaction centre to be used for photochemical charge separation. Alternatively, the excitation energy can be dissipated as heat, or reemitted as fluorescence. The sum of energy entering these three competing processes is equal to the absorbed light energy. Any change of photochemistry or dissipation results in a change of fluorescence, providing a direct insight into the energetics of PS II (Fig. 4).
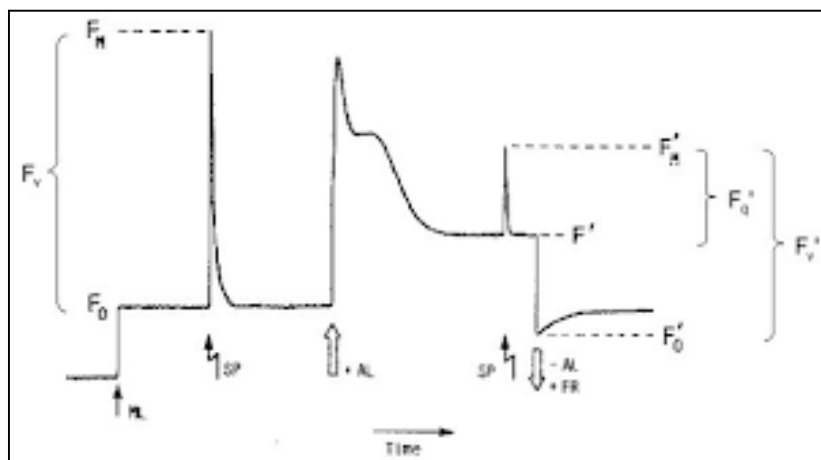
Figure 4 Scheme of the saturation pulse method

In the dark, all the reaction centres are in the so-called open state and the rate of photochemistry is maximal. The fluorescence yield in this state is low, designated as F_0. When PS II is exposed to a strong pulse of light, the reaction centres undergo charge separation and the electron is moved to the first electron acceptor Q_A. When Q_A is reduced, the reaction centres are in the closed state and photochemistry is transiently blocked. Since the yield of photochemistry is zero, the dissipation and fluorescence yields rise proportionally. The high fluorescence yield of the closed centres is described as F_M. Since the fluorescence yield rises proportionally to the level of the PS II closure, the open

reaction centre acts as a fluorescence quencher. This phenomenon is called photochemical quenching qP (Baker, 2008). The values of qP range from 0 to 1 reflecting the relative level of Q_A oxidation.

The difference between F_m' and F' reflects the photochemical part of fluorescence quenching which is expressed in the fluorescence parameter F_q'/F_m' (Genty et al., 1989). The ratio between the variable fluorescence and maximum fluorescence ranges from 0.7 to 0.8 in dark-adapted green microalgae. This ratio is frequently used as a convenient estimate of the photochemical yield of PS II. The yield varies significantly, depending on the irradiance regime and physiological treatment.

The application of a saturating flash in the presence of actinic light allows the determination of the maximum fluorescence in the light-adapted state (F_m'). A decrease in F_m' as compared to F_m indicates the presence of non-photochemical quenching (Bilger and Björkman, 1990), and indicates an increased heat dissipation of excitation. In principle, NPQ is inversely related to photochemistry and is considered a safety valve protecting PSII reaction centres from damage by excess irradiance.

2.2 Organic carbon metabolisms

Few microalgal species are able to use their heterotrophic growth capacity in the absence of light, replacing the fixation of atmospheric CO_2 of autotrophic cultures with organic carbon sources dissolved in the culture media. The basic culture medium composition for heterotrophic cultures is similar to the autotrophic culture with the sole exception of adding an organic carbon (Tsavalos and Day, 1994).

Mixotrophy is growth in which CO_2 and organic carbon are assimilated simultaneously and, hence, both respiratory and photosynthetic metabolism have to operate concurrently. For some microalgae, photosynthesis and the oxidative phosphorylation of organic carbon substances seem to function independently. The growth rate in mixotrophic conditions is approximately the same as the sum of the growth rate in the photoautotrophic and heterotrophic cultures, such as *Chlorella regularis* (Endo et al., 1977), and *Spirulina platensis* (Marquez et al., 1993). However some microalgal species are not truly mixotrophs, but have the ability of switching between phototrophic and heterotrophic metabolisms, depending on environmental conditions (Kaplan et al., 1986).

Under heterotrophic growth conditions, respiration rates equal or exceed the theoretical minimum cost of biomass synthesis and during heterotrophic growth conditions it can proceed at nearly the maximal theoretical efficiency, since CO_2/C ratios for autotrophic growth are much lower than values for heterotrophic growth (Raven, 1976).

On the other hand, in mixotrophic conditions, organic carbon metabolism may exert an opposing influence on photosynthesis. Glucose can reduce the apparent affinity for CO_2 in CO_2 fixation in the species such as *Chlorella* sp., and *C. vulgaris* (Martínez and Orús, 1991). Glucose can also depress photosynthetic O_2 evolution: in the study of Steinmüller and Zetsche, (1984), glucose was shown to have a strong inhibitory effect on the synthesis of the Calvin cycle enzyme ribulose bisphosphate

carboxylase/oxygenase (RuBPCase) and the light gathering proteins phycocyanin (PC) and allophycocyanin (APC) in *Cyanidium caldariam*, mainly by modulation of levels of translatable messenger RNA for these proteins. Oesterhelt et al., (2007) also showed that glucose could reduce photochemical efficiency of photosystem II (PSII) and levels of the PSII reaction centre protein D1. Many studies of photosynthesis have been carried out with acetate-grown *C. reinhardtii* cells. Growth of cells under increasing concentrations of acetate culture reduced the photosynthetic CO_2 fixation and net O_2 evolution, without effects on respiration and PSII efficiency (Heifetz et al., 2000). Acetate can also reduce carbonic anhydrase (CA) activity and expression of cah-1 encoding CA (Fett and Coleman, 1994). However, there are also some exceptions. Glucose could enhance the net photosynthesis rate in *Synechococcus* sp. This might be because the glucose promoted the donation of electrons to the plastoquinone pool from the respiratory substance, and the transforming of energy was promoted by photosynthetic system, which provided the energy needed by anabolism of cells caused by the glucose added to the medium (Kang et al., 2004).

From a practical point of view, the addition of small quantity continuously of organic carbon substrate, such as acetate is performed in open ponds cultivation processes. This is to support higher biomass concentration and moreover to prevent excessive bacteria growth, which would be the case if the organic substrate were added in large quantity in the culture medium. During the night, addition of organic carbon substrate is usually stopped, as the fast growing bacteria would compete out the algae under heterotrophic culture condition. This fed batch culture process is often limited to one culture cycle, in order to limit the level of microbial contaminants (Lee, 2001).

2.3 Nitrogen assimilation

Microalgae use a wide variety of organic and inorganic nitrogen composts. However, ammonium and nitrate salts are the primary sources for algae growth. When ammonium and nitrate are supplied together, microalgae preferentially utilize ammonium-nitrogen first. Absorption of the NH_4^+ leads to an increase in the pH of the medium, whereas consumption of NH_4^+ ion leads a decrease in pH.

Carbon and nitrogen metabolism are linked in microalgae because they share (a) carbon supplied directly from respiration of fixed CO_2 (autotrophic growth) or assimilated organic carbon (heterotrophic growth) and (b) the energy generated in the tricarbossilic acid cycle (TCA) and in the mithocondrial electron transport chain. The primary assimilation of inorganic nitrogen (ammonium) to form amino acids requires carbon skeletons in the form of keto-acids (2-oxaloglutarate and oxaloacetate) and energy in the form of ATP and NADPH to synthesize the amino acids glutamate, glutamine and aspartate. In both autotrophic and heterotrophic growing cells the TCA cycle provides keto-acids, ATP, and NADPH. The metabolic pathways involved in nitrogen assimilation are depicted in Fig. 5 (Fernandez and Galvan, 2007).
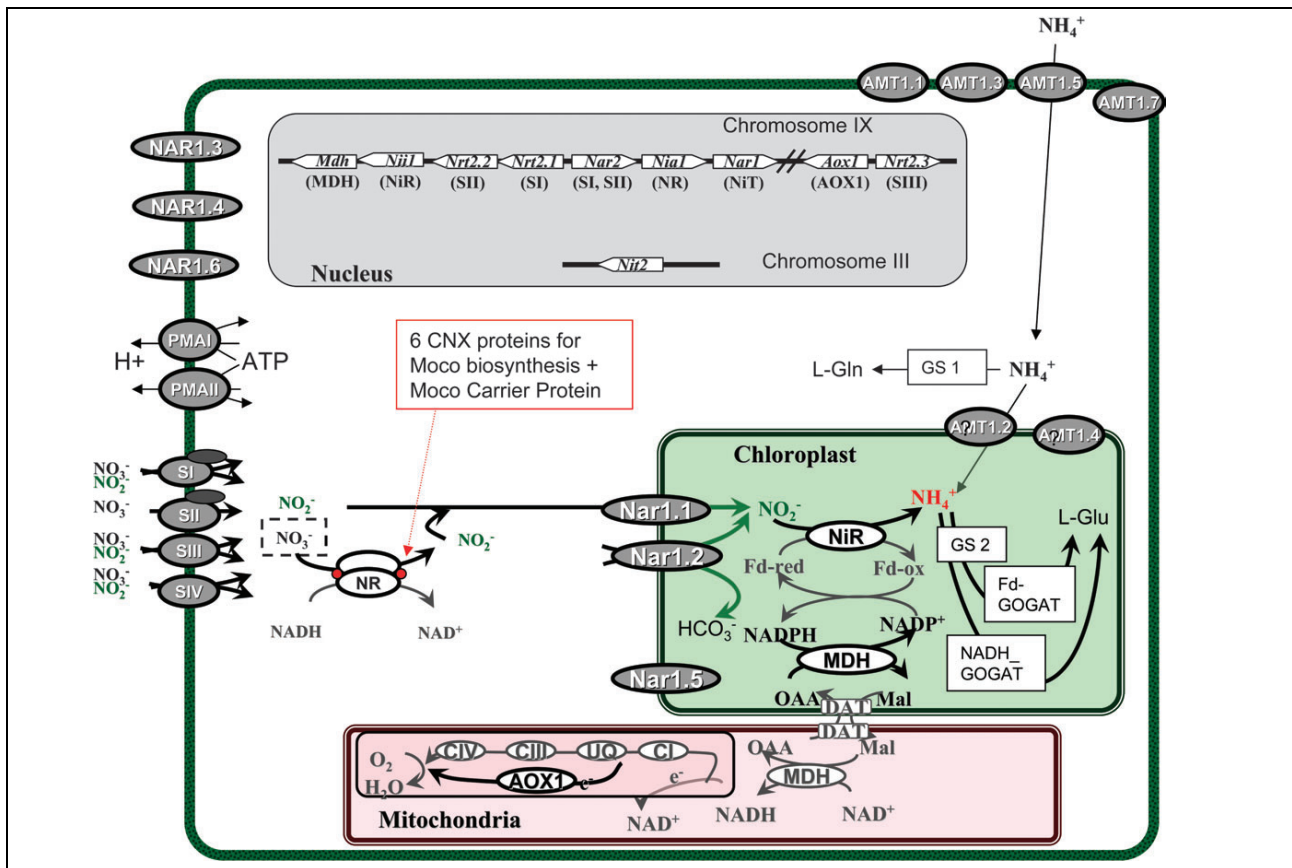
Figure 5 Scheme of nitrate and ammonium assimilation in *Chlamidomonas*. Components of the nitrate and the ammonium pathway are indicated, together with the relevant genes and gene for the nitrate pathway. The most probable localization of the proteins is indicated.
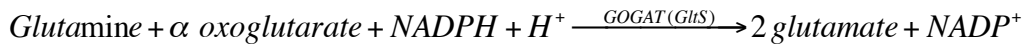
Ammonium assimilation

Assimilation of inorganic nitrogen in the form of ammonium is catalyzed by glutamine synthetase (GS, EC 6.3.1.2) and glutamate synthase (GOGAT, GltS, EC 1.4.1.14) in a combined way (Lu et al., 2005; Tischner, 1984). Alternately, ammonium is incorporated into glutamate by the reversible reductive amination of α-oxoglutarate, which is catalyzed by glutamate dehydrogenase (GDH, EC 1.4.1.2). The GS/GltS pathway is regarded as the primary pathway for ammonium assimilation, while the function of GDH pathway remains obscure; and could be active under conditions of stress (Lu et al., 2005).

The glutamine is formed by adding NH_4^+ into glutamic acid in the following reaction (Ahmad and Hellebust, 1993; Gawronski and Benson, 2004; Hutson and Schmidt, 1995; Turner et al., 1981):

$$NH_+^4 + glutamate + ATP \xrightarrow{\quad GS \quad} gluta\min e + ADP + Pi$$

One specific and important characteristic of this enzyme is its high affinity for ammonia, thus its ability to incorporate ammonia efficiently into amino acids (Miflin and Habash, 2002).

Glutamate synthases are crucial enzymes in ammonia assimilation in plants and bacteria, where they catalyze the formation of two molecules of L-glutamine from L- glutamine and α-oxoglutarate (Vanoni

18

and Curti, 2008) :

$$Gluta\mathrm{mine} + \alpha\,oxoglutarate + NADPH + H^+ \xrightarrow{\ GOGAT(GltS)\ } 2\,glutamate + NADP^+$$

In the alternative pathway, glutamate dehydrogenase (GDH, NADH oxidoreductase, EC 1.4.1.2) catalyzes the formation of glutamate directly from NH_4^+ and α-oxoglutarate.

$$NH_4^+ + \alpha\,oxoglutarate + NADPH + H^+ \xleftarrow{\ GDH\ } L\ glutamate + NADP + H_2$$
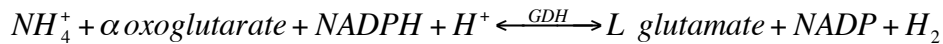
Glutamine synthetase has a high affinity for ammonium (30 µM), compared with glutamate dehydrogenase (40 mM), which means that GS functions even at very low levels of ammonium.

Nitrate reduction

When nitrate is the nitrogen source, microalgae reduce it to ammonium before incorporation into organic compounds. Studies in both higher plants and microalgae suggest that only two enzymes, nitrate reductase (NR) and nitrite reductase (NiR), working sequentially, catalyze the entire reduction of nitrate to ammonium (Kaplan et al. 1986). Assimilatory nitrate reductase (NR, EC 1.6.6.1-3) catalyzes the reduction of nitrate to nitrite, using reduced pyridine nucleotides as physiological electron donors:

$$NO_3^- + NADPH + H^+ \xrightarrow{\ NR\ } NO_2^- + NADP + H_2O$$
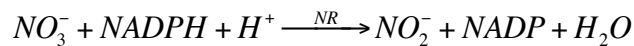
Nitrite reductase (NiR, EC 1.7.7.1) catalyzes the second step of nitrate assimilation, the reduction from nitrite to ammonium, using ferredoxin as the electron donor in a reaction that involves the transfer of six electrons (López-Ruiz et al., 1991):

$$NO_2^- + 6Fdred + 8H^+ \xrightarrow{\ NiR\ } NH_4^+ + 6\,fdox + 2H_2O$$
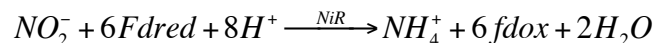
NiR is a chloroplastic enzyme, located specifically in the pyrenoid of green algae (Lopez-Ruiz et al. 1991). In all microalgae, nitrate reductase (NR) is fully expressed in cells growing in nitrate as the only nitrogen source, and it is repressed in cells growing in media containing excess ammonium or a mixture of ammonium plus nitrate (Sherman and Funkhouser, 1989).

3. MAIN FACTORS AFFECTING ALGAL PRODUCTIVITY AND CELL COMPOSITION

Many microalgae, regardless of their origins, possess a similar biochemical composition (on a basis of total organic carbon in the cells), particularly the relative amounts of protein, lipid, and carbohydrate, when grown rapidly under favorable culture conditions. This default setting, however, can be readily changed by environmental factors to give maximum flexibility on microalgae to permit proliferation or survival in the changing environment.

For instance the Chlorophyceae group shows the following typical biochemical composition: 30–50% proteins, 20–40% carbohydrate, and 8–15% of lipids under favorable environmental factors. But these species, however, can accumulate under stress up to 80% of lipids, 80% of hydrocarbons, and 40% of glycerol, on the basis of the dry weight.

Environmental conditions not only affect photosynthesis and productivity of cell biomass but also influence the pattern, pathway, and activity of cellular metabolism and thus dynamic cell composition. The major environmental factors that affect microalgae cultivation are: light, temperature, macronutrient, carbon dioxide and pH.

3.1. Light

One of the most important issue involved in mass cultivation of microalgae concerns effective use of light for photosynthetic productivity of cell mass and secondary metabolites. Efficient use of light means that for a given light intensity, microalgae can maximize the conversion of light energy absorbed into biochemical energy stored in the forms of carbohydrate, protein, and lipid. This is particularly true for mass cultivation of microalgae outdoors, in which effective use of strong solar energy is a foundation stone on which the prospects for this biotechnology rest. A common trend of cellular response to decreasing light intensity is to increase chlorophyll *a* and other light-harvesting pigments (such as chlorophyll *b*, chlorophyll *c*, and phycobiliproteins). Conversely, in response to high light intensity, however, chlorophyll *a* and other light-harvesting pigments directly involved in photosynthesis decrease, while the secondary carotenoids (e.g., β- carotene, lutein, astaxanthin), which serve as photoprotective agents, increase. On the other hand light limitations apparently leads to lower lipids (Cuhel and Lean, 1987; Scott, 1980).

When nutrients are provided in excess and light is the growth-limiting factor, most algal species display a remarkable consistency in their cellular phosphorous content, ca. 1% of dry weight (Goldman, 1979).

3.2 Temperature

Among the many factors that affect the growth and the metabolism of microbial cultures, temperature is a factor to which the cells are inescapably subjected. For instance, Lee et al., (1985) observed that

temperature strongly affected the growth and the bioenergetics of photosynthesis in *Chlorella vulgaris* cultures.

The effect of temperature is visible also on biochemical composition, and it works mainly through two distinct mechanisms: (1) temperature-dependent rate of chemical and biochemical reactions and (2) temperature-dependent partitioning of photosynthetic fixed carbon into various kinds of macro-molecules. Several studies (James et al., 1989; Oliveira et al., 1999; Thompson, 1999) demonstrated that fatty acid, protein and carbohydrate are largely influenced by the temperature but the response of microalgal chemical composition to high and low growth temperatures varies from species to species and there is no overall trend in gross biochemical composition for all species under study (Renaud et al., 2002; Thompson et al., 1992)

3.3 pH and Carbon Dioxide

Carbon comprises 45-50% of algal dry weight, so that 1.65-1.83 g CO_2 is theoretically required for the biosynthesis of 1 g of biomass. Carbon is stored in nutrient solution as a free carbon dioxide, bicarbonate or carbonate. The relative amount of each species is pH-dependent (Fig.6).

Carbon dioxide is responsible for the physiological processes and influences the buffering capacity and electrolyte balance of the nutrient solution. Although HCO_3^- is easily absorbed by the chlorococcal cells, it is a poor source of carbon compared to CO_2 (Goldman et al., 1981). Alkaline bicarbonate alone cannot provide sufficient carbon to optimize biomass yield due to the formation of chemical precipitates and, therefore, CO_2 must be added to the cultures. *Arthrospira* is the only microalga produced in large scale that can use carbonate or bicarbonate. Martínez et al., (2000) founded that as the nutrient medium becomes more complex the less the CO_2 absorption becomes a process only of physical absorption. Chemical reactions between CO_2 and the OH^- H_2O and NH_3 species, the influence of CO_2 supply in the pH of the nutrient medium, and the influence of the pH on the inorganic carbon species present in the medium make this form of CO_2 supply a complex physicochemical system that often limits growth (Talbot et al., 1991) and influences the biochemical composition.
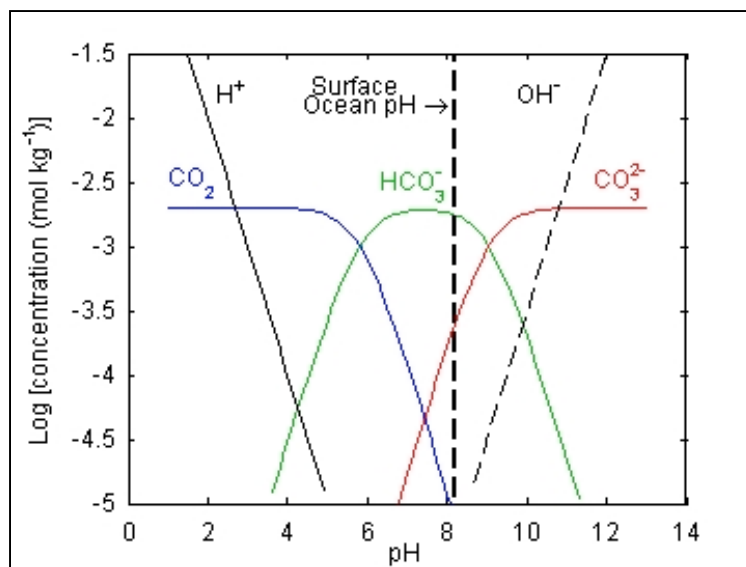
Figure 6 Distribution of total carbon dioxide, bicarbonate and carbonate vs pH

3.4 Inorganic macronutrients

Nutrients are essential elements for microalgal growth. Algae require a variety of both inorganic and organic nutrients. Phosphates and nitrates are taken up in the greatest abundance and are termed macronutrients. In addition to carbon, hydrogen and oxygen, algae use some additional elements to grow and reproduce. Algae grown under nutrient limitation exhibit considerable variation in their biochemical composition depending on the limiting nutrient and the degree of limitation. Consequently, biochemical constituents of microalgae vary in concentration depending upon the nutrient availability. The Redfield ratio 106 C: 16 N: 1 P is considered to be the standard elemental ratio for non-limited algal cultures, but it has been observed that the optimal elemental ratios of any given species of algae could vary from the Redfield ratio substantially, and freshwater species have a much greater deviation from the ratio than marine organisms. Nitrogen, an essential component of all structural and functional proteins in algal cells, generally accounts for about 7–10% of cell dry weight. A wide variety of nitrogen sources, such as nitrate, ammonia, and urea, each can be used as a sole source of nitrogen for sustaining algal growth. Phosphorus is another major macronutrient that plays an important role in cellular metabolic processes by forming many structural and functional components required for normal growth of microalgae. Orthophosphate is incorporated into organic components through various types of phosphorylation. Inorganic phosphate may also occur in the cells in the form of polyphosphates, accumulating in distinct polyphosphate granule. Among essential trace mineral elements, iron plays an important role in cellular biochemical composition because of its redox properties and implication in fundamental processes such as photosynthesis, respiration, nitrogen fixation, and DNA synthesis.

Healey and Hendzel, (1979) compared cellular contents of lipids, carbohydrates and proteins of batch cultures grown under phosphorous (P) and nitrogen (N) limited and non-limited conditions for

several algal species. Their results showed that carbohydrates were the major storage product under P limitation for cryptomonads, while diatoms and green algae had higher amounts of lipids and carbohydrates than non-limited cells. N-limited algae had lower protein contents and higher carbohydrates, but the resume for lipids was such variable. Indeed N-limited green algae had either somewhat higher or unchanged lipid contents compared to non-limited cells. However, analyses of 18 freshwater and 11 marine algal species showed in most cases an increased lipid content at N limitation, often two to three times higher than cultures with replete N (Shifrin and Chisholm, 1981). Geider et al., (1998) found that N limitation greatly reduced the synthesis of chloroplastic proteins, and among the pigments, chl *a* decreased, whereas carotinoids increased.

3.5 Biostimulants

Plant hormones, also known as plant growth substances, are naturally occurring chemicals that control plant growth and development. At present, our knowledge of the algal hormonal system is still rather fragmentary and the presence of the full-value hormonal system in algae and the correspondence of their biological activities to those of higher plant hormones are debate. Despite the information about the hormone metabolism and action mechanisms in algae is extremely scarce, currently, essentially all known phytohormones are found in the members of various algal groups and although the functioning of a comprehensive hormonal system in these organisms is not finally proven yet, the role of phytohormones in the regulation of key metabolic processes in algae is no longer in doubt (Basu et al., 2002; Jacobs, 1993; Kobayashi et al., 1997; Stirk et al., 2003). Attempts to improve microalgal biomass productivity using alternative means such as phytohormones and micronutrients have been reported a few times since the 1930's, earlier studies indicate that biochemical stimulants such as phytohormones, plant extracts, polyamines, and chemicals offer significant potential to enhance microalgae productivity.

The stimulating effect of phytohormones and their analogs on microalgal growth and metabolite production (i.e., carotenoid, lipid, carbohydrate and protein) has been reported (Bajguz and Piotrowska-Niczyporuk, 2014; Czerpak and Bajguz, 1997). Park et al., (2013) observed morphological changes in microalgae cells under the influence of phytohormones. Indole-3-acetic acid (IAA) is the most abundant and naturally occurring auxin in plants, which controls important physiological processes including cell enlargement and division, tissue differentiation and responses to light and gravity (Leveau and Lindow, 2005).

Auxins

Auxins are a group of natural and synthetic compounds and the first of the major plant hormones discovered. All auxins are compounds with an aromatic ring and a carboxylic acid group. This phythormones are responsible in plants for apical dominance and phototropism. They promote cell

growth and elongation of the plant. In the elongation process, auxin alters the plant wall plasticity making it easier for the plant to grow upwards. Auxins also influence rooting formations (Went, 1935).

Cytokinins

Another class of plant growth substances is represented by citokinins. They are divided in two functional groups: adenine-type (kinetin and zeatin) and phenylurea-type (diphenylurea and thidiazuron). Cytokinins are involved in root and shoot development, leaf senescence, nutrient mobilization, breaking of bud dormancy, and seed germination and on a cellular level, they play an essential role in cell division (Letham, 1974). Some research showed that the effect of cytokinin on the content of photosynthetic pigments increases in vascular plant and algae (Czerpak et al., 1994).

Gibberellins

All known gibberellins are diterpenoid acids that are synthesized by the terpenoid pathway and than modified in the endoplasmic reticulum and cytosol until they reach their biologically active form. All the gibberellins are based on a kaurene carbon skeleton. There are two fundamentally different forms of gibberellins: one with 19 carbon atoms and one class with 20 C. Gibberellins have several effects on plant as well. They promote the cell division for the elongations of internodes, cell elongation, change in leaf shape or size and retardation of root growth (Bostrack and Struckmeyer, 1967). Endogenous gibberellins have been quantified in 24 axenic microalgae strains from the Chlorophyceae, Trebouxiophyceae, Ulvophyceae and Charophyceae microalgae strains after 4 days in culture (Stirk et al., 2013).

Polyamines

Di- and polyamines (PA) as polycation nitrogen compounds commonly occur in the cells of all pro- and eucaryotic organisms. They belong to the specific cellular regulators of growth and physiological metabolic processes but differ essentially in their action from the typical hormones.

As we know, polyamines occur in almost all cellular organelles chiefly in the nuclei, chloroplasts, ribosomes, cytoplasmic membranes and the walls of young cells. A markedly higher level of polyamines was noted in decondensed regions of chromosomes and nucleoli replicatively and transcriptively active so, they participate in the activation of replication, transcription and translation. In chloroplasts polyamines stimulate the photosynthetic process in both its phases: the dark and the light as well as activating the primary and secondary growth of the cell wall. These compounds stimulate the active transport of metabolites, the functioning of enzymes and ion pumps localised in the cellular membrane (Kotzabasis et al., 1999).
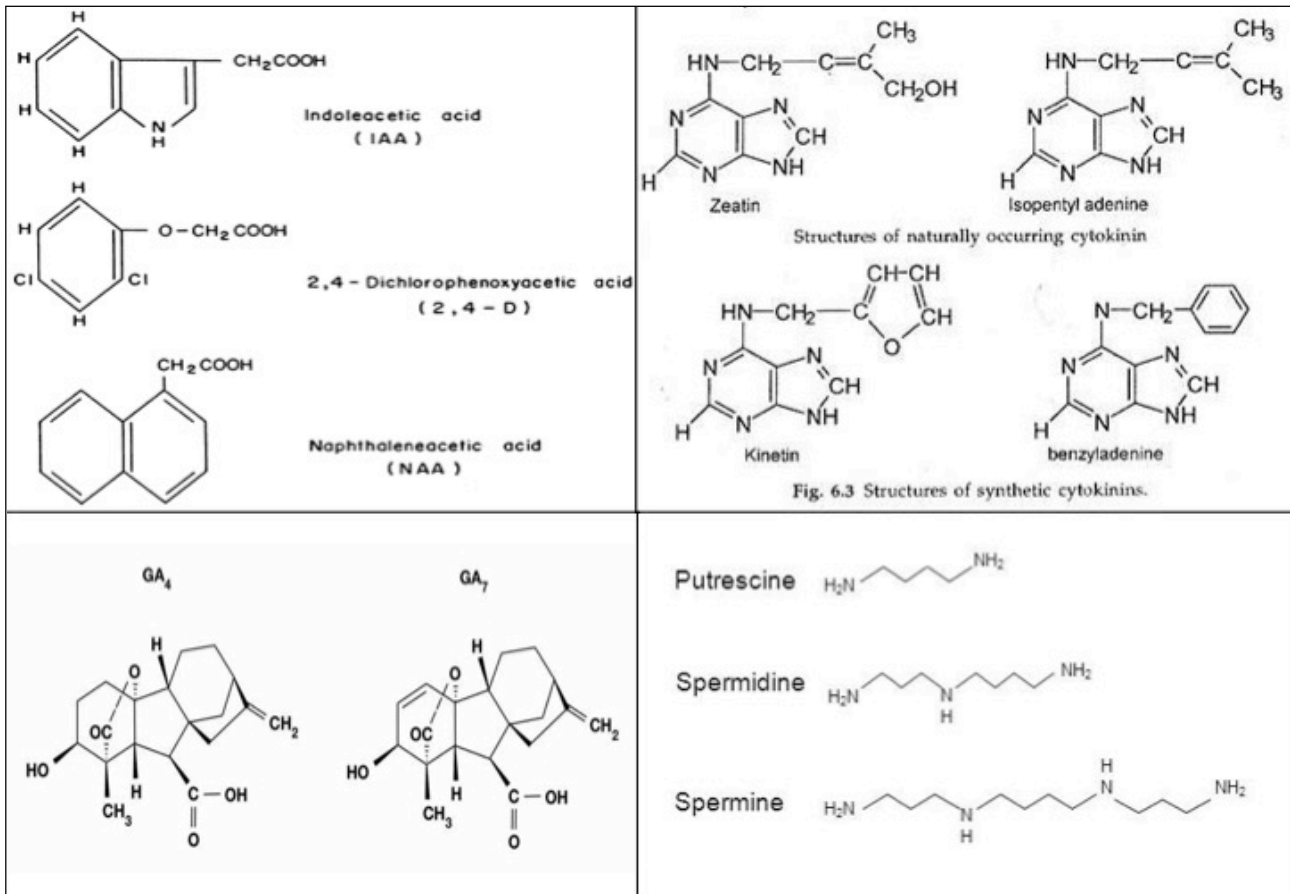
Figure 7 Example of biomolecules: auxins, cytokinins, gibberellins and polyamines

4. MASS CULTURE: SYSTEMS AND METHODS

Today, microalgae and cyanobacteria for commercial exploitation are either harvested from natural habitats or obtained through more or less controlled cultivation processes in open ponds or photobioreactors (PBRs) (Tredici, 2004; Tredici, 2010). For a long time, naturally grown microalgae were harvested from natural sources for human and animal consumption. Although this approach is still practised by a small number of commercial health food companies and aquaculture hatcheries with no cost for the cultivation of the biomass, the productivity and product quality (both biological and toxicological) cannot be assured. For this reason various physiological and technological approaches have been proposed (Fig. 8) and investigated for maximising productivity in mass algal culture systems (Grobbelaar, 2000; Richmond, 2008, 2000).

Open pond culture system

Depth (cm)	Location	Alga	Highest productivity		Ref.
			$(g/m^2/day)$	$(g/L/day)$	
13–15	Israel	*Spirulina platensis*	27.0	0.18	1
1	Czech	*Chlorella* sp.	25.0	2.50	2

Enclosed photobioreactors

Bioreactor	Orientation	ID (cm)	Location	Alga	Highest productivity		Ref.
					$(g/m^2/day)*$	$(g/L/day)$	
Tubular	Horizontal	12.3	Italy	*Spirulina maxima*	25.0	0.25	3
		2.6	Italy	*Spirulina* sp.	27.8	–	3
		2.5	Israel	*Spirulina platensis*	27.0	1.60	4
		2.6	Spain	*Isochrysis galbana*	–	0.32	5
		6.0	Spain	*Phaeodactylum*	–	2.02	6
		3.0	Spain	*Phaeodactylum*	–	2.76	7
		6.0	France	*Porphyridium cruentum*	25.0	0.36	8
	Inclined	2.5	Singapore	*Chlorella pyrenoidosa*	72.5	2.90	9
		1.2	Singapore	*Chlorella pyrenoidosa*	130.0	3.64	10
Coil	Vertical	2.4	Australia	*Tetraselmis chuii*	–	1.20	11
Column	Vertical	20.0	Spain	*Phaeodactylum*	–	0.69	12
		2.6	Israel	*Isochrysis galbana*	–	1.60	13
Flat plate	Inclined	10.4	Israel	*Spirulina platensis*	33.0	0.30	14
		1.3	Israel	*Spirulina platensis*	51.0	4.30	14
		3.2	Italy	*Spirulina platensis*	24.0	0.80	15

*Productivity per land (foot-print) area per day.
References: 1. Richmond et al., 1990; 2. Doucha & Livansky, 1995; 3. Torzillo et al., 1986; 4. Richmond et al., 1993; 5. Grima et al., 1994; 6. Fernandez et al., 1998; 7. Grima et al., 1996; 8. Chaumont et al., 1988; 9. Lee et al., 1995; 10. Lee & Low, 1991; 11. Borowitzka, 1997; 12. Miron et al., 1999; 13. Hu & Richmond, 1994 14. Hu et al., 1996; 15. Tredici et al., 1991.

Figure 8 Biomass productivity and cultivation systems depending on microalgae specie (Lee, 2001)

3.1 Enclosed photobioreactors

Enclosed photobioreactors have been developed to achieve high cell concentrations and higher biomass productivity, in addition to maintain monoculture for microalgae that grow in mild culture conditions, as the majority of microalgae do not require a specific growth environment or a selective medium. PBRs provide a close environment that protects the culture from direct fallout and invasion by unwanted species and allow a more accurate control of culture parameters (pH, temperature, pO_2, etc.), ensuring the cultivation of specific strains (Tredici, 2004).

PBRs are classified in terms of both design and mode of operation: (a) flat or tubular, (b) horizontal, inclined, vertical or spiral, (c) manifold or serpentine, (d) hybrid, (e) floating and (f) biofilm reactors. Here a brief overview of the main PBRs that are used nowadays for mass cultivation.

Polyethylene bags (sleeves) suspended from a framework or supported within a mesh frame and mixed by air bubbling are the most common cultivation devices used in hatcheries for the production of algal biomass (from 50 to 500 L in volume, such reactors are mostly used indoors with artificial illumination). Vertical columns are made of rigid transparent cylinders (typically 2–2.5 m in height and 30–50 cm in diameter), with mixing achieved by air bubbling or by an airlift. They are extensively used in hatcheries even if, because of their low S/V, volumetric productivities are rather low (typically below $0.1 \, \mathrm{g \, L^{-1} \, d^{-1}}$) (Fulks and Main, 1991).

Tubular PBRs are the most common design available and the preferred one in commercial algae production. These reactors are usually constructed with either glass or plastic tubes in which the culture is circulated with pumps or preferably by means of airlift systems. This category can be subdivided into three main groups: (i) serpentine, (ii) manifold, and (iii) helical PBRs (Tredici, 2010).

Vertical or inclined flat reactors represent very promising culture devices. They can be oriented and tilted at different angles so as to modify the intensity of impinging light and use diffuse and reflected light, which plays an important role in productivity (Qiang et al., 1998). Flat panels also offer the possibility to be closely packed together and thus attain, by a sort of "lamination" of the culture, high photosynthetic efficiencies (Carlozzi, 2003; Wijffels et al., 2010).

Figure 9 Photobioreactors: (a) Polyethylene bags, Le fremer Institute; (b) Vertical columns, University of Cape Town; (c) Tubular photobioreactor at the IGB Biotech; (d) Flat pannel developed by Subitech GmbH (Germany).

There is no "best reactor" that allows maximum productivity to be achieved with minimum operation costs in any situation, since the choice of the most suitable PBR is dictated by the cultivated species, the location, and the final product. However, the principles leading to maximum productivities of algae culture systems are well known (Posten, 2009):

- adequate mixing to provide a suitable light–dark cycle to the cells and avoid gradients and biofouling;
- high mass-transfer capacity to efficiently supply CO_2 and prevent O_2 buildup;
- high surface-to-volume ratio (S/V) to increase cell concentration and volumetric productivity;
- control of temperature at or near the optimum for the cultivated organism;
- accurate control of pH, CO_2, and nutrient concentrations;
- adequate harvesting regime to maintain the optimal population density;
- appropriate PBR inclination/orientation to reduce photoinhibition and maximize photosynthetic efficiency

3.2 Open pond

Open systems have so far been the method of choice to produce commercially microalgal biomass. In the open-pond culture system, monoculture of algae is usually achieved by maintaining an extreme culture environment, such as high salinity, high alkalinity and high nutritional status (Lee, 1986). Thus, a limited range of microalgae could be maintained as monoculture in open ponds in long term

operation. Today, only *Dunaliella* (high salinity), *Spirulina* (high alkalinity) and *Chlorella* (high nutrition) have been successfully mass cultured and marketed commercially (Richmond, 1999). It must be stressed that such approaches do not necessary exclude bacteria and other biological contaminants (e.g. protozoa), thus a major short coming of the open culture system. Starting from the fifties, with the realising of the potential of microalgae as source of biomass and biomolecules, microalgae are mass cultured in several open pond systems: circular, raceway open-ponds and open cascades or inclined. Japan, Taiwan and Indonesia still use the circular ponds with a rotating scraper. The pond could be shallow (less than 5 cm) but the size of the pond is limited by the strain of water resistance on the rotating motor and the largest pond reported is 50 m (Lee, 1997). Raceway-shape ponds are frequently used in Israel, the United States of America, China and other countries. Raceway ponds are the most common commercial system in use. They consist of a circuit of parallel channels, in which the microalgae suspension is circulated by a paddle wheel. The system is built in concrete or dug in the ground and may be covered with a plastic liner. Evaporation, as well as temperature fluctuations, is significant, and rainfalls can dilute the available nutrients and the biomass concentration (Tredici, 2010). With this pond biomass concentration of about 0.5 g L^{-1} could be maintained and a productivity of about 25g m^{-2} d^{-1} had been reported (Richmond et al., 1990).The production of algal biomass, with the above mentioned ways of ponds, is marketed as health food, speciality feed and source of pigments. Another way to cultivate with an open system is the open cascade, which with a culture depth of less than 1 cm developed in Czech Republic for cultivation of Chlorella has achieved a higher cell density of 10 g $^{-1}$L, but a comparable areal productivity of 25 g m^{-2} d^{-1} (Šetlík et al., 1970).

Figure 10 Example of opend ponds: (a) Circular ponds in Asia; (b) Raceway ponds in Israel.

5. WATER-POLLUTION AND BIOREMEDIATION

5.1. Water pollution

Humans now strongly influence almost every major aquatic ecosystem, and their activities have dramatically altered the fluxes of growth-limiting nutrients from the landscape to receiving waters. It is truism nowadays to recognize that, pollution associated problems, are a major concern of society. Environmental laws are given general applicability and their enforcement has been increasingly stricter. So, in term of health, environment and economy, the fight against pollution has become a major issue.

Although the strategic importance of fresh water management can be found almost in every scientific, social, or political agenda all over the world, water resources seem to face severe quantitative and qualitative threats. The pollution increase, industrialization and rapid economic development, impose severe risks to availability and quality of water resources, in many areas worldwide.

Pollution is a man-made phenomenon, arising either when the concentration of naturally occurring substances are increased or when non-natural synthetic compounds (xenobiotics) are released into the environment. Organic and inorganic substances which are released into the environment as a result of domestic, agricultural and industrial water activities lead to organic and inorganic pollution (Mouchet, 1986) .

Different sources of pollutants include "Discharge of either raw or treated sewage from towns and villages; discharge from manufacturing or industrial plants; run-off from agricultural land; and leachates from solid waste disposal sites" these sites of pollution have problems so that a solution is sought (Horan, 1989). The composition of waste-water is a reflection of the life styles and technologies practiced in the producing society, it is a complex mixture of natural organic and inorganic materials as well as man-made compound. We can find organic carbon under several forms as carbohydrates, fats, proteins, amino acids, and volatile acids, on the other hand inorganic constituents include large concentrations of sodium, calcium, potassium, magnesium, chlorine, sulphur, phosphate, bicarbonate, ammonium salts and heavy metals (Horan, 1989; Lim et al., 2010).

At last but not the least aquatic environment is often exposed to various pollutants including heavy metals owing to increasing industrial and/or agricultural wastes. The danger of heavy metal pollution is due to its ability to circulate within aquatic and near-shore ecosystems for a prolonged length of time.

5.2 Eutrophication

The rapid intensification of agriculture is one of the major cause in nutrient water pollution. The global production of agricultural fertilizer alone released < 10 million metric tonnes of nitrogen in 1950, but may exceed 135 million metric tonnes of N by the year 2030. In addition, humans use flowing waters

as convenient wastewater disposal systems, and the loading of N and P to the world's surface waters is very strongly influenced by human population density and land use(Smith, 2003).

Moreover a huge contribute is given by industrialized countries that generate a great volume of urban and industrial wastewaters. These effluents should not be dumped directly into rivers, lakes or the sea before treatment to reduce contaminants to environmentally safe levels. Special attention is required for inorganic substances, which encourage vegetal growth, such as ammonium, nitrates and phosphates, contributing to the eutrophication of the bodies of water receiving the effluents.

The eutrophication of both freshwater and coastal marine systems causes a wide array of undesiderable symptoms that are either directly or indirectly related to the nuisance growth of aquatic plants. The excessive increase in available nitrogen, phosphorus and carbon through human activities causes an enhancement of algal production, which can lead to conspicuous, and sometimes toxic, algal blooms and possibly to changes in species composition and seasonality. Eutrophication is a process linked to the massive algal growth, which physically impede waterways and increase oxygen demand during the decay phase. The effects of eutrophication are many and include: decreases in dissolved oxygen that can reach anaerobic levels, shifts in species and reduction in species diversity, degradation of the habitat from decreased light transmittance due to increases in phytoplankton growth. This process can disrupt the marine habitat, damaging the planktonic and benthic fauna and lead to serious economic losses.

5.2. Phytoremediation

Phytoremediation is an emerging technology that utilizes plants and other photosynthetic microorganisms to remove, transform, or contain toxic chemicals located in soils, sediments, ground water, surface water, and even the atmosphere. Currently, phytoremediation is used for treating many classes of contaminants including petroleum hydrocarbons, chlorinated solvents, pesticides, explosives, heavy metals and radionuclides, and landfill leachates.

The advantages due to the phytoremediation are: (1) the modification of the physical and chemical properties of contaminated soils; (2) the release of root exudates, thereby increasing organic carbon; (3) improving aeration by releasing oxygen directly to the root zone, as well as increasing the porosity of the upper soil zones; (4) intercepting and retarding the movement of chemicals; (5) effecting co-metabolic microbial and plant enzymatic transformations of recalcitrant chemicals; and (6) decreasing vertical and lateral migration of pollutants to ground water by extracting available water and reversing the hydraulic gradient (Chang and Corapcioglu 1998). Moreover when contaminants are in low concentration, phytoremediation alone may be the most economical and effective remediation strategy (Jones, 1991).

The use of algae in purification facilities to eliminate nutrients continues to be studied widely, (Horan, 1989; Olguín et al., 2007; Samorì et al., 2014; Tredici et al., 1992). Employing microalgae and

cyanobacteria offers a valuable alternative to the conventional purification treatments and provides several advantages in this technology: (i) rests on the principles of natural ecosystems and therefore is not environmentally dangerous; (ii) causes no secondary pollution if the biomass produced is reused; (iii) enables the efficient recycling of nutrients contained in the secondary effluents, since the microalgae are highly effective not only at using the inorganic N and P for growth but also at purifying the waste by producing oxygen and removing heavy metals and xenobiotic substances.

For instance the green microalga *Scenedesmus obliquus* has shown extraordinary vitality in urban wastewaters, registering growth rates similar to those reported for a complete synthetic medium. This freshwater alga tolerates a wide range of temperature and pH, making it versatile for sewage purification (Kessler, 1991).

Microalgae in phytoremediation can have good results in heavy metal bioabsorption, for example green unicellular freshwater algae with high metabolism rates play the basic role in the primary production and concentration of heavy metals, because of the algal cell wall, which is composed of a fiber-like structure and amorphous embedding matrix of various polysaccharides (Peña-Castro et al., 2004).

References

Adul Rahman, R.N., Jongsareejit, B., Fujiwara, S., Imanaka, T., 1997. Characterization of recombinant glutamine synthetase from the hyperthermophilic archaeon *Pyrococcus* sp. strain KOD1. Appl. Environ. Microbiol. 63, 2472–2476.

Ahmad, I., Hellebust, J.A., 1993. Partial characterization of enzymes of nitrogen metabolism in *Chlorella autotrophica* Shihira & Krauss. New Phytol.Apt, K.E., Behrens, P.W., 1999. Commercial Developments in Microalgal Biotechnology. J. Phycol. 35, 215–226.

Baker, N.R., 2008. Chlorophyll fluorescence: a probe of photosynthesis in vivo. Annu. Rev. Plant Biol. 59, 89–113.

Ball, S., Colleoni, C., Cenci, U., Raj, J.N., Tirtiaux, C., 2011. The evolution of glycogen and starch metabolism in eukaryotes gives molecular clues to understand the establishment of plastid endosymbiosis. J. Exp. Bot. 62, 1775–1801.

Basu, S., Sun, H., Brian, L., Quatrano, R.L., Muday, G.K., 2002. Early Embryo Development in *Fucus distichusIs* Auxin Sensitive. Plant Physiol. 130, 292–302.

Becker, E.W., 2007. Micro-algae as a source of protein. Biotechnol. Adv. 25, 207–210.

Bilger, W., Björkman, O., 1990. Role of the xanthophyll cycle in photoprotection elucidated by measurements of light-induced absorbance changes, fluorescence and photosynthesis in leaves of *Hedera canariensis*. Photosynth. Res. 25, 173–185.

Borowitzka, M.A., 1999. Commercial production of microalgae: ponds, tanks, and fermenters, in: R. Osinga, J.T., J. G. Burgess and R. H. Wijffels (Ed.), Progress in Industrial Microbiology, Marine Bioprocess Engineering Proceedings of an International Symposium Organized under Auspices of The Working Party on Applied Biocatalysis of the Eurpean Federation of Biotechnology and The European Society for Marine Biotechnology. Elsevier, pp. 313–321.

Bostrack, J.M., Struckmeyer, B.E., 1967. Effect of gibberellic acid on the growth and anatomy of *Coleus blumei*, *Antirrhinum majus* and *Salvia splendens*. New Phytol. 66, 539–544.

Carlozzi, P., 2003. Dilution of solar radiation through "culture" lamination in photobioreactor rows facing south-north: a way to improve the efficiency of light utilization by cyanobacteria (*Arthrospira platensis*). Biotechnol. Bioeng. 81, 305–315.

Chang, Y., Corapcioglu, M.Y., 1998. Plant-enhanced subsurface bioremediation of nonvolatile hydrocarbons. J. Environ. Eng., 112, pp. 162–169.

Chisti, Y., 2007. Biodiesel from microalgae. Biotechnol. Adv. 25, 294–306.

Cuhel, R.L., Lean, D.R.S., 1987. Influence of Light Intensity, Light Quality, Temperature, and Daylength on Uptake and Assimilation of Carbon Dioxide and Sulfate by Lake Plankton. Can. J. Fish. Aquat. Sci. 44, 2118–2132.

Czerpak, R., Bajguz, A., Białecka, B., Wierzchołowska, L.E., Wolańska, M.M., 1994. Effect of auxin precursors and chemical analogues on the growth and chemical composition in *Chlorella pyrenoidosa* Chick. Acta Soc. Bot. Pol. 63, 279–286.

Endo, H., Sansawa, H., Nakajima, K., 1977. Studies on *Chlorella regularis*, heterotrophic fast-growing strain II. Mixotrophic growth in relation to light intensity and acetate concentration. Plant Cell Physiol. 18, 199–205.

Fernandez, E., Galvan, A., 2007. Inorganic nitrogen assimilation in *Chlamydomonas*. J. Exp. Bot. 58, 2279–2287.

Fett, J.P., Coleman, J.R., 1994. Regulation of Periplasmic Carbonic Anhydrase Expression in *Chlamydomonas reinhardtii* by Acetate and pH. Plant Physiol. 106, 103–108.

Fulks, W., Main, K.L., 1991. Rotifer and microalgae culture systems: proceedings of a U.S.-Asia workshop,

Honolulu, Hawaii, January 28-31, 1991. Oceanic Institute.

Gawronski, J.D., Benson, D.R., 2004. Microtiter assay for glutamine synthetase biosynthetic activity using inorganic phosphate detection. Anal. Biochem. 327, 114–118.

Geider, R., Macintyre, Graziano, L., McKay, R.M., 1998. Responses of the photosynthetic apparatus of *Dunaliella tertiolecta* (Chlorophyceae) to nitrogen and phosphorus limitation. Eur. J. Phycol. 33, 315–332.

Genty, B., Briantais, J.-M., Baker, N.R., 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim. Biophys. Acta BBA - Gen. Subj. 990, 87–92.

Goldman, J.C., 1979. Outdoor algal mass cultures—II. Photosynthetic yield limitations. Water Res. 13, 119–136.

Goldman, J.C., Dennett, M.R., Riley, C.B., 1981. Inorganic carbon sources and biomass regulation in intensive microalgal cultures. Biotechnol. Bioeng. 23, 995–1014.

Graham, L.E., Graham, J.M. & Wilcox, L.W. (2009) *Algae*, 2nd edn. 616 pp. Benjamin Cummings, San Francisco, CA.

Grobbelaar, J.U., 2000. Physiological and technological considerations for optimising mass algal cultures. J. Appl. Phycol. 12, 201–206.

Grossman, A.R., Bhaya, D., Apt, K.E., Kehoe, D.M., 1995. Light-Harvesting Complexes in Oxygenic Photosynthesis: Diversity, Control, and Evolution. Annu. Rev. Genet. 29, 231–288.

Guschina, I.A., Harwood, J.L., 2006. Lipids and lipid metabolism in eukaryotic algae. Prog. Lipid Res. 45, 160–186.

Healey, F.P., Hendzel, L.L., 1979. Indicators of Phosphorus and Nitrogen Deficiency in Five Algae in Culture. J. Fish. Res. Board Can. 36, 1364–1369.

Heifetz, P.B., Förster, B., Osmond, C.B., Giles, L.J., Boynton, J.E., 2000. Effects of Acetate on Facultative Autotrophy in *Chlamydomonas reinhardtii* Assessed by Photosynthetic Measurements and Stable Isotope Analyses. Plant Physiol. 122, 1439–1446.

Horan, N.J., 1989. Biological wastewater treatment systems: theory and operation. viii + 310 pp.

Hunter, P.A., Darby, G.K., Russel, N.J., 1996. Fifty Years of Antimicrobials: Past Perspectives and Future Trends. J. Eukaryot. Microbiol. 43, 160–162.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., Darzins, A., 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J. Cell Mol. Biol. 54, 621–639.

Hutson, R.G., Schmidt, R.R., 1995. Cell Cycle Timing of Replication of the Nuclear Gene Encoding Chloroplastic NADP-Specific Glutamate Dehydrogenase Isoenzymes in *Chlorella sorokiniana*. Plant Cell Physiol. 36, 397–407.

Jacobs, W.P., 1993. A Search for Some Ang1osperm Hormones and Their Metabolites in *Caulerpa Paspaloides* (chlorophyta)1. J. Phycol. 29, 595–600.

James, C.M., Al-Hinty, S., Salman, A.E., 1989. Growth and ω3 fatty acid and amino acid composition of microalgae under different temperature regimes. Aquaculture 77, 337–351.

Kang, R., Wang, J., Shi, D., Cong, W., Cai, Z., Ouyang, F., 2004. Interactions between organic and inorganic carbon sources during mixotrophic cultivation of *Synechococcus* sp. Biotechnol. Lett. 26, 1429–1432.

Kaplan, D., Richmond, A.E., Dubinsky, Z., Aaronson, S., 1986. Algal nutrition. In: Richmond, A. (Ed.), Handbook for Microalgal Mass Culture. CRC Press, Boca Raton, FL., USA, pp. 147e198.

Kawachi, M., Inouye, I., Honda, D., O'Kelly, C.J., Bailey, J.C., Bidigare, R.R., Andersen, R.A., 2002. The Pinguiophyceae classis nova, a new class of photosynthetic stramenopiles whose members produce large

amounts of omega-3 fatty acids. Phycol. Res. 50, 31–47.

Kessler, E., 1991. Scenedesmus: Problems of a Highly Variable Genus of Green Algae. Bot. Acta 104, 169–171.

Khozin-Goldberg, I., Iskandarov, U., Cohen, Z., 2011. LC-PUFA from photosynthetic microalgae: occurrence, biosynthesis, and prospects in biotechnology. Appl. Microbiol. Biotechnol. 91, 905–915.

Kobayashi, M., Hirai, N., Kurimura, Y., Ohigashi, H., Tsuji, Y., 1997. Abscisic acid-dependent algal morphogenesis in the unicellular green alga *Haematococcus pluvialis*. Plant Growth Regul. 22, 79–85.

Kotzabasis, K., Strasser, B., Navakoudis, E., Senger, H., Dörnemann, D., 1999. The regulatory role of polyamines in structure and functioning of the photosynthetic apparatus during photoadaptation. J. Photochem. Photobiol. B 50, 45–52.

Kretovich, W.L., Evstigneeva, Z.G., Tomova, N.G., 1970. Effect of nitrogen source on glutamate dehydrogenase and alanine dehydrogenase of *Chlorella*. Can. J. Bot. 48, 1179–1183.

Lee, Y.-K., 1986. Enclosed bioreactors for the mass cultivation of photosynthetic microorganisms: the future trend. Trends Biotechnol. 4, 186–189.

Lee, Y.-K., 1997. Commercial production of microalgae in the Asia-Pacific rim. J. Appl. Phycol. 9, 403–411.

Lee, Y.-K., 2001. Microalgal mass culture systems and methods: Their limitation and potential. J. Appl. Phycol. 13, 307–315.

Lee, R.E. (2008) *Phycology*, 4th edn. 547 pp. Cambridge University Press, Cambridge, MA.

Lee, Y.-K., Tan, H.-M., Hew, C.-S., 1985. The effect of growth temperature on the bioenergetics of photosynthetic algal cultures. Biotechnol. Bioeng. 27, 555–561.

Letham, D.S., 1974. Regulators of Cell Division in Plant Tissues. Physiol. Plant. 32, 66–70.

Lim, S.-L., Chu, W.-L., Phang, S.-M., 2010. Use of *Chlorella vulgaris* for bioremediation of textile wastewater. Bioresour. Technol. 101, 7314–7322.

López-Ruiz, A., Verbelen, J.-P., Bocanegra, J.A., Diez, J., 1991. Immunocytochemical Localization of Nitrite Reductase in Green Algae 1. Plant Physiol. 96, 699–704.

Lu, B., Yuan, Y., Zhang, C., Ou, J., Zhou, W., Lin, Q., 2005. Modulation of key enzymes involved in ammonium assimilation and carbon metabolism by low temperature in rice (Oryza sativa L.) roots. Plant Sci. 169, 295–302.

Marquez, F.J., Sasaki, K., Kakizono, T., Nishio, N., Nagai, S., 1993. Growth characteristics of Spirulina platensis in mixotrophic and heterotrophic conditions. J. Ferment. Bioeng. 76, 408–410.

Martínez, F., Orús, M.I., 1991. Interactions between Glucose and Inorganic Carbon Metabolism in *Chlorella vulgaris* Strain UAM 101. Plant Physiol. 95, 1150–1155.

Martínez, M.E., Sánchez, S., Jiménez, J.M., El Yousfi, F., Muñoz, L., 2000. Nitrogen and phosphorus removal from urban wastewater by the microalga *Scenedesmus obliquus*. Bioresour. Technol. 73, 263–272.

Masojídek, J., Vonshak, A., Torzillo, G., 2010. Chlorophyll Fluorescence Applications in Microalgal Mass Cultures, in: Suggett, D.J., Prášil, O., Borowitzka, M.A. (Eds.), Chlorophyll a Fluorescence in Aquatic Sciences: Methods and Applications, Developments in Applied Phycology. Springer Netherlands, pp. 277–292.

Miflin, B.J., Habash, D.Z., 2002. The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. J. Exp. Bot. 53, 979–987.

Mouchet P. Algal reactions to mineral and organic micropollutants, ecological consequences and possibilities for industrial scale application; a review. Water Res. 1986;20:399–412.

Nakamura, Y., Ikawa, T., 1993. Purification and Properties of NADH: Nitrate Reductase from the Red Alga Porphyra yezoensis. Plant Cell Physiol. 34, 1239–1249.

Oesterhelt, C., Schmälzlin, E., Schmitt, J.M., Lokstein, H., 2007. Regulation of photosynthesis in the unicellular acidophilic red alga *Galdieria sulphuraria*. Plant J. 51, 500–511.

Olaizola, M., 2003. Commercial development of microalgal biotechnology: from the test tube to the marketplace. Biomol. Eng., Marine Biotechnology: Basics and Applications 20, 459–466.

Olguín, E.J., Sánchez-Galván, G., Pérez-Pérez, T., 2007. Assessment of the Phytoremediation Potential of *Salvinia minima* Baker Compared to *Spirodela polyrrhiza* in High-strength Organic Wastewater. Water. Air. Soil Pollut. 181, 135–147.

Oliveira, M.A.C.L. de, Monteiro, M.P.C., Robbs, P.G., Leite, S.G.F., 1999. Growth and Chemical Composition of *Spirulina Maxima* and *Spirulina Platensis* Biomass at Different Temperatures. Aquac. Int. 7, 261–275.

Peña-Castro, J.M., Martínez-Jerónimo, F., Esparza-García, F., Cañizares-Villanueva, R.O., 2004. Heavy metals removal by the microalga *Scenedesmus incrassatulus* in continuous cultures. Bioresour. Technol. 94, 219–222.

Posten, C., 2009. Design principles of photo-bioreactors for cultivation of microalgae. Eng. Life Sci. 9, 165–177.

Prunkard, D.E., Bascomb, N.F., Robinson, R.W., Schmidt, R.R., 1986. Evidence for Chloroplastic Localization of an Ammonium-Inducible Glutamate Dehydrogenase and Synthesis of Its Subunit from a Cytosolic Precursor-Protein in *Chlorella sorokiniana*. Plant Physiol. 81, 349–355.

Pulz, O., Scheibenbogen, K., Groß, W., 2001. Biotechnology with Cyanobacteria and Microalgae, in: Rehm, H.-J., Reed, G. (Eds.), Biotechnology. Wiley-VCH Verlag GmbH, pp. 105–136.

Qiang, H., Faiman, D., Richmond, A., 1998. Optimal tilt angles of enclosed reactors for growing photoautotrophic microorganisms outdoors. J. Ferment. Bioeng. 85, 230–236.

Raven, J.A., 1976. The Quantitative Role of "Dark" Respiratory Processes in Heterotrophic and Photolithotrophic Plant Growth. Ann. Bot. 40, 587–602.

Renaud, S.M., Thinh, L.-V., Lambrinidis, G., Parry, D.L., 2002. Effect of temperature on growth, chemical composition and fatty acid composition of tropical Australian microalgae grown in batch cultures. Aquaculture 211, 195–214.

Richmond, A. (1999) Physiological principles and modes of cultivation in mass production of photoautotrophic microalgae. In: *Chemicals from Microalgae* (ed. Z. Cohen), pp. 353–386. Taylor & Francis, London

Richmond, A., 2000. Microalgal biotechnology at the turn of the millennium: A personal view. J. Appl. Phycol. 12, 441–451.

Richmond, A., 2008. Handbook of Microalgal Culture: Biotechnology and Applied Phycology. John Wiley & Sons.

Richmond, A., Lichtenberg, E., Stahl, B., Vonshak, A., 1990. Quantitative assessment of the major limitations on productivity of *Spirulina platensis* in open raceways. J. Appl. Phycol. 2, 195–206.

Samorì, G., Samorì, C., Pistocchi, R., 2014. Nutrient removal efficiency and physiological responses of *Desmodesmus communis* at different HRTs and nutrient stress condition using different sources of urban wastewater effluents. Appl. Biochem. Biotechnol. 173, 74–89.

Scott, J.M., 1980. Effect of growth rate of the food alga on the growth/ingestion efficiency of a marine herbivore. J. Mar. Biol. Assoc. U. K. 60, 681 – 702.

Šetlík, I., Šust, V., Málek, I., 1970. Dual Purpose Open Circulation Units for Large Scale Culture of Algae in Temperate Zones. I. Basic Design Considerations and Scheme of a Pilot Plant. Algol. Stud. Für Hydrobiol. Suppl. Vol. 111–164.

Sherman, T.D., Funkhouser, E.A., 1989. Induction and synthesis of nitrate reductase in *Chlorella vulgaris*. Arch. Biochem. Biophys. 274, 525–531.

Shifrin, N.S., Chisholm, S.W., 1981. Phytoplankton Lipids: Interspecific Differences and Effects of Nitrate, Silicate and Light-Dark Cycles1. J. Phycol. 17, 374–384.

Singh, A., Nigam, P.S., Murphy, J.D., 2011. Mechanism and challenges in commercialisation of algal biofuels. Bioresour. Technol. 102, 26–34.

Smith, V.H., 2003. Eutrophication of freshwater and coastal marine ecosystems a global problem. Environ. Sci. Pollut. Res. 10, 126–139.

Steinmüller, K., Zetsche, K., 1984. Photo- and Metabolite Regulation of the Synthesis of Ribulose Bisphosphate Carboxylase/Oxygenase and the Phycobiliproteins in the Alga *Cyanidium caldarium*. Plant Physiol. 76, 935–939.

Stirk, W.A., Novák, O., Strnad, M., Staden, J. van, 2003. Cytokinins in macroalgae. Plant Growth Regul. 41, 13–24.

Talbot, P., Gortares, M.P., Lencki, R.W., de la Noüe, J., 1991. Absorption of CO2 in algal mass culture systems: A different characterization approach. Biotechnol. Bioeng. 37, 834–842.

Thompson, P., 1999. The Response of Growth and Biochemical Composition to Variations in Daylength, Temperature, and Irradiance in the Marine Diatom *Thalassiosira Pseudonana* (bacillariophyceae). J. Phycol. 35, 1215–1223.

Thompson, P.A., Guo, M., Harrison, P.J., 1992. Effects of Variation in Temperature. I. on the Biochemical Composition of Eight Species of Marine Phytoplankton1. J. Phycol. 28, 481–488.

Tischner, R., 1984. Evidence for the participation of NADP-Glutamate dehydrogenase in the ammonium assimilation of *Chlorella sorokiniana*. Plant Sci. Lett. 34, 73–80.

Tredici, M.R. (2004) Mass production of microalgae: photo- bioreactors. In: *Handbook of Microalgal Cultures, Biotech- nology and Applied Phycology* (ed. A. Richmond), pp. 178– 214. Blackwell, Oxford.

Tredici, M.R., 2010. Photobiology of microalgae mass cultures: understanding the tools for the next green revolution. Biofuels 1, 143–162.

Tredici, M.R., Margheri, M.C., Zittelli, G.C., Biagiolini, S., Capolino, E., Natali, M., 1992. Nitrogen and phosphorus reclamation from municipal wastewater through an artificial food-chain system. Bioresour. Technol. 42, 247–253.

Tsavalos, A.J., Day, J.G., 1994. Development of media for the mixotrophic/heterotrophic culture of *Brachiomonas submarina*. J. Appl. Phycol. 6, 431–433.

Turner, K.J., Bascomb, N.F., Lynch, J.J., Molin, W.T., Thurston, C.F., Schmidt, R.R., 1981. Evidence for messenger ribonucleic acid of an ammonium-inducible glutamate dehydrogenase and synthesis, covalent modification, and degradation of enzyme subunits in uninduced *Chlorella sorokiniana* cells. J. Bacteriol. 146, 578–589.

Vanoni, M.A., Curti, B., 2008. Structure–function studies of glutamate synthases: A class of self-regulated iron-sulfur flavoenzymes essential for nitrogen assimilation. IUBMB Life 60, 287–300.

Wang, Z.T., Ullrich, N., Joo, S., Waffenschmidt, S., Goodenough, U., 2009. Algal Lipid Bodies: Stress Induction, Purification, and Biochemical Characterization in Wild-Type and Starchless *Chlamydomonas reinhardtii*. Eukaryot. Cell 8, 1856–1868.

Went, F.W., 1935. Auxin, the plant growth-hormone. Bot. Rev. 1, 162–182.

Wijffels, R.H., 2008. Potential of sponges and microalgae for marine biotechnology. Trends Biotechnol. 26, 26–31.

Wijffels, R.H., Barbosa, M.J., Eppink, M.H.M., 2010. Microalgae for the production of bulk chemicals and biofuels. Biofuels Bioprod. Biorefining 4, 287–295.

Aim

The aims of this thesis are focused to study the physiological and biochemical effects of different environmental factors, mainly macronutrients, lights and temperature on microalgae. Microalgal species have been selected on the basis of their potential in biotechnologies and nitrogen occurs in all chapters, due to its importance in physiological and applicative fields.

A physiological point of view is necessary to provide and optimize already existing biotechnologies and applications with microalgae.

The main goals are: (i) to measure the kinetic parameters and the nutrient removal efficiencies for a selected and local strain of microalgae; (ii) to study the biochemical pathways of the microalga *D. communis* in presence of nitrate and ammonium; (iii) to improve the growth and the removal efficiency of a specific green microalga in mixotrophic conditions; (iv) to optimize the productivity of some microalgae with low growth-rate conditions through phytohormones and other biostimulants; and (v) to apply the phyto-removal of ammonium in an effluent from anaerobic digestion.

This thesis is based on 5 chapters, and all of them are ready or in preparation to be submitted. Technical and scientific supports have been provided by Professor Rossella Pistocchi of the Algal Biology and Physiology Lab at the University of Bologna, and Professor John Beardall of the Beardall Research Group at the Monash University.

Chapter 1

Batch kinetic parameters of nitrogen and scaling up of a *Scenedesmus* species

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Introduction

The concentration of nitrogen and phosphorus in growth media has a direct influence on algal growth kinetics, which relates closely to their capacity for nutrient removal and lipid accumulation. Nitrate and ammonium are the major N sources supporting phytoplankton growth. Inorganic N assimilation is a key and apparently simple process in mineral nutrition. For nitrate utilization, two reduction steps, separated into the cytosol ($NO_3^- \rightarrow NO_2^-$) and the chloroplast ($NO_2^- \rightarrow NH_4^+$), are necessary, while ammonium does not require any transformation as it is the N form incorporated into carbon skeletons by the glutamine synthetase/glutamate synthase pathway (Crawford, 1995; Crawford and Arst, 1993).

Since uptake through transporters is the main mechanism for nutrient removal from water by microalgae, the microalgal population growth rate directly affects the nutrient removal rate. In addition, nitrogen and phosphorus can be simultaneously utilized and removed efficiently only if the N/P ratio in the medium is in an appropriate range. The growth kinetics of microalgae also influences the biochemical composition of cells: for instance the microalgal population growth rate affects lipid accumulation rate, and the nutrient conditions determine the lipid productivity and the lipid content per unit microalgal biomass. Normally microalgae contain four main kinds of organic substance required for growth: proteins, carbohydrates, nucleic acids and lipids. Under environmental pressure the cessation of microalgal cell division is observed and the synthesis of CO_2 is switched to lipid or starch (depending on species) as energy storage compounds thus the lipid/starch content per microalgal biomass is increased (Xin et al., 2010).

Wastewater rich in nutrients (but not in heavy metals or toxic substances) are the product of agroindustrial and domestic sewage (González and A, 1997; Lazarovits et al., 2001) and are a major threat to the environment because sewage is produced in vast quantities. Wastewater usually contains an excess of nutrients (nitrogen and phosphorus) after secondary treatment of organic matter (de la Noue and de Pauw, 1988). Although these nutrients are essential for the growth of plants and microalgae, when in excess, as in this case, they represent the main source of eutrophication in natural aquatic ecosystems. Thus, removal of nutrients is a priority for any reuse of the discharged waters and for avoiding environmental contamination. The bio-treatment of wastewater using microalgae cultures has been well documented (de la Noue and de Pauw, 1988; González and A, 1997).

The principles of microalgae cultivation in shallow open ponds, or engineered raceways, and in closed PBRs were put in place by the 1950s. These systems have been refined in the intervening decades involving cross-disciplinary research and technological development encompassing biology, process engineering, mathematics and physics. The

potential of culturing microalgae for the purposes of effluent bioremediation and biofuel production has been reviewed by Chisti (2007). However, existing commercial applications for microalgae remain limited to relatively low-volume/high-value markets for speciality food and feed ingredients (Spolaore et al., 2006), whether as whole cell preparations (e.g. *Arthrospira* sp., *Chlorella* sp.) or extracts such as β-carotene and astaxanthin.

One microalgal genus widely used for nitrogen and phosphorus removal is the green microalga *Scenedesmus.* In order to improve its use for this purpose it is important to know the effect of different nitrogen compounds on growth and nutrient removal because wastewaters may contain different forms of nitrogen. Moreover nitrogen and phosphorus removal efficiencies vary depending on the media composition and environmental conditions such as initial nutrient concentration, light intensity, nitrogen/phosphorus ratio, light/dark cycle or algae species (Aslan and Kapdan, 2006). It has been already observed that ammonium is more easily absorbed by algae than nitrate (Hyenstrand et al., 2000), but the influence of different nitrogen sources on cultivation of members of the Scenedesmaceae and their nutrient removal efficiency has been seldom reported. For these reasons the selection and the characterization of a strain of Sphaeropleales has been conducted. Specifically, at first the removal rate for ammonium or nitrate was measured, then the capacity of the species *Desmodesmus communis* to grow on the above mentioned nitrogen sources with CO_2 supply was investigated and, finally, a scaling up of the culture system with biomass characterization has been set up.

Materials and method
Microalgae isolation and identification
Three strains were obtained by collecting samples from different geographic and morphological water bodies and periods: 1) artificial fresh water pond in the province of Forlì-Cesena (Emilia Romagna, Italy) in February 2009 (strain DC); 2) wastewater collected from the tertiary treatment sedimentation pond of the wastewater reclamation facility (WRF) of Ravenna (Italy) in July 2011 (strain S11); 3) water sample collected from an artificial well 5 m deep in the province of Ravenna, in June 2012 (strain S12). Each strain was isolated using a capillary pipette method (Hoshaw and Rosowski, 1973). Identification at family level was carried out through morphological methods for each strain, using information in Standard Methods and other identification materials. Molecular analyses to identify the species of *Desmodesmus communis* was performed only for the DC strain (Samorì et al., 2013).

Culture and experimental conditions
The research presented in this study is a compilation of five experiments:

- A preliminary screening to determine which strain belonging to the order Sphaeropleales presented the fastest growth rate. Each strain was cultivated in 500 mL flasks with 400ml of medium with nitrate and ammonium as nitrogen source and monitored daily through dry weight and cell count (Tab. 1)

- An experiment to measure the maximum uptake rate and the half saturation concentrations for nitrate and ammonium. Five different concentrations of ammonium or nitrate were added to *D. communis* culture in nitrogen starvation, for a pre-determined time of incubation, and nutrient concentrations in the filtered medium was determined after 10 minutes for ammonium and 30 minutes for nitrate. No biomass variance was detected over these short periods

- A growth experiment with ammonium or nitrate without pH control. 28 mg L⁻

^{1} of nitrogen as ammonium or nitrate were added to the medium, 1L of *D. communis* was grown in 1L bottles and aerated continuously with filtered (0.22 μm) air bubbling from the bottom of the bottles with an aeration rate of 142 mL min^{-1}. Dry weight, photosynthetic efficiency and inorganic nutrient removal were monitored.

- Same conditions of the above-mentioned experiment were applied, but a stable pH value of 7.5 was maintained. KOH and HCl 1N were used to adjust the medium pH daily. Daily monitoring was conducted to determine dry weight, nutrient uptake, while cellular metabolites and elemental composition were performed at the end of the exponential phase.

- Scaling-up of the system through 70 L indoor vertical photo-bioreactors in which *D. communis* was cultivated in fed-batch mode with ammonium as nitrogen source. During the cultivation, nutrient concentration was monitored and, when expired, their resupply was done in order to reinstate the CHU 13 original concentrations.

All inocula and all experiments were performed in CHU 13 medium (Largeau et al., 1980) with KNO$_3$ or NH$_4$Cl as nitrogen source, and at pH 7.5. The batch cultures were maintained at ambient temperature of 20-25 °C, with a photoperiod of 12:12 light-dark cycle and a light intensity of 100-110 μE m^{-2} s^{-1}. All cultures were mixed with a magnetic stirrer and aerated with a mixture of air/CO$_2$ 98/2 v/v.

Each treatment was performed in triplicate, and the parameters measured were given as the mean with respective standard deviations for each set of triplicates shown in the figures. The cultures in the PBRs were kept in the same conditions as described above, except for the light intensity which was set at around 300 μmol photons m^{-2} s^{-1}, in order to supply the appropriate light intensity, considering the PBR type, and were performed in duplicate.

Cell growth measurement

The biomass concentration was measured as dry cell weight (g l^{-1}). The samples were filtered onto pre-weighed fiber glass membranes of 1.2 μm pore size (Whatman GF/C) and the filter was then dried at 105°C in a oven for 1 hour or until a constant value was measured. After cooling down of the membrane, the final dry weight was measured. The culture growth was also evaluated through cell counting using an Optical Microscope (Axiovert S 100) at 32x following the Uthermöhl protocol (Hasle et al., 1978).

Photosynthetic efficiency

A pulse-amplitude modulated fluorometer (101-PAM connected to a PDA-100 data acquisition system. H. Walz, Effeltrich, Germany) was used to measure the effective quantum yield of the PSII, as an indirect measure of photosynthetic efficiency. 3 mL samples were dark adapted for 20 min in order to the electron transport chain and determine maximal quantum efficiency Fv/Fm. The culture was exposed to a continuous actinic light, with the same intensity used for growth, for 5 minutes. This time was previously stabilized. The effective quantum yield Φ'_{PSII} was obtained from Genty et al., (1989):

$$\Phi'_{PSII} = \frac{F'_m - F}{F'_m} = \frac{\Delta F}{F'_m}$$

The photochemical quenching (qP) and the non-photochemical quenching (NPQ), were calculated as follow (Schreiber et al., 1986):

$$qP = \frac{F'_m - F}{F'_m - F'_0}$$

$$NPQ = \frac{F_m - F'_m}{F_m - F'_0}$$

All parameters were obtained through induction curve measurements. After dark adaptation, a short saturating pulse of 3000 μmol m^{-1} s^{-1} for 0.8 s induced the maximal fluorescence yield (F$_m$). The change of the fluorescence yield (F) was induced by administering the actinic light. Simultaneously the change in the maximal fluorescence yield (F'$_m$) was induced by saturating pulses given periodically every 60 s. F'$_0$ was measured at the end, when the actinic light was turned off and a far-red light was applied.

Determination of macro-nutrients
Inorganic nutrient uptake was measured in the pre-filtered medium by using Whatman GF/C glass fiber filters first and then in samples collected along the growth curve. 1 mL sample was filtered with a 0.22 μm syringe filter and injected into an Ion chromatograph (883 Basic IC plus, Metrohm) with an anionic column (Metrosep A Supp 4 – 250 / 4) to measure nitrate and phosphate, and into a cationic column (Metrosep C 4 – 150 / 4.0) to measure ammonium.

Cellular constituents
Lipids, polysaccharides and proteins were determined by collecting the cultures at the end of the exponential phase. Total lipids were extracted from 50 mg of lyophilized algal pellet with 15 mL of 2:1 v/v chloroform-methanol in a Soxhlet extractor apparatus at 80°C. Every 2 hours the supernatant was filtered through Whatman GF/F filters and 15 mL of solvent were added to the residue. This procedure was repeated twice. After complete solvent evaporation, total lipids were measured gravimetrically (Bligh and Dyer, 1959).
For cell protein concentration determination, the pellet from 10 mL of culture was suspended in NaOH 0.1 N and sonicated for 30 s x 3 cycles (Samorì et al., 2013). The quantitative determination of protein was then performed with the Folin Phenol reagent as described in Lowry et al. (1951).

Total polysaccharides were extracted by adding 30 mL of absolute ethanol to 15 mL of culture and incubated at -20°C for 24 h according to Myklestad et al. (1972). The analysis was performed on the pellet, obtained by centrifuging the solution at 5000 rpm for 20 min at 4° C, and resuspending it in 6 mL distilled water, by the Phenol Sulfuric Method (DuBois et al., 1956),

Cell elemental composition
Carbon and nitrogen cell contents were determined using 2-4 mg of lyophilized biomass. The analysis was conducted using a ThermoFisher organic elemental analyser (Flash 2000) with a copper/copper oxide column. C/N, C/P and N/P ratio were calculated in terms of atoms.
The phosphorus cell content was determined after extraction performed following Ge et al., (2011): 10 mg of lyophilized biomass was digested with potassium persulfate in an autoclave at 121°C for 30 min. The total P was than determined by using the Ascorbic Acid Method and a UV spectrophotometer (7800, Jasco) at 885 nm (APHA Method 4500 E, 1995).

Calculation
Biomass productivity (g L^{-1}d^{-1}) was calculated in the batch cultures following the equation:

$$P = \frac{B_t - B_0}{t_t - t_0}$$

where B$_t$ and B$_0$ are the biomass dry weight (g L^{-1}) on selected days t$_t$ and t$_0$.
The growth rate μ (d^{-1}) was calculated using cell counts, with the following equation:

$$\mu = \frac{\ln(X_t / X_0)}{t_t - t_0}$$

where X$_t$ and X$_0$ are the cell number on days t$_t$ and t$_0$ respectively.

Table 1 Cell and growth characteristics of three different strains belonging to the family Scenedesmaceae

strain	B_{MAX} (g/l)	# cell $_{MAX}$ (cell/ml)	Biovol. (μm^3)	μ (d^{-1})
DC	0.38 ± 0.02	$2.4 \cdot 10^6 \pm 1.4 \cdot 10^5$	256 ± 87	0.231 ± 0.016
S11	0.31 ± 0.01	$1.3 \cdot 10^6 \pm 1.0 \cdot 10^5$	434 ± 10	0.200 ± 0.024
S12	0.13 ± 0.01	$5.0 \cdot 10^6 \pm 1.9 \cdot 10^5$	91 ± 45	0.198 ± 0.005

The cell volume V was calculated with the assumption of prolate spheroid shape using the following equations:

$$V = \frac{\pi}{6} b^2 a$$

where a is the length and b is the width of the cell.

Values of half-saturation constant K_s and maximal velocity V_{max} for nutrient uptake were calculated following the protocol suggested by Flynn et al. (1997) and the Microsoft Office Excel's supplement indicated by Bezerra et al. (2013):

$$V_t = \frac{V_{max}[S]}{K_m + [S]}$$

where V_t is the rate at time of incubation t, [S] is the substrate added at different concentrations.

The nutrient ($N-NO_3^-$ or $N-NH_4^+$ and $P-PO_4^{2+}$) uptake (U) was calculated according to Lim et al. (2006). Nitrate (μmol $N-NO_3^-$ g biomass^{-1} d^{-1}), ammonium (μmol $N-NH_4^+$ g biomass^{-1} d^{-1}) and phosphate (μmol $P-PO_4^{2-}$ g biomass^{-1} d^{-1}) uptake rates were calculated from the residual nutrient concentration in the medium (C) and the change in biomass densities (γ) over a period of time ($\Delta t = t_1 - t_0$), when the depletion of nutrients was linear. Δt therefore differs depending on nutrient sources and algal conditions.

$$U = -\frac{C_t - C_0}{\gamma \Delta t}$$

$$\gamma = \frac{B_t - B_0}{\ln B_t - \ln B_0}$$

where B_t and B_0 are the biomass of *D. communis* at time t and 0 respectively.

Statistical analysis

Statistical analyses were performed with PAST 2.17 software. Levene and Tukey tests were performed to verify the homogeneity of the variance and to do pairwise comparisons respectively. All the data are reported as means ± standard deviations of triplicates.

Results and discussion

Sphaeropleales screening

Table 1 shows the characteristics, in terms of cell volume and growth, of three different strains belonging to the order Sphaeropleales. DC was the strain reaching the highest biomass value of 0.38 g L^{-1}, in 15 days. S11 showed a slightly lower value of 0.31 g L^{-1}, while S12 was characterized by a much lower value of 0.13 g L^{-1}. On the other hand, S12 at day 15 reached a final number of cells of $5.0 \cdot 10^6$ cell mL^{-1} which was significantly ($p < 0.005$) higher than those reached by the others two strains; the finding that this strain was characterized by a smaller cell volume, could explain the lower value of maximum biomass reached. The other two strains had a cell volume in the order S11>DC. These results showed that *Desmodesmus communis* possessed the best characteristics for biomass production, which were very similar to what already observed by Samorì et al. (2013) and it was therefore selected for the following experiments.

Nutrient uptake kinetics

The kinetics parameters for NH_4^+-N and NO_3—N removal capacity by *D. communis* were determined by incubating the algae with increasing concentration of nitrate or ammonium. The range of concentrations was chosen through preliminary assays in order that very low reaction rates as well as saturating rates were measured.

Fig. 1 shows the plots of the uptake rate versus $[NO_3^-]$ and $[NH_4^+]$ that allowed measurement of two important kinetic parameters: V_{max} and K_s for ammonium and nitrate transport. The maximum uptake rate, $V_{max,}$ for nitrate was lower compared with that for ammonium: *D. communis* supplied with ammonium was shown to transport up to 98.0 ± 13.1 µmol of nitrogen per gram (g) of biomass per litre
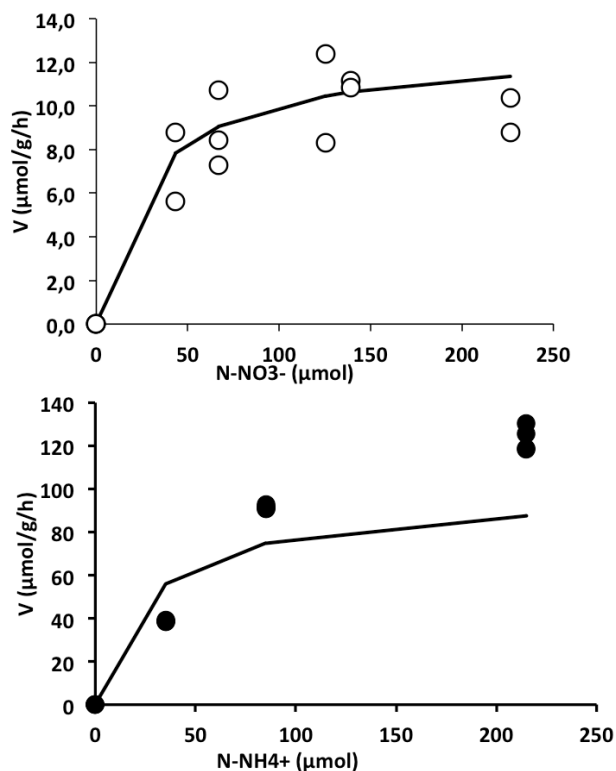
Figure 1 Uptake rates for nitrate (white spots) and ammonium (black spots) in *D. communis*. The black lines are the calculated values, while the spots are the experimental measures.

versus 12.7 ± 0.2 µmol g^{-1}L^{-1} of nitrate. This high rate has already been observed by Aslan and Kapdan (2006), but with an estimation of

the above mentioned parameters during the growth curve. The constant K_s, showed low values both for NH_4^+-N and NO_3—N, of 27.0 and 21.6 µM, respectively. As the K_s is inversely related to the apparent affinity of the transporter for its substrate, a low numerical value attests that the interaction between the transporter and its substrate in *D. communis* occurs with very high affinity (Bezerra et al., 2013). Following the above described protocol, it was shown that it is easier to avoid typical errors commonly encountered in the attempts to determine the V_{max} and the K_s, as described in Flynn et al., (1997). It is though true that kinetic values depend on the starvation state of the cell, the level of free amino-acids and the N:P ratio inside the cell (RHEE, 1978).

Desmodesmus communis *batch cultures with variable pH*

To determine the optimal nitrogen source for cell growth, 28 mg L^{-1} of nitrate (N) or ammonium (A) were added to the artificial medium supplied with 2% CO_2. As shown in Fig. 2*a,* ammonium chloride can support the growth of *D. communis* to a lesser extent than does potassium nitrate. In the latter condition the biomass was higher and approximately 0.76 g L^{-1} at the end of the exponential phase. Higher biomass values in *Scenedesmus* species grown with nitrate compared with ammonium were already observed by Xin et al., (2010), Lin and Lin, (2011) and Arumugam et al., (2013) and, in different species, by Li et al. (2008) and Wu et al. (2013).

Despite these results we could observe that the effective photosynthetic yield (Φ'_{PSII}) of PSII remained constant at optimal values only in the ammonium treatment, while in the microalgae grown with nitrate the yield started to slightly decrease from day 9 (Fig. 2b). The higher photosynthetic yield of microalgae in presence of ammonium can be due to the fact that cell grown with NH_4^+ have a much greater content of the chloroplast enzyme ribulose diphosphate carboxylase and a correspondingly greater capacity for photosynthetic O_2 evolution (Paasche, 1971).
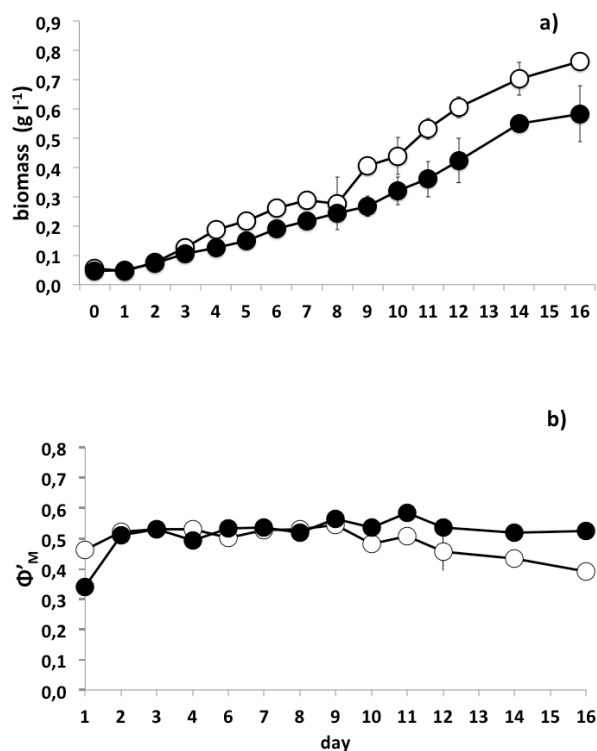
Figure 2 Growth curves (a) and effective quantum yield (b) of *D. communis* grown under different nitrogen conditions: nitrate (white spots) and ammonium (black spots) with 2% CO_2 supply without pH control. (Data points are means +/- standard deviation, n=3)

As Φ'_{PSII} values provide an indication of the amount of energy used in photochemistry and is related to the moles of carbon fixed through photosynthesis per mole of photons, low Φ'_{PSII} values can reflect the higher energy costs involved in the uptake of nitrate and carbon than that are necessary in cells utilizing ammonium (Sakshaug et al., 1997).

Nitrogen and phosphorus consumption over time in batch cultures of *D. communis* with CO_2 supply and variable pH is presented in Fig. 3. Generally 28 mg L^{-1} nitrogen was completely removed from the media in 8 days (Fig 3a).

However the N source that first disappeared was ammonium, which was removed in only 5 days, although its decrease could be influenced also by the stripping caused by the lower pH and not only by the cell uptake (Martínez et al., 2000). On the other hand, the trend for P removal (Fig. 3b) was completely different in the two conditions as the added amount of 9 mg L^{-1} was not

completely taken up in 14 days and a faster depletion occurred in cultures grown with nitrate compared with those with ammonium, following the growth trend. A lower P removal efficiency in the presence of ammonium was observed also by Xin et al. (2010), who observed after 13 days of cultivation of *Scenedesmus* sp. LX1, with nitrate, ammonium or urea as nitrogen sources, that phosphorus removal efficiencies were >99%, 76.4% and >99%, respectively and, for the cultures with ammonium, the low percentage was related to the low algal density in the stationary phase.
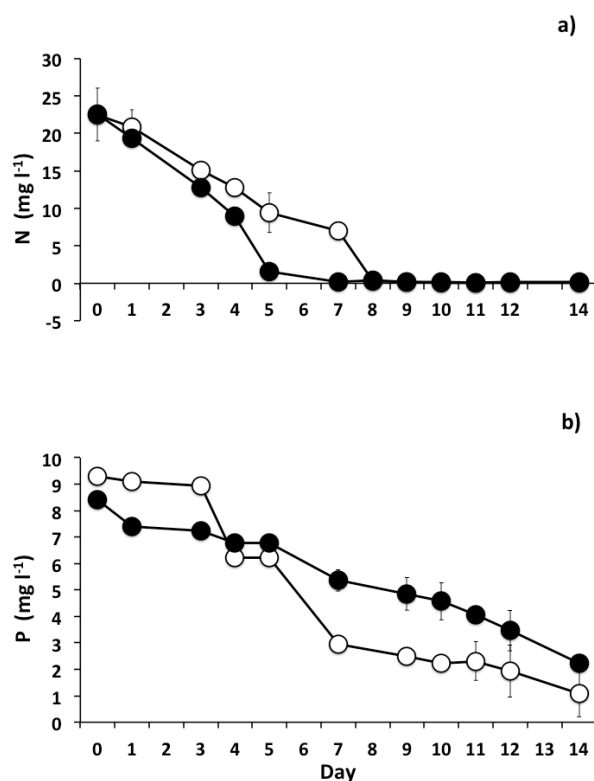
Figure 3 Nitrogen and phosphate consumption from the medium by *D. communis* grown in nitrate (white spots) or ammonium (black spots) as nitrogen source and with 2% CO_2 supply, without pH control. . (Data points are means +/- standard deviation, n=3)

Desmodesmus communis *batch cultures with pH control*

The ammonia and nitrate removal rate can affect *D. communis* growth and can determine changes in the pH of the medium, affecting metabolic pathways, as was already demonstrated by Martínez et al. (2000) and Xin et al. (2010). For this reason measures for pH

stabilization were applied in the following batch experiments, having the same environmental conditions of those above described. Fig. 4 and 5 show *D. communis* growth, in terms of biomass, the photosynthetic efficiency and the external nutrient concentration variations. Potassium nitrate and ammonium chloride, as N sources, resulted in similar trends of growth (Fig. 4a), and the cultures reached a biomass of around 1 g L^{-1} in 18 days with the same productivity value of 0.07 g $L^{-1}d^{-1}$.
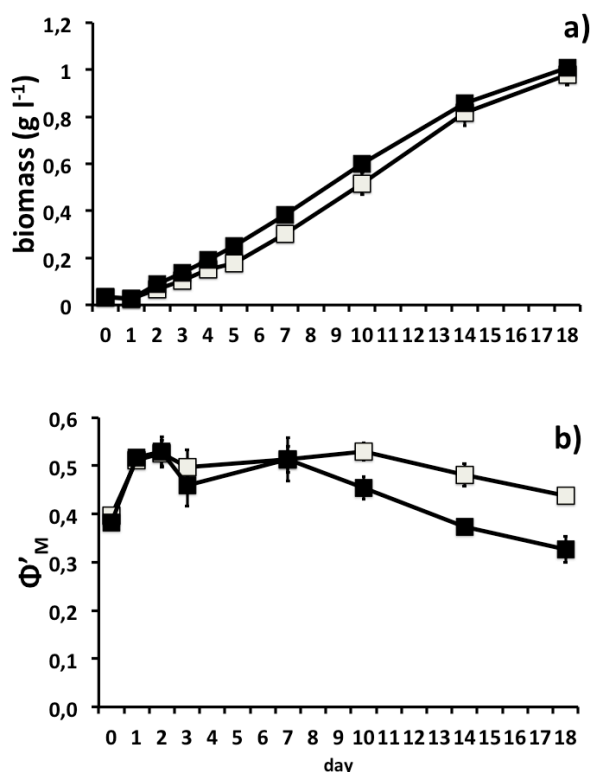

Figure 4 Growth curve of *D. communis* grown under different nitrogen source, nitrate (white squares) and ammonium (black squares) with 2% CO_2 supply at constant pH. .(Data points are means +/- standard deviation, n=3)

Even though the two different nutrient conditions induced the same growth responses in *D. communis*, differences in Φ'_{PSII} values were observed. Starting from day 7, in the treatment with ammonium the effective quantum yield showed a decreasing trend (Fig. 4b), probably due to the higher carbon fixation induced by a higher nitrogen uptake rate that still remained in the ammonium conditions. It is well known that the operational photosynthetic yield can be influenced by many factors such as nitrogen

uptake, carbon fixation, loss of functional reaction centres, cyclic electron flow and the packaging of pigments inside the cell (Falkowski and Kolber, 1995; Vassiliev et al., 1995; Sakshaug et al., 1997). However the nitrogen removal (Fig. 5b) trend didn't change, and the uptake rate for nitrate was higher than for ammonium, being 67.3 μmol $g^{-1}h^{-1}$ and 15.1 μmol $g^{-1}h^{-1}$, respectively. In this experiment at constant pH it is clear that ammonium removal is not influenced by medium acidification, and that *D. communis* has good NH_4^+ removal capacity.
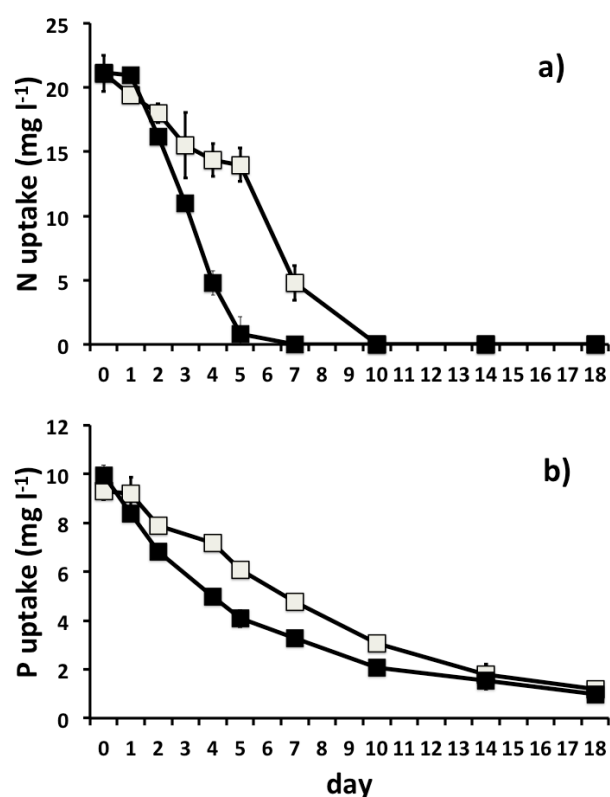

Figure 5 Nutrient consumption from the medium of *D. communis* grown with nitrate (white squares) or ammonium (black squares) with 2% CO_2 supply and constant pH . .(Data points are means +/- standard deviation, n=3)

Fig. 5b shows that the two conditions displayed the same trend, in terms of P removal, observed in the previous experiment as phosphorus was depleted faster in the treatment with nitrate. This result could probably be due to the smaller volume of cells in nitrate conditions (data not shown) so that a bigger surface/volume ratio was available for P absorption as already observed by several

authors (Boyd and Musig, 1981; Khummongkol et al., 1982; Okada et al., 1982). The biochemical composition was determined at the beginning of the stationary growth phase (day 18), with the culture medium practically free of N and P (Fig. 6 and Tab. 2).
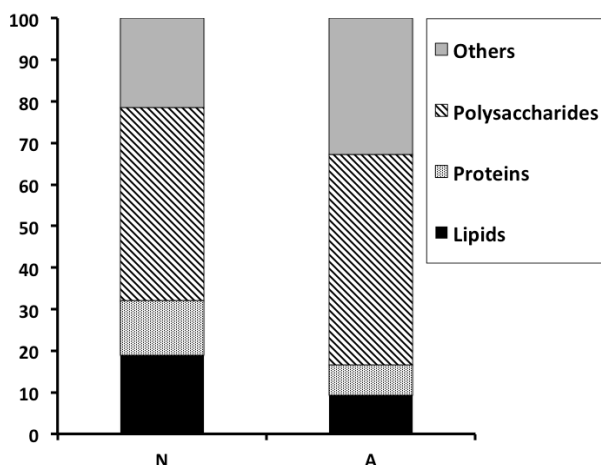
Figure 6 Cell composition obtained at the beginning of the stationary phase in *D. communis* in the experiment with stable pH. N = treatment with nitrate, A = treatment with ammonium

Nitrate treated cells showed significantly higher values of lipids, while there were no significant differences ($p > 0.005$) in polysaccharide and protein contents; similarly no differences were detected in terms of elemental composition.

**Table 2 Elemental composition in *D. communis* grown with different nitrogen sources, 2% of CO_2 and constant pH**

condition	C	N	P
N	46.0 ± 0.2	6.7 ± 0.1	1.0 ± 0.1
A	45.3 ± 1.3	5.7 ± 0.2	1.1 ± 0.0

The similar amounts can be probably attributable to the time of sampling, with all N and P assimilated, and to the stable pH. In this experiment lower protein and carbohydrate contents have been found compared with data obtained in the same algae grown in real wastewater by Samorì et al. (2013). Conversely the internal C, N and P composition did not differ substantially from the elemental formula

for biomass proposed by Harrison (1967): $CH_{1:64} N_{0:16} O_{0:52} S_{0:0046} P_{0:0054}$.

Fed-batch cultures in PBR
D. communis growth in fed-batch culture with ammonium as nitrogen source, CO_2 supply and stable pH was followed for 55 days. In Fig. 7 the growth curve is reported: a lower biomass of 1 g L^{-1} was obtained in 45 days, compared with 2.5 g L^{-1} dry weight obtained growing *D. communis* in the same conditions but with nitrate as nitrogen source (Pezzolesi et al., unpublished). A lower productivity, of 0.02 g L^{-1}d^{-1}, and higher pH fluctuations (Fig. 8) were obtained during the cultivation compared to the daily pH adjustment performed in the previous batch experiment.
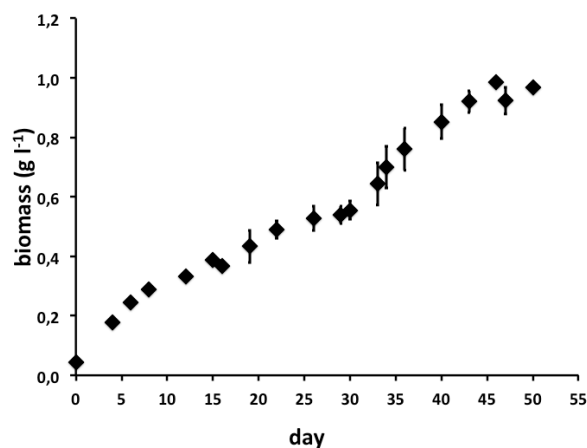

Figure 7 Growth curve of *D. communis* in PBRs as fed-batch cultures supplied with ammonium as nitrogen source. (Data points are means +/- standard deviation, n=2)
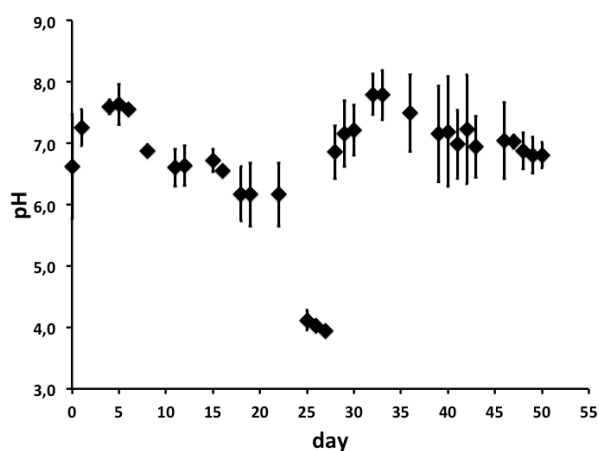
Figure 8 pH in *D. communis* fed-batch cultures in PBRs, measured daily with a fixed pH electrode . (Data points are means +/- standard deviation, n=2)

The photosynthetic parameters of these cultures are shown in Fig. 9. The Effective quantum yield (Fig. 9a) was 0.2 and 0.4 mole C (mole photon)$^{-1}$ for the first 25 days, probably do to the low density of the culture and the adaptation phase, while it increased to a range of 0.4 – 0.5 for the last growth period in which the biomass was still growing and the dry weight increased up to 0.5 g L^{-1}.
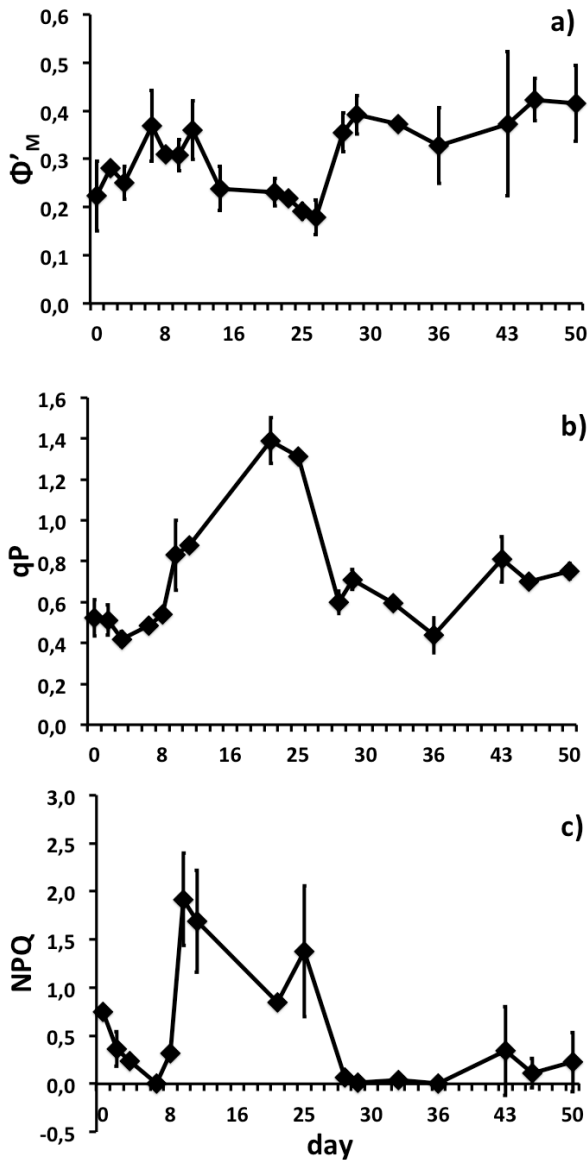
Figure 9 Effective quantum yield (a), photochemical quenching (b), and non photochemical quanching (c) in *D. communis* grown in semi-continuous cultures in PBRs, with ammonium as nitrogen source. (Data points are means +/- standard deviation, n=2)

The photochemical quenching (Fig. 9b) was high, between 0.8 and 1.2, when *D. communis* was able to use a high proportion of

light excitation energy for photochemical processes. All quenching processes, not related to photochemistry (Fig. 9c), occurred only between day 8 and 25 when a reduction of the effective photosynthetic yield was observed. This was probably due to the high light intensity which determined the necessity for the cells to discharge the excessive light (Juneau et al., 2002) until they reached high concentrations.
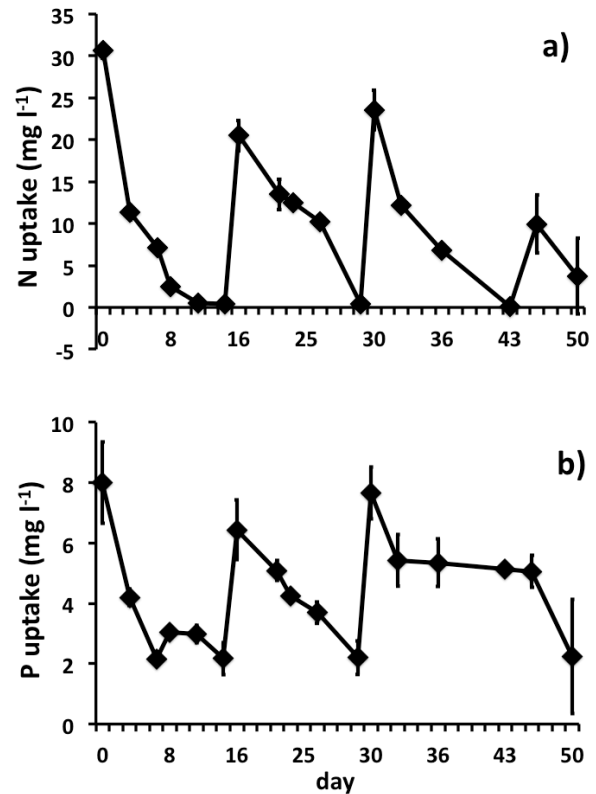
Figure 10 Ammonium (a) and Phosphate (b) consumption by *D. communis* in semi-continuous cultures ina PBR. . (Data points are means +/- standard deviation, n=2)

Nutrient concentrations in the medium are reported in Fig. 10. Nitrogen was depleted every 15 days while phosphorus concentration did not decrease below 2 mg L^{-1} in the same period. The results of this study in terms of NH$_4^+$-N removal is consistent with other studies. For instance, ammonium was completely removed in 10 days by *C. vulgaris* from an initial concentration of 21.2 mg L^{-1} (Aslan and Kapdan, 2006), and 97% removal in 8 days was achieved by *Scenedesmus obliquus*

for a starting nitrogen concentration of 27.4 mg L^{-1} (Martínez et al., 2000).

Table 3 Cell and elemental composition of *D. communis* grown in a PBR with ammonium, measured at the end of the exponential phase, day 50. Each value is in percentage w/w, and it is the mean of two replicates ± standard deviation.

Content	% w/w
Polysaccharides	56.3 ± 0.8
TFA	12.7 ± 0.6
Proteins	18.9 ±1.6
C	47.2 ± 1.6
N	8.9 ± 0.6
P	0.9 ±0.1

Finally, the cellular composition for *D. communis* grown with ammonium at the end of exponential phase is reported in Table 3. A high percentage of polysaccharids, with low percentages of proteins and total fatty acids were obtained. A similar biomass composition was observed by Samorì et al. (2014) in *D. communis* grown in a semi-continuous system with a high ammonium level. The cell C percentage did not show any differences from those measured in the previous experiments, probably due to the constant CO_2 supply; the increment in nitrogen content was probably due to constant nutrient concentrations.

Conclusions

The kind of nitrogen source could significantly affect the growth, the nutrient removal and the photosynthetic characteristics of *D. communis*. Faster nitrogen removal rate has been determined for *D. communis* grown with ammonium compared with cultures grown with nitrate, in all the experiments at small volume scale and in photobioreactors. It is worth noticing that the measured K_s and V_{max} perfectly described the nutrient uptake characteristics of the selected strain, showing that this could be an easy way to select the right species for the desired application.

D. communis grew faster with nitrate despite the fact that the ammonium uptake was higher. By keeping the pH stable, the same productivity level was obtained with either ammonium or nitrate; on the other hand N-NH_4^+ uptake still occurred at a faster rate than that of N-NO_3^-. This aspect was more evident in the photo-bioreactor culture, with stable pH and ammonium as nitrogen source. In conclusion it is clear how *D. communis* can be an ideal strain for wastewater treatment with ammonium however, in order to optimize the biomass productivity, it is necessary to examine in depth the nitrogen assimilation and the carbon allocation of this genus in presence of different nitrogen sources.

References

APHA (American Public Health Association), 1995. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington, DC.

Arumugam, M., Agarwal, A., Arya, M.C., Ahmed, Z., 2013. Influence of nitrogen sources on biomass productivity of microalgae *Scenedesmus bijugatus*. Bioresour. Technol. 131, 246–249.

Aslan, S., Kapdan, I.K., 2006. Batch kinetics of nitrogen and phosphorus removal from synthetic wastewater by algae. Ecol. Eng. 28, 64–70.

Bezerra, R.M.F., Fraga, I., Dias, A.A., 2013. Utilization of integrated Michaelis–Menten equations for enzyme inhibition diagnosis and determination of kinetic constants using Solver supplement of Microsoft Office Excel. Comput. Methods Programs Biomed. 109, 26–31.

Bligh, E.G., Dyer, W.J., 1959. A Rapid Method of Total Lipid Extraction and Purification. Can. J. Biochem. Physiol. 37, 911–917.

Boyd, C.E., Musig, Y., 1981. Orthophosphate uptake by phytoplankton and sediment. Aquaculture 22, 165–173.

Chisti, Y., 2007. Biodiesel from microalgae. Biotechnol. Adv. 25, 294–306.

Crawford, N.M., 1995. Nitrate: nutrient and signal for plant growth. Plant Cell 7, 859–868.

Crawford, N.M., Arst, H.N., 1993. The Molecular Genetics of Nitrate Assimilation in Fungi and Plants. Annu. Rev. Genet. 27, 115–146.

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De la Noue, J., de Pauw, N., 1988. The potential of microalgal biotechnology: a review of production and uses of microalgae. Biotechnol. Adv. 6, 725–770.

DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric Method for Determination of Sugars and Related Substances. Anal. Chem. 28, 350–356.

Falkowski, P., Kolber, Z., 1995. Variations in Chlorophyll Fluorescence Yields in Phytoplankton in the World Oceans. Funct. Plant Biol. 22, 341–355.

Flynn, K.J., Fasham, M.J.R., Hipkin, C.R., 1997. Modelling the interactions between ammonium and nitrate uptake in marine phytoplankton. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 352, 1625–1645.

Genty, B., Briantais, J.-M., Baker, N.R., 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim. Biophys. Acta BBA - Gen. Subj. 990, 87–92.

Ge, Y., Liu, J., Tian, G., 2011. Growth characteristics of *Botryococcus braunii* 765 under high CO2 concentration in photobioreactor. Bioresour. Technol., Special Issue: Biofuels - II: Algal Biofuels and Microbial Fuel Cells 102, 130–134.

González, L.E., A, R.B., 1997. Efficiency of Ammonia and Phosphorus Removal from a Colombian Agroindustrial Wastewater by the Microalgae *Chlorella Vulgaris* and *Scenedesmus Dimorphus*.

Hasle, G. R. (1978) The inverted microscope method. In Sournia, A. (ed.), Phytoplankton Manual. Monographs on Oceanographic Methodology, Vol. 6. UNESCO, Paris.

Harrison, J. S., 1967. Process. Biochem. 2, 41.

Hoshaw, R.W., Rosowski, J.R., 1973. Methods for microscopic algae. In: Stein, J.R. (Ed.), Handbook of Phycological Methods. Cambridge University Press, New York, pp. 53e67.

Hyenstrand, P., Burkert, U., Pettersson, A., Blomqvist, P., 2000. Competition between the green alga *Scenedesmus* and the cyanobacterium *Synechococcus* under different modes of inorganic nitrogen supply. Hydrobiologia 435, 91–98.

Juneau, P., Berdey, A.E., Popovic, R., 2002. PAM Fluorometry in the Determination of the Sensitivity of *Chlorella vulgaris, Selenastrum capricornutum,* and *Chlamydomonas reinhardtii* to Copper. Arch. Environ. Contam. Toxicol. 42, 155–164.

Khummongkol, D., Canterford, G.S., Fryer, C., 1982.

Accumulation of heavy metals in unicellular algae. Biotechnol. Bioeng. 24, 2643–2660.

Largeau, C., Casadevall, E., Berkaloff, C., Dhamelincourt, P., 1980. Sites of accumulation and composition of hydrocarbons in *Botryococcus braunii.* Phytochemistry 19, 1043–1051.

Lazarovits, G., Conn, K., Tenutaemail, M., Soltani, N., 2001. Control of Plant Pathogenic Bacteria in Soil with Organic Amendments, in: Boer, S.H.D. (Ed.), Plant Pathogenic Bacteria. Springer Netherlands, pp. 291–295.

Lim, P.-T., Leaw, C.-P., Usup, G., Kobiyama, A., Koike, K., Ogata, T., 2006. Effects of Light and Temperature on Growth, Nitrate Uptake, and Toxin Production of Two Tropical Dinoflagellates: *Alexandrium tamiyavanichii* and *Alexandrium minutum* (dinophyceae)1. J. Phycol. 42, 786–799.

Lin, Q., Lin, J., 2011. Effects of nitrogen source and concentration on biomass and oil production of a *Scenedesmus rubescens* like microalga. Bioresour. Technol. 102, 1615–1621.

Li, Y., Horsman, M., Wang, B., Wu, N., Lan, C.Q., 2008. Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. Appl. Microbiol. Biotechnol. 81, 629–636.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Martínez, M.E., Sánchez, S., Jiménez, J.M., El Yousfi, F., Muñoz, L., 2000. Nitrogen and phosphorus removal from urban wastewater by the microalga *Scenedesmus obliquus*. Bioresour. Technol. 73, 263–272.

Myklestad, S., Haug, A., Larsen, B., 1972. Production of carbohydrates by the marine diatom *Chaetoceros affinis* var. willei (Gran) Hustedt. II. Preliminary investigation of the extracellular polysaccharide. J. Exp. Mar. Biol. Ecol. 9, 137–144.

Okada, M., Sudo, R., Aiba, S., 1982. Phosphorus uptake and growth of blue-green alga, *Microcystis aeruginosa*. Biotechnol. Bioeng. 24, 143–152.

Paasche, E., 1971. Effect of Ammonia and Nitrate on Growth, Photosynthesis, and Ribulosediphosphate Carboxylase Content of *Dunaliella tertiolecta*. Physiol. Plant. 25.

Rhee GY (1978) Effect of N:P atomic ratio and nitrate limitation on algal growth, cell composition

and nitrate uptake. Limnol Oceanogr 23:10–25

Sakshaug, E., Bricaud, A., Dandonneau, Y., Falkowski, P.G., Kiefer, D.A., Legendre, L., Morel, A., Parslow, J., Takahashi, M., 1997. Parameters of photosynthesis: definitions, theory and interpretation of results. J. Plankton Res. 19, 1637–1670.

Samorì, G., Samorì, C., Guerrini, F., Pistocchi, R., 2013. Growth and nitrogen removal capacity of *Desmodesmus communis* and of a natural microalgae consortium in a batch culture system in view of urban wastewater treatment: Part I. Water Res. 47, 791–801.

Samorì, G., Samorì, C., Pistocchi, R., 2014. Nutrient removal efficiency and physiological responses of *Desmodesmus communis* at different HRTs and nutrient stress condition using different sources of urban wastewater effluents. Appl. Biochem. Biotechnol. 173, 74–89.

Schreiber, U., Schliwa, U., Bilger, W., 1986. Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. Photosynth. Res. 10, 51–62.

Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A., 2006. Commercial applications of microalgae. J. Biosci. Bioeng. 101, 87–96.

Vassiliev, I.R., Kolber, Z., Wyman, K.D., Mauzerall, D., Shukla, V.K., Falkowski, P.G., 1995. Effects of Iron Limitation on Photosystem II Composition and Light Utilization in *Dunaliella tertiolecta*. Plant Physiol. 109, 963–972.

Wu, L.F., Chen, P.C., Lee, C.M., 2013. The effects of nitrogen sources and temperature on cell growth and lipid accumulation of microalgae. Int. Biodeterior. Biodegrad. 85, 506–510.

Xin, L., Hong-ying, H., Ke, G., Jia, Y., 2010. Growth and nutrient removal properties of a freshwater microalga *Scenedesmus* sp. LX1 under different kinds of nitrogen sources. Ecol. Eng. 36, 379–381.

Chapter 2

Effects of different nitrogen sources on C fixation and N assimilation in *Scenedesmus* sp.

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Abstract

Microalgal biomass is strongly dependent on the composition of the culture medium and, after carbon, nitrogen is quantitatively the most important element contributing to the amount and composition of dry matter. The aim of this study was to investigate the physiological responses of an Australian isolate of the freshwater algae *Scenedesmus* sp. grown with two different nitrogen sources, nitrate and ammonium, under four different conditions: (N) with 28 mg/l of $N-NO_3^-$, (A) with 28 mg/l of $N-NH_4^+$, (AA) with 28 mg/l of $N-NH_4^+$ and air bubbling as carbon source, and (DA) with 56 mg/l of $N-NH_4^+$. From previous studies it was well known that, even though ammonium represents the most energetically efficient nitrogen source, nitrate is the most commonly used source, permitting cells to attain the highest biomass. Our results confirmed that the highest growth of the cultures occurred under nitrate which reached twice the cell density at the end of exponential phase compared with those supplied with ammonium. The uptake of dissolved inorganic carbon (DIC) was also measured and a faster consumption of all the three sources (HCO_3^-, CO_3^{2-}, CO_2) of carbon was observed cultures with ammonium. Measurements of the intra- and extracellular pH were also performed in order to verify the effect of ammonium on the observed pH rise in the medium, due to the release of protons associated with ammonium assimilation. Results showed that both internal and external pH were lower in the culture with the highest growth, so that a change in pH value is unlikely to be the cause for the lower growth in the presence of ammonium. In relation to the supply of different nitrogen sources, at different concentrations and in the absence or presence of air as inorganic carbon source, our findings suggest that the algae grown under ammonium have a faster uptake of the nitrogen source, and consequently a faster depletion of cellular carbon skeletons.

Introduction

Nowadays microalgae are studied, and a number of species already mass-cultivated, for their application in many fields: food and feed, chemicals, pharmaceutical, phytoremediation and renewable energy. Phytoremediation, in particular, can become a valid integrated process in many algae biomass production systems. As a novel "green technology", microalgae have many advantages as tools in the removal of nitrogen and phosphorus from waste water, including the following (Aslan and Kapdan, 2006; Vergara-Fernández et al., 2008):1) low cost due to sufficient solar energy , 2) simultaneous fixation of CO_2, 3) lack of a requirement for extra organic carbon, 4) discharge of oxygenated effluents into water bodies, 5) avoidance of sludge handling problems, and 6) high economic potential of harvested algal biomass (for feedstock, fertilizers, biogas, biofuels and so on). *Scenedesmus*, *Arthrospira* and *Chlorella* are the most widely studied microalgae species for nitrogen and phosphorus removal (Hernandez et al., 2006; Olguín et al., 2003; Shi et al., 2007). There are many different kinds of human activity that generate wastewater containing large quantities of ammonium, for instance the petrochemical, pharmaceutical, fertilizer and food industries and leachates produced by

anaerobic digestion of either plant or animal wastes. Disposal of this type of waste is a serious environmental problem because the free ammonium ion, diluted in water, is one of the worst contaminants of aquatic ecosystems (Effler et al., 1990). Even though nitrogen compounds are essential elements for living organisms, when they are in excess they can accelerate eutrophication, leading to the depletion of dissolved oxygen and to plant and animal toxicity in receiving water. The common ammonium removal processes consist of air stripping, chemical treatment, selective ion exchange and biological nitrification-denitrification (Metcalf & Eddy, 2003; Kelly, 1996;de-Bashan et al., 2004).

From a physiological point of view, nitrogen is quantitatively the most important element, after carbon, hydrogen and oxygen, contributing to the dry matter of microalgal cells, accounting from 1 to 10% dry weight. Nitrogen can be absorbed by the algae in different forms; as ammonium, urea, nitrate as well as yeast extract, peptone, amino acids and purines. Due to the lower energy expenditure on the assimilation of ammonium by algae than is needed for nitrate, the most preferred nitrogen source is ammonium (Goldman and Ryther, 1976; Syrett and Morris, 1963). Moreover it is well known from previous research (Cromar et al., 1996; Hyenstrand et al., 2000) that ammonium is more easily assimilated by some algae than nitrate, due to the lack of nitrate reductase in the algal cells. In addition in the estimation of kinetic parameters for the transport of nitrate and ammonium into phytoplankton, a higher maximum transport rate for ammonium than for nitrate has been shown (Flynn, 1998; Mazzotti et al., in prep.).

Nevertheless, ammonium has not been commonly utilized in algal biomass production, due to difficulties in sterilization and to the lethal effect on cells caused by the severe drop in pH after ammonium has been consumed (Shi et al., 2000).

Though it is well known that the consumption of either nitrate or ammonium causes great changes in medium pH as the culture grows, there are still some doubts on why, if ammonium is taken up faster by the cell and is energetically the most convenient source, its addition to algal cultures is not coupled to a higher biomass production than occurs with nitrate. The aim of this study was therefore to determine the physiological effects of different nitrogen sources on *Scenedesemus* sp. and to understand how to improve biomass production using wastewaters rich in ammonium. To address this, we investigated pH changes in the medium and inside the cells, dissolved inorganic carbon uptake, cell macromolecular composition and their photosynthetic activity.

Methods

Culture maintenance and experimental conditions

The *Scenedesmus* strain used in this work was originally isolated from the Murray River, near Albury on the NSW/Victoria border, Australia, in 2010 by Dr Daryl P. Holland. An axenic culture has been maintained in MLA medium (Bolch and Blackburn, 1996) at Monash University at 25° in an environmentally controlled room with 100 µmol photons m^{-2} s^{-1} of irradiance and 12:12 h photoperiod.

Scenedesmus sp. was adapted to the different nitrogen sources by culturing it in 400 mL of CHU 13 modified by adding the usual amount of nitrogen (28 mg/L of TN) half as nitrate and half as ammonium. After 3 growth cycles, batch cultures with either nitrate or ammonium were inoculated with 10^5 cell ml^{-1} from a pre-adapted culture in stationary phase under the above experimental conditions for 15 days.

Experiments were run three times and each condition (Table 1) was grown in triplicate batch cultures. The air flow which was bubbled in the AA conditions was at air-equilibrated levels of CO_2, i.e. 0.04% (v/v) CO_2.

Table 1 Experimental set –up conditions. There are two different sources of nitrogen as ammonium and nitrate, and two different concentrations of total nitrogen in the medium. The third parameters is the supply of air

Culture	N source	TN mg L^{-1}	Air flow mL min^{-1}
N	Nitrate	28	-
A	Ammonium	28	-
AA	Ammonium	28	5 -10
DA	Ammonium	56	-

Growth

Scenedesmus growth was monitored by cell counts in a Neubauer haemocytometer at 100x magnification using a Zeiss Axio Scope optical microscope. Ten microliters of cultures were added to each side of the haemocytometer, and the cells were counted. The specific growth rate (μ, d^{-1}) was calculated during the exponential phase using the following equation:

$$\mu = \ln(n_1/n_0)/(t_1-t_0)$$

Where n_1 and n_0 are the number of cell (cell mL^{-1}) at time t_t and t_0.

Cellular content analysis

Chlorophyll and protein analyses were performed on pellets obtained by centrifuging 10 ml of algal cultures. Because of the hard cell wall characterizing the *Scenedesmus* genus, the extraction was performed as follows. Briefly 3 ml of 0.5 N NaOH were added to the pellet then incubated at 90°C for 60 minutes, and the procedure repeated 3 times. The protein content of the combined NaOH-extracts was estimated with the Folin Phenol reagent (Lowry et al., 1951) using bovine serum albumin as standard, while chlorophyll *a* concentration in the NaOH supernatant was analysed spectrophotometrically according to Strickland and Parsons (1972). Cellular content of total lipids and total proteins was also analyzed using Fourier-transform infrared (FTIR) spectroscopy, using mid-infrared absorbance spectra from dried microalgal samples. Spectra were collected on a FTIR microspectrometer (Spectrum 2000 System, Perkin-Elmer Life and Analytical Sciences Inc.) fitted with a liquid-N_2-cooled mercury-cadmium-tellurium detector. The resulting spectra were processed following Heraud et al. (2008).

Oxygen evolution

Rates of photosynthetic oxygen evolution as a function of irradiance (P vs. I curve) and dark respiration (R_d) were measured with a Clark-type oxygen electrode (Hansatech, Norfolk, UK) at 25°C. Electrodes were calibrated by bubbling distilled water in the chambers with oxygen-free N_2 (zero) and air (272 µM). For each condition, 2 ml samples were placed in the electrode chamber. Firstly the chamber was darkened for estimates of dark respiration then, after 10 minutes, it was illuminated at 350 µmol photons m^{-2} s^{-1}. The gross O_2 evolution is the result of the net O_2 evolution plus the dark respiration. All data are calculated on the basis of 10^6 cells.

Rapid Light Curves

Fluorescence measurements of PSII activity were performed using a Phyto-PAM Phytoplankton Analyzer (Heinz Walz, Effeltrich, Germany). A sample of 2 ml culture was collected by filtration and resuspended in 3 mL of fresh culture medium. After 15 min of dark acclimation the sample was provided with a measuring light beam of intensity low enough to avoid photochemistry, as evidenced by a stable F_0 value, and then exposed to a saturating beam of red light to estimate the maximum fluorescence (F_m) and the maximum quantum yield of photosynthetic energy conversion in PSII (F_v/F_m). A pulse of maximal intensity and duration of 350 ms was found to be sufficient to cause fluorescence yield to plateau (Pierangelini et al., 2014). Subsequently measurements were made of effective quantum yields [$(F_m' - F_0')/F_m'$] under a series of increasing background (actinic) light intensities and relative electron

transport rate (rETR) calculated for each actinic light intensity as follows:

$$rETR = [(F_m' - F_o')/F_m'] * \text{photon flux} * 0.5$$

Values of ETR estimated in this way assume that the light absorption properties of the cells are unaltered as the light intensity changes during each set of measurements (Beardall et al. 2001).

The photosynthetic parameters α (light harvesting efficiency), $rETR_{max}$ (light saturated relative electron transport rate) and E_k (the light intensity at with rETR begins to saturate) were then determined as described by Pierangelini et al (2014).

Nutrient determination
Inorganic nitrogen uptake was measured by quantifying nitrate and ammonium with a portable spectrophotometer (DR/2010; Hach, Colorado, USA). Briefly, 25 ml algae culture were filtered every 2 or 3 days with Whatman GF/C filters. Nitrate and ammonium was measured in the filtrate using the Nessler Method and the Cadmium Reduction Method, respectively (APHA).

Internal and medium pH measurement
Internal pH was determined using the silicone oil centrifugation technique with 5-5-dimethyl-2[^{14}C]-oxazolidine-2,4-dione (DMO) as described in Burns & Beardall (1987). Cells were harvested and then resuspended to 10^6 cell mL^{-1} density in their media. The cellular plus external water was estimated using ^3H_2O and [^{3}H] dextran respectively (Burns & Beardall (1987).

DIC, and carbon speciation
The total dissolved Carbon Dioxide (TCO$_2$) was measured using an infrared gas analyser (IRGA) technique, using a LiCor LI-820 sensor. A sample of medium, after filtration through a glass fiber syringe filter of 0.22 μm, was injected in a volumetric flask containing 20 ml

of 0.1 M HCl. Calibrations were run, using standard bicarbonate solutions, between analysis of media from each growth treatment. DIC speciation was calculated from pH, calculated TCO$_2$, salinity, temperature and atmospheric pressure values by the software "CO2Calc", using the CO_2 constant of Millero et al. (1979), the K SO$_4$ constant of Dickson (1990b), the Free scale (mol/kgSW) for pH, and values of Wanninkhof (1992) for the Air-sea flux.

Statistical analysis
Variability between replicates is expressed by standard error. Statistical significance was analysed between N conditions by ONE-way ANOVA using PAST 2.17 software. Levene's test was applied to test the homogeneity of variance and when the results showed significant differences the Tuckey test was applied for a pairwise comparison. Tests yielding $p > 0.05$ were deemed to indicate no significant differences. All data are the means of three independently replicated cultures for each condition.

Results and discussions
Growth
The effect of the two different nitrogen forms on *Scenedesmus* sp. growth is shown in Figs. 1 and 2. It can be observed that the microalgae attained a higher final cell density when nitrate was used as the nitrogen source in the media (N cultures) than when cultures were grown with ammonium (A cultures). However the condition with the fastest growth rate and the highest final number of cells was the culture with ammonium plus carbon supply (AA), with values, respectively, of 0.33 d^{-1} and 2.04•10^6 cell mL^{-1}. The addition of a double concentration of ammonia (DA) did not stimulate growth; indeed, the growth rate in this condition showed the lowest value of 0.07 d^{-1}.
From these results it can be hypothesized that the lower density of the culture grown with ammonium as nitrogen source instead of
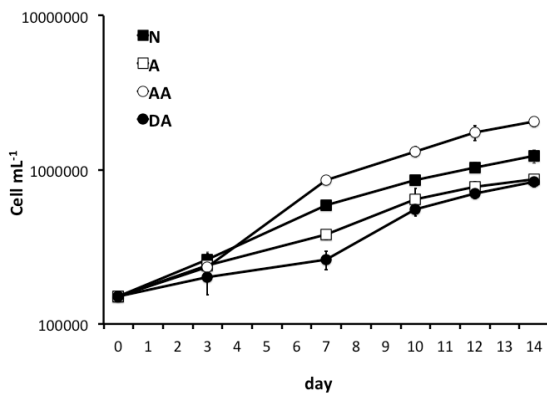
Figure 1 Growth curves for *Scenedesmus* grown under different nitrogen sources and aeration regimes. The double-sided bars represent the standard deviation of the mean (n=3).

nitrate was not caused by faster ammonium (compared to nitrate) depletion.

As reported in Xin et al. (2010), green microalgae such as those of the *Scenedesmus* genus, show a tendency to have faster exponential growth in the presence of ammonium as nitrogen source, but, after few days the culture growth slows down and final cell numbers are lower than in cultures having nitrate as nitrogen source.
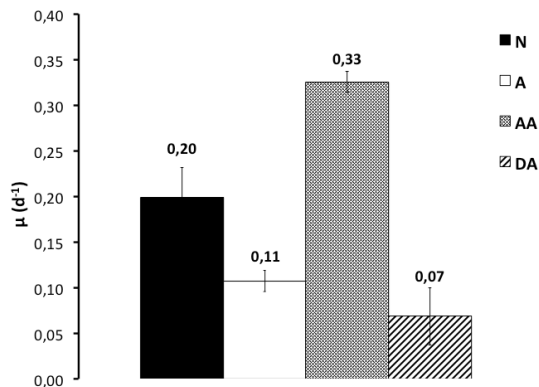

Figure 2 Specific growth rate. Standard error is presented as a double-sided bar, n=3 (P < 0.05).

Nutrient removal

Results shown in Figure 3 report the consumption of nitrogen, as ammonium or nitrate, in the medium under all conditions. *Scenedesmus* sp. showed a faster uptake of nitrogen when this was available as

ammonium, especially if there was an improved carbon supply through air bubbling.
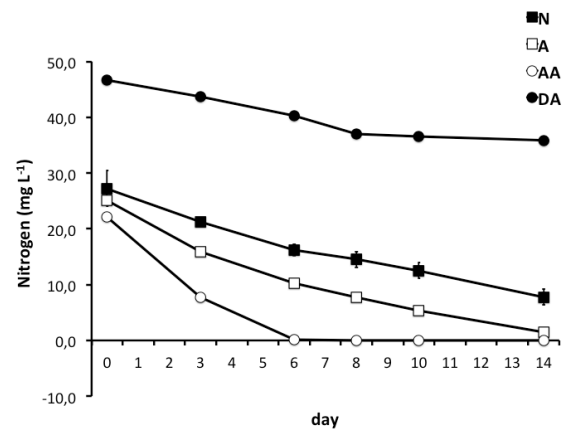
Figure 3 Total nitrogen in the medium for cultures of *Scenedesmus* grown under different nitrogen sources and aeration. The double-sided bars represent the standard deviation of the mean (n=3).

This phenomenon could be associated with the inability of plant cells to control NH_4^+ influx at high external NH_4^+ concentrations (Britto et al., 2001a, 2001b). In particular our data show that this microalga is able to remove 15 mg L^{-1} without air bubbling and more than 20 mg L^{-1} of $N-NH_4^+$ in only 6 days of culture, thus displaying a high removal efficiency if we consider that *C. sorkiniana*, an efficient microorganism in ammonium removal from wastewater, can remove only up to 10 mg L^{-1} under optimal conditions (de-Bashan et al., 2008). An high ammonium absorption was also confirmed in a previous study (Mazzotti et al., in prep.) on *D. communis.* The microalgae had an almost ten times greater velocity of uptake of nitrogen as ammonium than nitrate.

Dissolved inorganic carbon depletion

All cultures with ammonia as nitrogen source showed the same trend in the dissolved inorganic carbon uptake (Fig. 4). The growth conditions that were characterized by a fast nitrogen uptake, i.e. cultures with N in the form of ammonium, resulted in a fast inorganic carbon uptake, with a rapid drop of the carbon concentration in the medium. *Scenedesmus*

grown with nitrate showed an initial rise in DIC until day 3 but thereafter displayed a stable concentration for the remaining growth period.
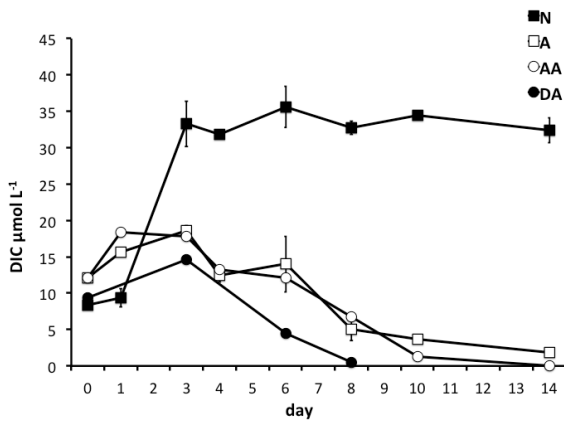

Figure 4 Dissolved inorganic carbon in the medium for cultures of *Scenedesmus* grown under different nitrogen sources and aeration. Data are means +/- standard deviation (n=3)

Figure 5 shows in detail the concentrations of the different inorganic carbon species dissolved in the medium. Low concentrations of CO_2, probably due to the alkalinity of the medium, were observed in all treatments. The most abundant species was HCO_3^-, especially from day 4, in the N condition. Moreover there was a gradual removal of the bicarbonate in all ammonium-cultures, with a faster depletion in the aerated one. However, it has been demonstrated that most of the microalgae examined so far have an efficient dissolved CO_2 concentrating mechanism (CCM), which permits them to use either CO_2 or HCO_3^- as external sources of DIC (Huertas et al., 2000).

There could be several reasons to explain the faster decrease of DIC in cultures with ammonium. Intense photosynthetic activity will decrease the DIC concentration significantly. As a result of photosynthetic activity, DIC levels are rarely as high as might be expected from air-equilibrium (Beardall & Raven, 2013). Carbon and nitrogen metabolism are linked in microalgae because they share carbon supplied directly from respiration of fixed CO_2. In addition the fast DIC uptake could

be a consequence of the high internal $[NH_4^+]$, to maintain a correct C:N ratio, thereby ensuring optimal operational capacity for the cell (Britto et al., 2001b; Howitt and Udvardi, 2000).

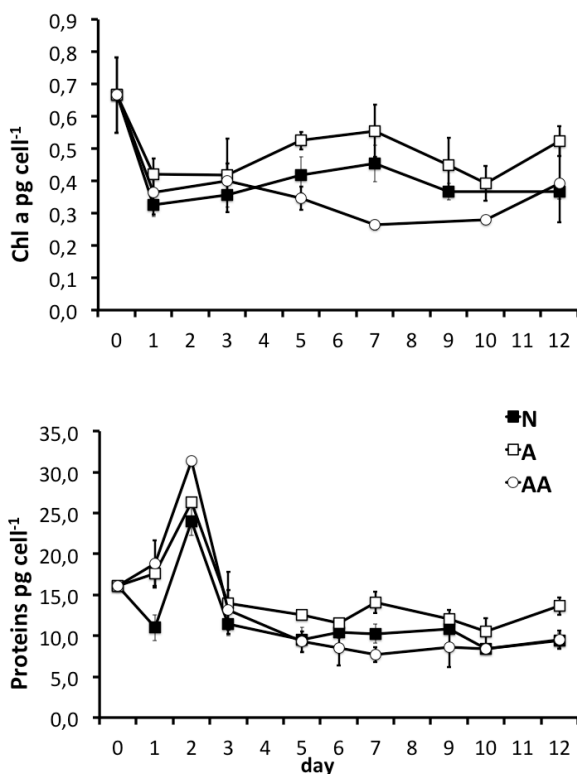The log pCO2 decreased linearly with increasing pH of the culture (Lívanský and Doucha, 1996) .
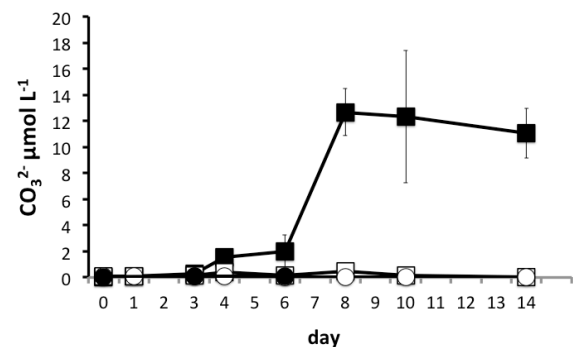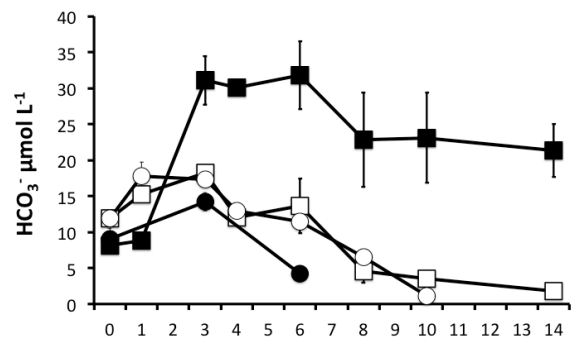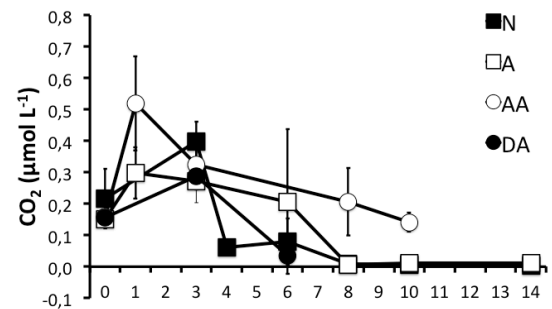
Figure 5 Carbon dioxide, Bicarbonate, and Carbonate concentrations in the culture medium under different nitrogen sources and aeration. The double-sided bars represent the standard deviation of the mean (n=3).
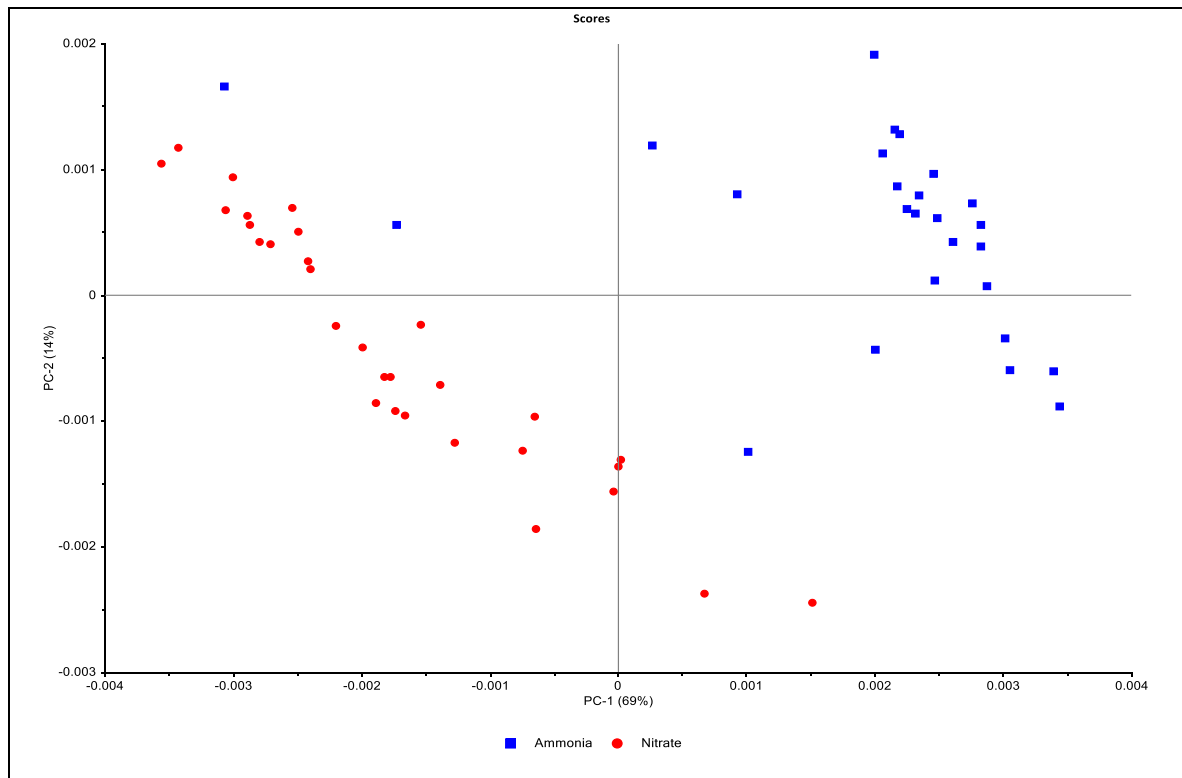
Cellular composition

Chlorophyll *a* and total protein contents (as pg cell^{-1}) are shown in Fig. 6. In the first 3 days the cultures supplied with ammonium (A) displayed an higher protein content than did those with nitrate (N). After day 3, chlorophyll *a* in particular decreased in cultures characterized by the highest growth rate. The reduction in chlorophyll content would thus allow more light to penetrate deeper into the culture and be better utilised. This would therefore increase the efficiency of use of incident photons and areal productivity (Beardall and Raven, 2013), and this could be one of the reasons for higher biomass production in cultures with the lower chlorophyll content .

We also found a higher level of proteins in A cells than N cells. This could be due to a higher NH_4^+ uptake value that could enhance C assimilation. Enhancement of C assimilation is achieved mostly through an increase in the amount of the Rubisco protein (Giordano, 1997; Giordano and Bowes, 1997).

The FTIR analysis was done only on cells grown under N and A conditions, and at day 14, when the biggest difference in number of cells/mL was observed. From the PCA plot (Fig. 7) a net difference in terms of protein and lipids content between cells grown with ammonium compared to those grown with nitrate is clearly visible. This confirms the macromolecular changes in response to changes in nutrient status as seen in Giordano et al., (2001). *Scenedesmus* sp. grown with ammonium showed higher levels of lipids and proteins than those with nitrate as nitrogen source. From this data seems that the nitrogen, absorbed faster as ammonium, is directed to protein but, by day 14, a high proportion of the C assimilated is stored as lipid instead of being used by the algae for cell growth. In addition, from the comparison between the two different methods used to determine the macromolecular composition it is evident that the FTIR analysis permit a rapid, simultaneous measurement from very small quantities of algal sample allowing frequent monitoring of macromolecular changes in cultures of small volume, hitherto impossible using conventional chemical analyses (Heraud et al., 2008).

Figure 6 Chlorophyll *a* cell and protein content trend . The cholophyll cell content has been determined only for those three conditions with the highest growth. The double-sided bars represent the standard deviation of the mean (n=3).

Figure 7 PCA scores from FTIR analysis. The analysis has been preformed on day 14 between ammonia (blue square) and nitrate (red spot) conditions. Models were mean centred and generated using full cross validation. The PC1 axe represents the increment of lipids, while the PC2 represents the increment of proteins.
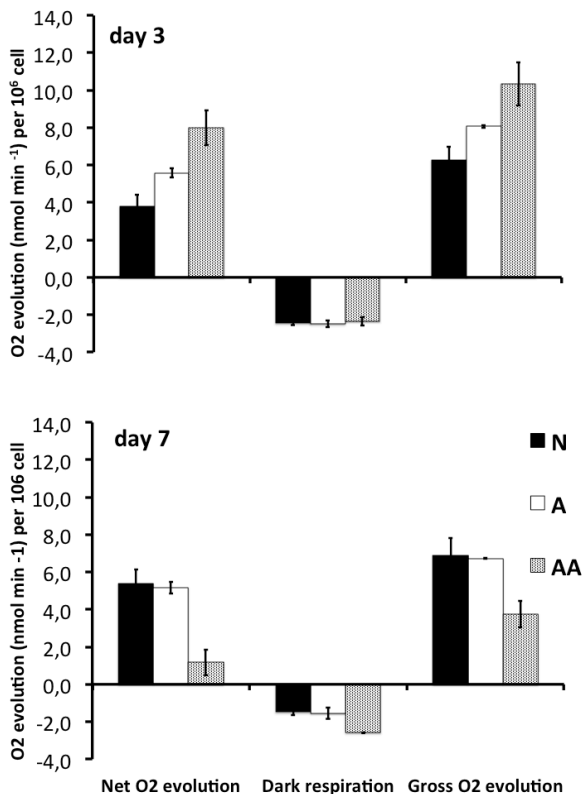
Table 2 Measurements of the photosynthetic response of *Scenedesmus* sp. under different nitrogen sources and aeration. Light harvesting efficiency (α); Maximum electron transport rate (ETR_{max}), light saturation parameter (Ik); Maximum quantum Yield (Fv/Fm); effective quantum yield ($\Phi PSII_e$). Each values is the mean of 3 replicates ± standard deviation.

| day	condition	α	ETR_{max}	Ik	Fv/Fm	$\Phi PSII_e$
3	**N**	0.295 ± 0.004	43.4 ± 1.5	146.3 ± 7.8	0.69 ± 0.02	0.58 ± 0.04
	A	0.266 ± 0.004	45.3 ± 0.4	170.4 ± 1.8	0.66 ± 0.01	0.57 ± 0.01
	AA	0.264 ± 0.005	49.6 ± 0.3	188.1 ± 4.1	0.66 ± 0.03	0.58 ± 0.01
	DA	0.239 ± 0.011	39.7 ± 2.1	165.3 ± 1.5	0.59 ± 0.03	0.50 ± 0.01
6	**N**	0.293 ± 0.006	44.1 ± 0.4	148.6 ± 3.4	0.71 ± 0.01	0.60 ± 0.01
	A	0.270 ± 0.005	41.6 ± 1.0	154.3 ± 3.6	0.59 ± 0.02	0.56 ± 0.01
	AA	0.284 ± 0.004	48.1 ± 0.7	169.3 ± 2.4	0.71 ± 0.01	0.60 ± 0.01
	DA	0.256 ± 0.011	43.1 ± 1.5	168.5 ± 2.1	0.63 ± 0.02	0.52 ± 0.03

Photosynthetic capacity

Photosynthetic capacity was determined on day 3 and 6, i.e. in the exponential phase, with a direct technique (the measurement of O_2 evolution, Fig. 5), and an indirect method (PAM fluorometry).

After 3 days in culture in the presence of ammonium plus aeration (AA cultures) *Scenedesmus* sp. showed the highest rate of gross O_2 evolution, mostly due to the high net O_2 evolution. An increase in the photosynthetic performance due to the chemical nature of the NH_4^+, had been already observed in *Chlamydomonas reinhardtii* where it was postulated that more energy should be available when N is present in the more reduced form, being however somehow related also to its concentration (Beardall and Giordano, 2002).On day 7 when the difference, in terms of growth, among the conditions was most evident, the gross O_2 evolution in AA cells

showed a sharp decrease reaching values almost half those of the other conditions. The O_2 evolution trend seemed to follow the N uptake trend (Fig 8). This can be explained by the necessity for cells to satisfy the demand for C-skeletons to support ammonium assimilation to glutamate, so that glycolysis and mitochondrial respiration are stimulated.
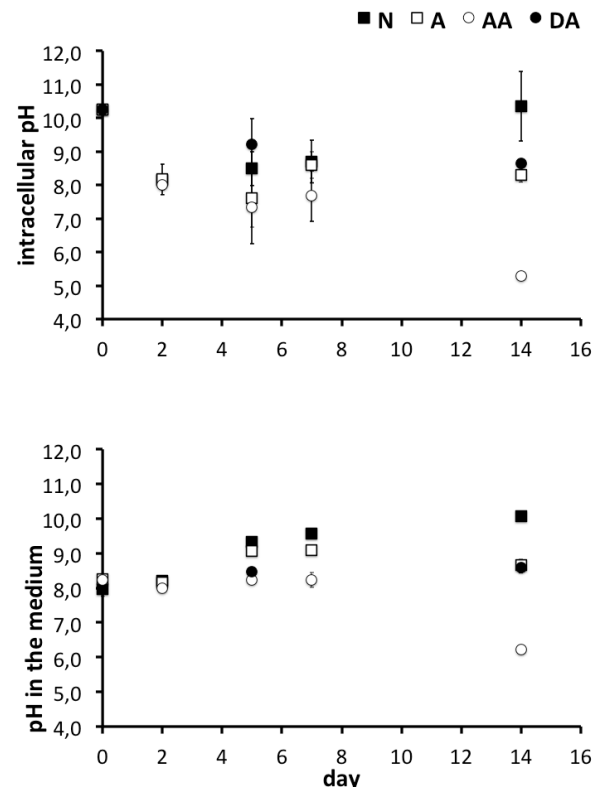
Figure 9 Intracellular and medium pH measured in pre-definited days for each experimental treatment. The double-sided bars represent standard deviation of the mean (n=3).

The uptake and assimilation of N and P both require the expenditure of energy as ATP and /or reducing equivalents. Consequently ATP derived from the light reactions of photosynthesis can be used for rapid uptake of the nutrient at the expense of C assimilation. If the ATP is derived from enhanced cyclic photophosphorylation, rather than linear electron transport, photosynthetic O_2 evolution will be diminished while nutrients are being taken up by the cells (Beardall et al., 2001).

Finally the dataset from the PAM measurements is given in table 2, and confirms (though measurements were only done on day

Figure 8 Net oxygen evolution rates, dark respiration rates and gross respiration rates at different experimental conditions in day 3 and day 7 of culture. The double-sided bars represent the standard deviation of the mean (n=3).

3 and 6) the trends in the O_2 evolution dataset, with a higher rETR max and a higher light saturation point (Ik) for cultures with ammonium and air, while the highest light harvesting efficiency values are in cultures grown with nitrate.

In summary, the parameters of photosynthetic O_2 evolution rate, F_v/F_m and electron transport rate which are all indicators of photosynthetic efficiency, similarly increased by day 3 as a consequence of the faster ammonium uptake occurring in the first days of growth, then decreased for the reduced nitrogen availability in AA conditions.

pH

Fig. 9 shows the pH trend both in the cultures and in the cells as a function of time. The external pH was affected by the nitrogen source in the medium, but there was no correlation between the pH in the medium, or intracellular pH, and the growth pattern. In the condition with ammonium and air bubbling, the pH dropped faster than in the other conditions and after 5 days the pH inside the cell decreased to 6, and after 12 days it was, both in the cells and in the medium. The condition with double nitrogen as ammonia didn't show differences also related to the pH measures, as the trend was the same as in cultures with standard ammonium concentration.

The pH data is in keeping with values reported by Xin et al.(2010), who also reported a pH rise directly correlated with the ammonium uptake. It is although true that the assimilation of ammonium in cell cytoplasm produces at least one H^+ per NH_4^+, and NO_3^- assimilation produces almost one OH^- per NO_3^-, the cytosolic pH regulation mostly achieved by transport of excess H^+ or OH^- out of the cytoplasm (Raven and Smith, 1976). Since the vacuole is a smaller sink than the external solution and its pH is always lower than that of the cytoplasm, it can only contain H^+ (Raven and Smith, 1974).

It is obvious that all the conditions showed rapid changes in pH level, due to the nutrient uptake, without the supply of buffer, as described in Shi et al., (2000), but the most interesting thing is that the conditions in which the highest number of cells and biomass was obtained, were also those with the fastest pH rise. On the other hand, a fast pH rise has previously been observed in cultures of *Nannochloris gaditana* and *Nannocloris maculata* with higher levels of DIC and consequentially higher growth rate, but with nitrate as nitrogen source (Huertas et al., 2000)

Conclusions

From this study it appears evident how *Scenedesmus* sp. growth is influenced by the nitrogen source because of the different carbon availability and the difference in the biomass value reached in the presence of nitrate or ammonium it is not caused by the pH level. Although pH fluctuation is dependent on the microalgal uptake rate for ammonium or nitrate, it doesn't affect the internal metabolism of the cell to the same extent as does the lack of carbon. From our data it is understandable how the cells grown with ammonium need more carbon skeletons to metabolize all the nitrogen which is more rapidly absorbed; in the occurrence of inorganic carbon depletion, the algal cells store all the nitrogen and stops the cells division, waiting for new carbon supply.

Our data also confirm the possibility of growing large volumes of microalgal biomass through a wastewater process. The removal of high concentrations of ammonium is possible if the sewage to be treated has the right concentration of inorganic carbon to support photosynthetic metabolism and permit to the algae to grow. One important aspect of the nitrogen removal from waste-water, is the improvement of carbon fixation, and so the CO_2 sequestration, only if the preferred nitrogen source as ammonium is provided. Through this process, accumulated carbon and nitrogen are metabolised into useful biochemical and biomolecules that can be used as high-value bioproducts.

References

APHA (American Public Health Association), 1995. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington, DC.

Aslan, S., Kapdan, I.K., 2006. Batch kinetics of nitrogen and phosphorus removal from synthetic wastewater by algae. Ecol. Eng. 28, 64–70.

Beardall, J., Giordano, M., 2002. Ecological implications of microalgal and cyanobacterial CO2 concentrating mechanisms, and their regulation. Funct. Plant Biol. 29, 335–347.

Beardall, J., Raven, J.A., 2013. Limits to Phototrophic Growth in Dense Culture: CO2 Supply and Light, in: Borowitzka, M.A., Moheimani, N.R. (Eds.), Algae for Biofuels and Energy, Developments in Applied Phycology. Springer Netherlands, pp. 91–97.

Britto, D.T., Glass, A.D.M., Kronzucker, H.J., Siddiqi, M.Y., 2001a. Cytosolic Concentrations and Transmembrane Fluxes of NH4 +/NH3. An Evaluation of Recent Proposals. Plant Physiol. 125, 523–526.

Britto, D.T., Siddiqi, M.Y., Glass, A.D.M., Kronzucker, H.J., 2001b. Futile transmembrane NH4+ cycling: A cellular hypothesis to explain ammonium toxicity in plants. Proc. Natl. Acad. Sci. U. S. A. 98, 4255–4258.

Cromar, N.J., Fallowfield, H.J., Martin, N.J., 1996. Influence of environmental parameters on biomass production and nutrient removal in a high rate algal pond operated by continuous culture. Water Sci. Technol., Water Quality International '96 Part 7: Agro-Industries Waste Management; Appropriate Technologies and Waste Stabilization Ponds; Wastewater Reclamation and Reuse Selected Proceedings of the 18th Biennial Conference of the International Association on Water Quality 34, 133–140.

de-Bashan, L.E., Hernandez, J.-P., Morey, T., Bashan, Y., 2004. Microalgae growth-promoting bacteria as "helpers" for microalgae: a novel approach for removing ammonium and phosphorus from municipal wastewater. Water Res. 38, 466–474.

de-Bashan, L.E., Trejo, A., Huss, V.A.R., Hernandez, J.-P., Bashan, Y., 2008. *Chlorella sorokiniana* UTEX 2805, a heat and intense, sunlight-tolerant microalga with potential for removing ammonium from wastewater. Bioresour. Technol., Exploring Horizons in Biotechnology: A Global Venture 99, 4980–4989.

Giordano, M., 1997. Adaptation of *Dunaliella salina* (Volvocales, Chlorophyceae) to growth on NH4+ as the sole nitrogen source. Phycologia 36, 345–350.

Giordano, M., Bowes, G., 1997. Gas Exchange and C Allocation in *Dunaliella salina* Cells in Response to the N Source and CO2 Concentration Used for Growth. Plant Physiol. 115, 1049–1056.

Giordano, M., Kansiz, M., Heraud, P., Beardall, J., Wood, B., McNaughton, D., 2001. Fourier Transform Infrared Spectroscopy as a Novel Tool to Investigate Changes in Intracellular Macromolecular Pools in the Marine Microalga *Chaetoceros muellerii* (bacillariophyceae). J. Phycol. 37, 271–279.

Goldman, J.C., Ryther, J.H., 1976. Temperature-influenced species competition in mass cultures of marine phytoplankton. Biotechnol. Bioeng. 18, 1125–1144.

Heraud, P., Stojkovic, S., Beardall, J., McNaughton, D., Wood, B.R., 2008. Intercolonial Variability in Macromolecular Composition in P-Starved and P-Replete *Scenedesmus* Populations Revealed by Infrared Microspectroscopy1. J. Phycol. 44, 1335–1339.

Hernandez, J.-P., de-Bashan, L.E., Bashan, Y., 2006. Starvation enhances phosphorus removal from wastewater by the microalga *Chlorella* spp. co-immobilized with *Azospirillum brasilense*. Enzyme Microb. Technol. 38, 190–198.

Howitt, S.M., Udvardi, M.K., 2000. Structure, function and regulation of ammonium transporters in plants. Biochim. Biophys. Acta BBA - Biomembr. 1465, 152–170.

Huertas, E., Montero, O., Lubián, L.M., 2000. Effects of dissolved inorganic carbon availability on growth, nutrient uptake and chlorophyll fluorescence of two species of marine microalgae. Aquac. Eng. 22, 181–197.

Hyenstrand, P., Burkert, U., Pettersson, A., Blomqvist, P., 2000. Competition between the green alga *Scenedesmus* and the cyanobacterium *Synechococcus* under different modes of inorganic nitrogen supply. Hydrobiologia 435, 91–98.

Lívanský, K., Doucha, J., 1996. CO2 and O2 gas exchange in outdoor thin-layer high density microalgal cultures. J. Appl. Phycol. 8, 353–358. doi:10.1007/BF02178578

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Olguín, E.J., Galicia, S., Mercado, G., Pérez, T., 2003. Annual productivity of *Spirulina (Arthrospira)* and

nutrient removal in a pig wastewater recycling process under tropical conditions. J. Appl. Phycol. 15, 249–257.

Pierangelini, M., Stojkovic, S., Orr, P.T., Beardall, J., 2014. Photosynthetic characteristics of two *Cylindrospermopsis raciborskii* strains differing in their toxicity. J. Phycol. 50, 292–302.

Raven, J.A., Smith, F.A., 1974. Significance of hydrogen ion transport in plant cells. Can. J. Bot. 52, 1035–1048.

Raven, J.A., Smith, F.A., 1976. Nitrogen assimilation and transport in vascular land plants in relation to intracellular pH regulation. New Phytol. 76, 415–431.

Shi, J., Podola, B., Melkonian, M., 2007. Removal of nitrogen and phosphorus from wastewater using microalgae immobilized on twin layers: an experimental study. J. Appl. Phycol. 19, 417–423.

Shi, X.-M., Zhang, X.-W., Chen, F., 2000. Heterotrophic production of biomass and lutein by *Chlorella protothecoides* on various nitrogen sources. Enzyme Microb. Technol. 27, 312–318.

Syrett, P.J., Morris, I., 1963. The inhibition of nitrate assimilation by ammonium in *Chlorella*. Biochim. Biophys. Acta BBA - Spec. Sect. Enzymol. Subj. 67, 566–575.

Vergara-Fernández, A., Vargas, G., Alarcón, N., Velasco, A., 2008. Evaluation of marine algae as a source of biogas in a two-stage anaerobic reactor system. Biomass Bioenergy 32, 338–344.

Xin, L., Hong-ying, H., Ke, G., Jia, Y., 2010. Growth and nutrient removal properties of a freshwater microalga *Scenedesmus* sp. LX1 under different kinds of nitrogen sources. Ecol. Eng. 36, 379–381.

Chapter 3

Effect of glucose addition on inorganic nutrient removal by the green alga *Desmodesmus communis*

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Microalgal biomass production has been the subject of many studies because of the wide variety of its practical and potential applications, such as food supplements, pharmaceutical products, polymers, "green energy" resources and wastewater treatment.

Currently the most common procedure for microalgae mass cultivation is based on autotrophic growth in the presence of light and CO_2 as inorganic carbon source, and is performed on diverse mineral media and on synthetic or real wastewater. The supply of a sufficient natural or artificial light that will allow dense growth is the main objective of, and a limiting factor for, the cultivation. Photoautotrophic cultivation is considered the most convenient culture method as sunlight represents a "freely available" photosynthetic energy source. In addition, the photosynthetic efficiency of microalgae is substantially higher than plants (Chisti, 2007) .

A feasible alternative to autotrophic culture is to use a heterotrophic cultivation system in which organic carbon sources, such as sugars or organic acids, are added in the absence of light (Cheirsilp and Torpee, 2012) This condition shows several limitations, e.g. the increasing costs of adding organic substrates, higher risk of contamination, lack of light-induced metabolites and growth inhibition by excess organic matter (Perez-Garcia et al., 2011a). However it would allow growth of the algae in the absence of light, a source not always available in appropriate amounts, and in cheap containers as those designed for bacteria.

A mixotrophic growth regime is a variant of the heterotrophic one, where CO_2 and organic carbon are simultaneously assimilated and both respiration and photosynthetic metabolism operate concurrently. For instance in some open-pond cultivations, organic carbon sources, such as acetate and glucose, are added continuously in small quantities to support higher microalgal biomass production and avoid excessive bacterial growth. Many algal species display enhanced growth when cultivated under mixotrophic conditions, however, this behaviour appears highly variable even within the same species.

As practiced with other microbial communities producing economic products, open ponds that mimic the natural environments of microalgae are the most common option for mass cultivation (Oswald, 1992; Tredici 2004) but, notwithstanding their many benefits, have several disadvantages including for example poor light diffusion inside the pond. This aspect is aggravated when cultivation is intensive and the high cell density causes self-shading or when the cultivation is done with dark wastewater.

The rapid development of human activities has greatly increased the input of nitrogen and phosphorus into bodies of water. These inputs induce eutrophication and cause deterioration of natural water quality. As such, the removal of nitrogen and phosphorus from wastewater is a fundamental way to prevent eutrophication and algal blooms (Xin et al., 2010). Microalgae have high potential to remove inorganic nutrients from wastewater and to yield

biomass for many other applications, and because algal growth requires the availability of primary nutrients, such as carbon, nitrogen and phosphorus which can be costly, the use of wastewater can reduce the cost of algae production minimizing the addition of nutrients (Christenson and Sims, 2011).

Media formulation is important in the cultivation of microalgae to obtain high final concentrations of cells (Berges et al., 2001). Moreover the medium constituents must satisfy the basic requirements for cell build-up and metabolite production, by providing an adequate supply of energy for biosynthesis and cell maintenance. For heterotrophic cultivation of microalgae, glucose (Shi et al., 2000) has been successfully used as a carbon source. Growth of heterotrophic cultures of microalgae has also been found to be greatly influenced by the type and concentration of nitrogen sources supplemented to the medium (Shi et al., 2000).

Although much research has already been performed to understand the growth and accumulation of lipid in microalgae due to different nutrient conditions, in particular by supplying different combination of glucose, nitrate and ammonium (Chandra et al., 2014), there is still a lack of understanding regarding the combined effects, in terms of nutrient removal, of these three parameters supplied under different light intensities (Perez-Garcia et al., 2011). Our study was focused on this aspect in order to model the behaviour of a *D. communis* strain in synthetic wastewater treatment and biomass production to be performed in open ponds. In particular we wanted to focus on the inorganic and organic nutrient removal, on algal growth and productivity, and on microalgal stress evaluation.

Materials and methods

Microrganism

The autochtonous freshwater alga *Desmodesmus communis* (Chlorophyceae) used in the present work had been previously isolated from a local freshwater pond (Samorì et al., 2013) and kept in CHU13 mod. medium under a continuous PAR photon flux of 100 µmol photons m^{-2} s^{-1} with a light:dark photoperiod of 16:8h, at 20°C.

Experimental design

D. communis was cultivated in 500 mL Erlenmeyer flasks by using CHU13 mod. medium modified by using three different N-sources and C-sources, under two different light conditions (100 µmol photons m^{-2} s^{-1} for high light conditions and 10 µmol photons m^{-2} s^{-1} for low light conditions), to which it was allowed to acclimatize until the stationary phase.

Table 1 Experimental set up conditions

Culture	N-source	Metabolism	Carbon	Light
NAHL	Nitrate	Autotrophic	Inorganic	High
NMHL	Nitrate	Mixotrophic	Inorganic + Organic	High
NMLL	Nitrate	Mixotrophic	Inorganic + Organic	Low
NAAHL	Nitrate/2 + Ammonium/2	Autotrophic	Inorganic	High
NAMHL	Nitrate/2 + Ammonium/2	Mixotrophic	Inorganic + Organic	High
NAMLL	Nitrate/2 + Ammonium/2	Mixotrophic	Inorganic + Organic	Low
AAHL	Ammonium	Autotrophic	Inorganic	High
AMHL	Ammonium	Mixotrophic	Inorganic + Organic	High
AMLL	Ammonium	Mixotrophic	Inorganic + Organic	Low

All the cultures were incubated at 20°C, air-supplemented with a flow rate of 0.01 mL min^{-1} and stirred with a magnetic system.

In order to test the different growth conditions, in terms of nutrients and light, three experiments were run in batch cultures as reported in Table 1. Each culture condition was carried out in triplicate and each experiment has been replicated twice.

The three different N-sources were: NH_4Cl as ammonium source, KNO_3 as nitrate source, or a mix of both, with a total amount of nitrogen of 28 mg/l per condition; 1 g/L glucose was added in all the mixotrophic conditions as organic carbon source (Ogawa and Aiba, 1981), while in autotrophic and mixotrophic conditions the inorganic carbon source was the CO_2 introduced through the air flow.

All samples were collected for analysis according to the same schedule for each experiment, till day 15 when all cultures were in stationary phase. The culture volume withdrawn for sampling was not replaced.

Cell growth
Microalgal growth was monitored by following the increase in dry weight, OD_{750} and cell number.

The dry weight (g L^{-1}) was determined by filtering 30 mL of sample through a Whatman GF/C glass fiber filter and drying at 105°C for 1 hour.

The turbidity of the culture was estimated by measuring the optical density (OD) at 750 nm using a spectrophotometer (Jasco, V-650), after appropriate dilution.

The culture was also monitored through cell counting (n_{cell} mL^{-1}) in settling chambers following the Utermöhl method.

Mean daily biomass productivity (P, g L^{-1} d^{-1}) and specific growth rate (μ, d^{-1}) were calculated using the following equations, respectively:

$$P = [x_1-x_0]/[t_1-t_0]$$

where x_1 and x_0 are the biomass concentrations (g L^{-1}) at times t_1 and t_0.

$$\mu = \ln (OD_1/OD_0)/[t_1-t_0]$$

where OD_1 and OD_0 are the optical absorbances at a wavelenght of 750 nm at day t_1 and t_0

Quantification of nutrient removal
Nutrient (N-NO_3^-, N-NH_4^+, P-PO_4^{3-}, $C_6H_{12}O_6$) removal was analyzed by measuring the variation of each compound over time by using 30 ml of filtered culture (Millipore, GF/C) according to specific protocols.

Ammonium, nitrate and phosphate concentration in the medium were measured through ion-exchange chromatography (883 Basic IC plus, Metrohm) using a cationic column (Metrosep C 4 – 150 / 4.0) for the ammonium and an anionic column (Metrosep A Supp 4 – 250 / 4) for the latter two anions. All the samples were filtered through a 0.22 µm syringe filter (Teknokroma) before the injection into the chromatography system.

The glucose concentration in the filtered medium was determined by the Phenol Sulfuric Acid Method (DuBois et al., 1956).

The removal efficiency (RE) was calculated from the residual nutrient concentrations in the medium and the change in biomass (B) over a period of time ($\Delta t = t_1-t_0$) when nutrient depletion was linear. Δt values therefore differ depending on the nutrient composition of the medium.

$$U= -[C_1-C_0]/ B\Delta t$$

Where $B = [b_1-b_0]/[\ln b_1 \ln b_0]$

C_0 and C_1 are the nutrient concentrations (mg L^{-1}) at the time t_0 and t_1; and b_1 and b_0 are biomass dry weight (g L^{-1}) values in the same days (Fiori et al., 2013).

Pulse amplitude modulation (PAM) fluorimetry
The photosynthetic efficiency, in terms of effective quantum Yield of PSII, was measured daily through a Pulse Amplitude Modulation fluorometer (101-PAM connected to a PDA-100 data acquisition system, H. Walz, Effeltrich, Germany) provided with a high power LED

Lamp Control unit HPL-C and LED-Array–Cone HPL-470 to supply saturating pulses, US-L665 and 102-FR to supply far red light and measuring light respectively. 3 mL algal samples were added to a cuvette (10 x 10 mm) mounted on an optical unit ED-101US/M.

All samples were dark adapted for 20 minutes to quench all the reaction centres (CR) of the photosystem II (PSII). After the dark adaptation, 3 ml sample was exposed to a saturation pulse (3000 μmol photons m^{-2} s^{-1} for 0.8s) and then to an actinic light (100 μmol photons m^{-2} s^{-1} for high light conditions and 10 μmol photons m^{-2} s^{-1} for low light conditions) for 5 minutes (a time interval previously optimized in order to obtain a stabilized yield). From these two measures it was possible to calculate the maximum Φ_{PSII} and the operational Φ'_{PSII} quantum yield respectively:

$$\Phi_{PSII} = F_v/F_m = [F_m - F_0]/F_m$$

$$\Phi'_{PSII} = \Delta F/F_m = [F'_m - F]/F'_m$$

where F_0 is the minimal fluorescence after dark adaptation, F_m is the maximal fluorescence yield induced by the saturating pulse, F'_m is the effective fluorescence yield at the actinic light and F is the steady-state fluorescence (Genty et al., 1989).

Statistical analysis
The statistical significance of the results was evaluated by ONE-way ANOVA and PAST 2.17 software was used. The homogeneity of variance was tested with the Levene's test, and the Tukey test was applied for a pairwise comparison, when the results showed significant differences.

Results and discussion
Growth influenced by nitrogen, carbon and light sources
The growth pattern, the nutrient removal and the photosynthetic yield of *D. communis* were studied under photoautotrophic conditions, in cultures supplied with different nitrogen sources, and under mixotrophic cultivation, again with different nitrogen sources but supplied with glucose and exposed to different light intensities.

Figure 1 reports the results of *D. communis* growth, given as biomass (g L^{-1}) and cell numbers and displaying very similar patterns, with the exception of the stationary phase of the autotrophic cultures. It is possible to observe that the autotrophic condition resulted in higher biomass production with respect to the mixotrophic conditions and that the highest yield was obtained in cultures having nitrate as nitrogen source, followed by the condition with both nitrate and ammonium and then by those supplied with ammonium alone. This result is not surprising as several papers (Arumugam et al., 2013; Shi et al., 2000; Dortch, 1990) have already shown nitrate to be the best nitrogen source to obtain high biomass values in green microalgae. With regard to the mixotrophic conditions, with both high and low light, cell growth started at a high rate, without the lag phase observed in the autotrophic cultures, however cell growth stopped after only 4-8 days, reaching a biomass value 50 % less of the autotrophic conditions. Under mixotrophy a different effect on growth by the two nitrogen sources was not observed (in low light) or was very small (in high light); in the latter condition a slightly higher biomass value was determined by the addition of both nitrate and ammonium with respect to the cultures with either of two. The absence of a lag phase in cultures supplied with glucose was previously reported: glucose might be considered a "preferred substrate" for heterotrophic cultivation of microalgae, as it was observed that microalgal cells grown on different organic substrates require a lag period to develop the specific transport systems (Perez-Garcia et al., 2011a). In contrast to previous reports on *Chlorella* spp. (Cheirsilp and Torpee, 2012), in our cultures the mixotrophic condition did not enhance the biomass yield; this phenomena could be due to the addition of glucose in batch mode, instead of fed-batch, thus being not enough to induce a
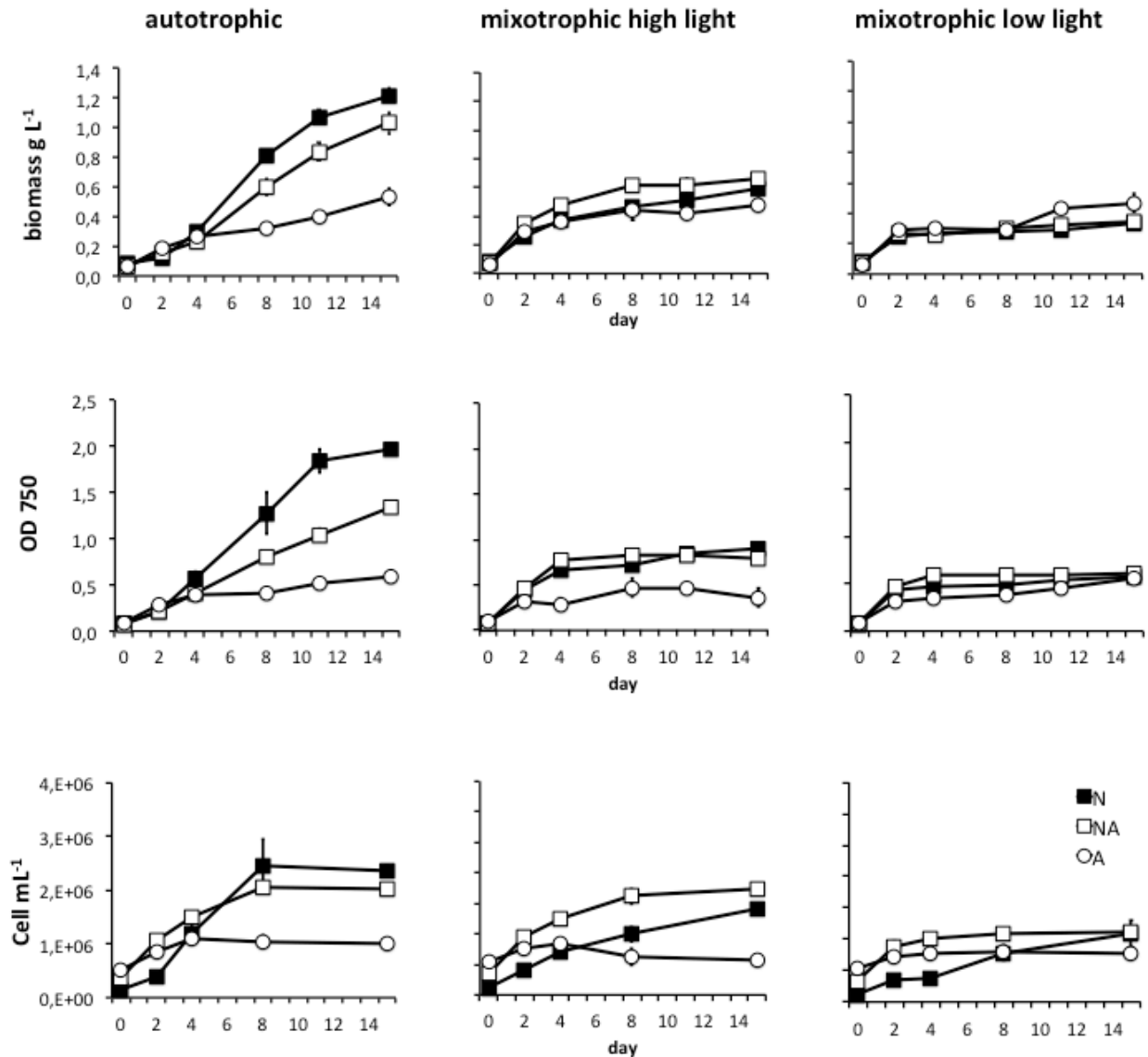
Figure 1 Effect of culture conditions on the growth of *D. communis* supplied with different N-source. (N= nitrate, NA= nitrate plus ammonium, A=ammonium). Data points are means +/- standard deviation, n=3.

Table 2 Productivity, maximum dry weight and growth rate measured for each growth conditions. Each value represents mean of three independent measurements ± standard deviation.

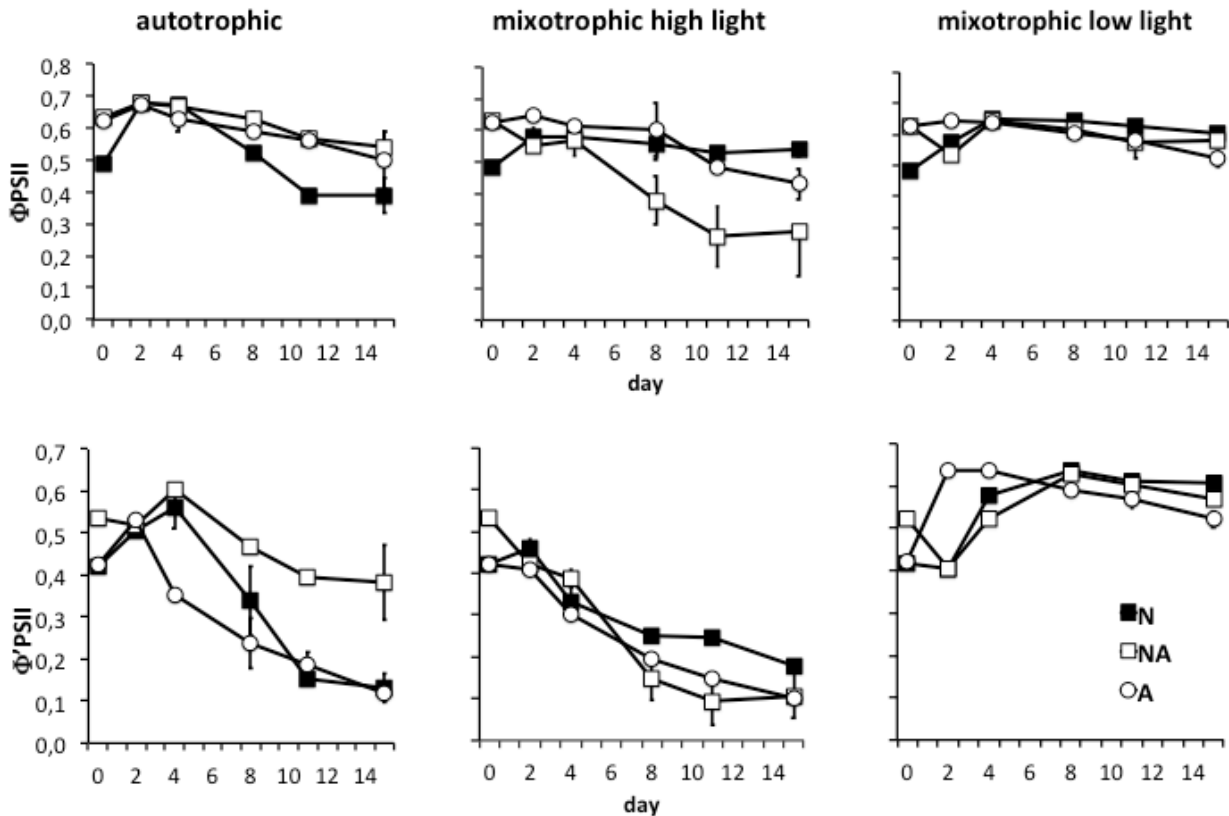| Culture	P $g\ l^{-1}d^{-1}$	DW_{max} $g\ l^{-1}$	$\mu\ d^{-1}$
NAHL	0.11 ± 0.01	1.22 ± 0.05	0.34 ± 0.02
NMHL	0.07 ± 0.01	0.59 ± 0.01	0.28 ± 0.01
NMLL	0.05 ± 0.00	0.34 ± 0.02	0.23 ± 0.01
NAAHL	0.08 ± 0.01	1.03 ± 0.07	0.29 ± 0.00
NAMHL	0.10 ± 0.01	0.66 ± 0.03	0.29 ± 0.00
NAMLL	0.05 ± 0.01	0.34 ± 0.01	0.26 ± 0.00
AAHL	0.05 ± 0.00	0.53 ± 0.06	0.19 ± 0.00
AMHL	0.07 ± 0.01	0.43 ± 0.11	0.20 ± 0.03
AMLL	0.06 ± 0.01	0.52 ± 0.13	0.18 ± 0.01

Figure 2 Photosynthetic efficiency measured as dark (ΦPSII) and effective (Φ'PSII) yield in *D. communis* grown with different N-source and light intensities. Data points are means +/- standard deviation, n=3

higher biomass production but only an initial faster growth.

Table 2 reports the productivity (g L^{-1} d^{-1}) and the growth rate (d^{-1}) measured during the exponential phase, and the maximum dry weight (g L^{-1}) measured when the cultures reached the plateau. The productivity of all the cultures grown under low light was the lowest, independently from the nutrient source, and corresponding at 0.06 ± 0.001 g L^{-1} d^{-1} with a maximum dry weight between 0.34 g L^{-1} and 0.52 g l^{-1}. With regard to the cultures supplied with ammonium, in the mixotrophic conditions productivity values were higher than those obtained in the autotrophic conditions supplied with only ammonium and inorganic carbon and not far from the maximum value measured in the autotrophic condition with nitrate. This behaviour could suggest that in the presence of ammonium as sole nitrogen source *D. communis* needs an extra carbon source to grow.

Pam fluorometry measurements

Due to the occurrence of two different metabolic pathways, photosynthesis and respiration of organic carbon substances, the photosynthetic quantum yield was investigated as a function of time, along the growth curve, to investigate the effect of nutritional status and the irradiance.

Figure 2 shows the maximum quantum yield (Φ) and the effective quantum yield (Φ') of the PSII, as indirect measures of real photosynthetic effort of the microalgae and of the effective efficiency of the process, respectively.

In mixotrophic cultures with low light it can be clearly seen how both the maximum and the effective yield, for all the nutritional conditions, remained high and constant during all the 15
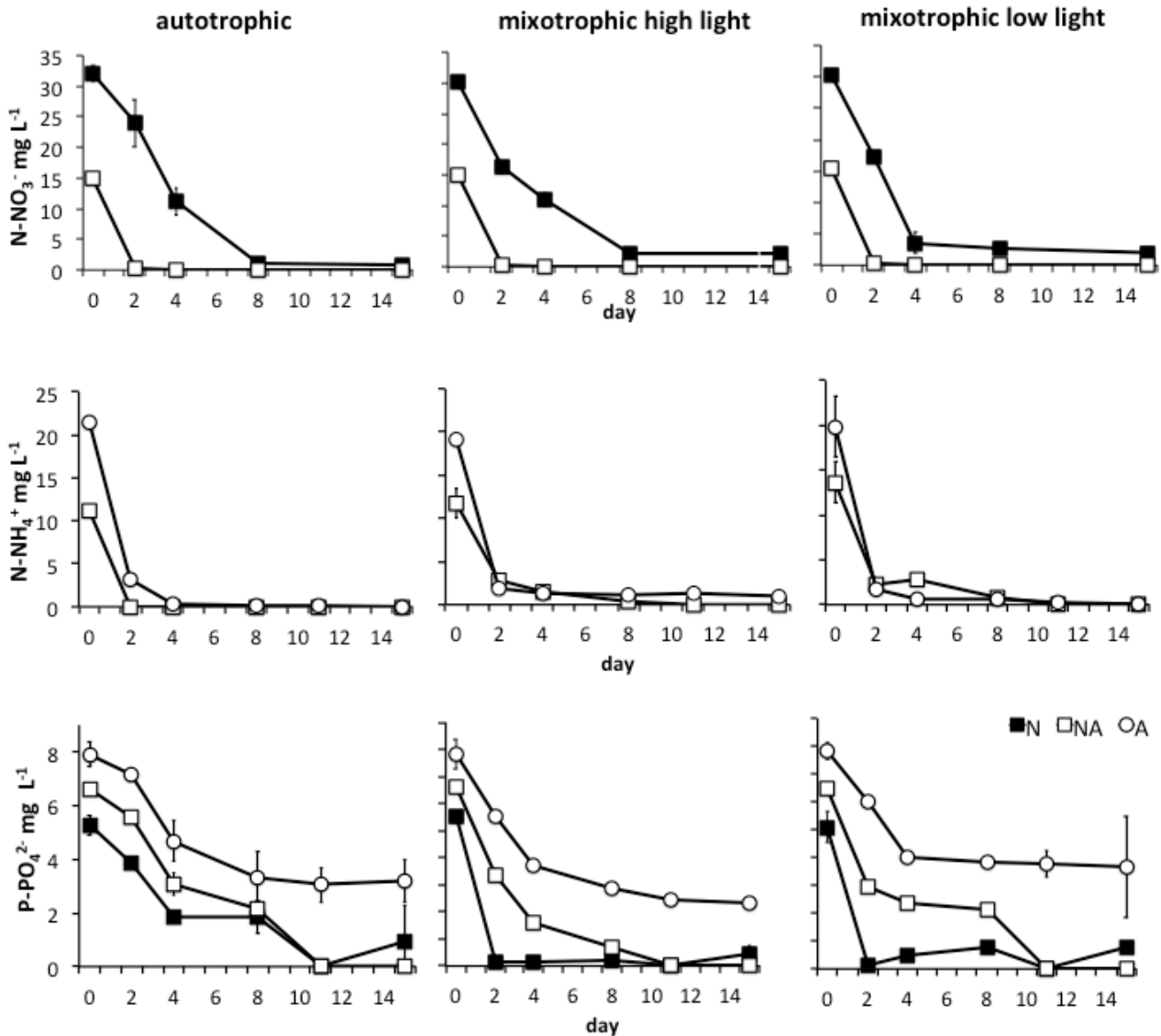
Figure 3 Nutrient removal in in *D. communis* grown with different N- source and light intensities. Data points are means +/- standard deviation, n=3

Table 3 Removal efficiency (RE) for macronutrients (nitrate, ammonium and phosphate) and for glucose. Each value is the mean of three independent measure ± standard deviation.

Culture	$N\text{-}NO_3^-$ mmol $g^{-1} d^{-1}$	$N\text{-}NH_4^+$ mmol $g^{-1} d^{-1}$	$P\text{-}PO_4^{2-}$ mmol $g^{-1} d^{-1}$	Glucose mmol $g^{-1} d^{-1}$
NAHL	12.7 ± 2.1	-	13.7 ± 4.1	-
NMHL	79.3 ± 1.8	-	14.6 ± 0.0	2.5 ± 0.2
NMLL	132.7 ± 2.8	-	15.5 ± 2.8	3.2 ± 0.6
NAAHL	22.8 ± 7.3	98.5 ± 14.3	23.2 ± 6.0	-
NAMHL	21.2 ± 0.5	12.9 ± 0.3	7.2 ± 0.9	1.5 ± 0.1
NAMLL	37.7 ± 2.6	28.1 ± 6.5	22.8 ± 6.3	2.5 ± 0.3
AAHL	-	93.9 ± 3.5	14.6 ± 3.4	-
AMHL	-	36.9 ± 1.5	11.0 ± 0.7	1.0 ± 0.2
AMLL	-	39.0 ± 4.2	15.4 ± 2.4	2.7 ± 0.8

days of the experiment, probably because of the slower consumption of nutrient and the maintenance of all the reaction centres. In cultures with a faster growth and a faster depletion of nutrients a faster drop of the Φ'_{PSII} occurred, as was reported by Rodolfi et al., 2009. It is also interesting to observe how in the condition yielding the highest biomass, that is autotrophic with nitrate, there was a faster drop of the maximum and the effective quantum yields, even though this was not the condition where nutrients first became depleted.

Finally, it is important to notice that, for what concerns the effective quantum yield, results showed significant differences between cultures at different light intensities, but there were no differences either between autotrophic and mixotrophic conditions or between N sources. It can be supposed that the increasing concentration of organic matter, as acetate, could reduce the photosynthetic CO_2 fixation and net O_2 evolution, without effects on respiration and PSII efficiency (Heifetz et al., 2000). Furthermore it is well known that in mixotrophic growth, CO_2 and organic carbon are assimilated simultaneously and, hence, both respiratory and photosynthetic metabolism have to operate concurrently (Marquez et al., 1993; Ogawa and Aiba, 1981). For some microalgae, photosynthesis and the respiration of organic carbon substances seem to function independently.

Nutrient removal efficiency

The evaluation of the RE in batch culture is a very important first step for future applications of microalgae for wastewater treatment. The effect of the glucose addition, media composition and light intensity on macronutrient (nitrate, ammonium, phosphate and glucose) efficiency removal is reported as mmol of nutrient removed per gram of biomass per day in Table 3, while Figure 3 shows the nutrient removal in mg L^{-1} with time.

The removal of nitrogen in the form of nitrate is clearly faster when it is the only nitrogen source in the media, and in low light condition with glucose supply, the nutrient removal can reach 132.7 nmol g^{-1} d^{-1}, but it is moreover obvious that if in this condition there is a lower algal density, there could be a lower nutrient removal.

Concerning ammonium removal, it is possible to observe a fast consumption within 2 days, and a removal rate that can reach values of 93.9 and 98.5 nmol g^{-1} d^{-1}, in autotrophic conditions supplied alone or in combination with nitrate, respectively. According to this result we confirm, as already reported by Perez-Garcia et al. (2011), that ammonium is the most preferred nitrogen source for algae, and it is also the most energetically efficient source, since less energy is required for its uptake. Our results also show that in mixotrophic conditions *D. communis* (fig. 3), showed a really fast depletion of the ammonium in the medium, both with low and high light. This can be explained by the fact that the addition of glucose will allow ammonium assimilation to continue, as well as aminoacid and protein synhtesis, as reported by Geider and Osborne (1989). The low value of RE for N and NA in mixotrophic conditions, shown in table 3, can be explained by the highest biomass productivity measured in the culture during the first 3 days.

Values reported in table 3 for phosphate assimilation, don't show significant differences (p > 0.05) between all the conditions, as phosphate RE is in the range between 11.0 nmol g^{-1} d^{-1} and 23.2 nmol g^{-1} d^{-1}, with no trend determined by the glucose addition or the nitrogen source. The only difference to be underlined is that in the presence of nitrate as the sole N source this was removed faster in the mixotrophic conditions, both with high and low light, with depletion occurring in 2 days instead of the 11 days necessary for autotrophic conditions. It is worthwhile noticing that, as in all the other conditions, *D. communis* was able to remove at least 50% of the inorganic P in 8 days (Fig 3), which confirms results from several studies (de la

Noue and de Pauw, 1988, González and A, 1997 and Voltolina et al., 1999).

Glucose uptake showed marked differences between conditions where media contained nitrate as nitrogen source. Both in low light and darkness, *D. communis* could remove all the glucose from the media in two days, while in the media where only ammonium was added, a complete depletion of the organic carbon source was not achieved in 15 days. The fastest uptake occurred in the LL mixotrophic condition for all the nitrogen condition: N, NA and A with 3.2, 2.5 and 2.7 mmol of glucose removed per gram of biomass per day respectively.

4. Conclusions

One of the most pursued industrial applications of microalgae is related to biofuel production and, for this reason, high biomass with elevated lipid content is the major objective of the cultivation. When considering phytoremediation applications, the most important result to be achieved consists in the fast depletion of nutrients in order to build up a process characterized by low residence times. These applications require microalgae to be able to grow in open ponds, to survive and use high ammonia concentrations and to resist changeable environmental conditions. One of the most challenging of wastewaters types is represented by anaerobic digestion effluents which are very rich in ammonia and very dark in colour. *D. communis* is a robust microalga able to outcompete other microalgae and to thrive in extreme temperature values. Our results show that the best conditions to obtain high *D. communis* biomass is autotrophic growth with nitrate. However, this condition is rarely fulfilled in cultures based on wastewaters, as mentioned above. The *D. communis* strain we used did not show enhanced growth in mixotrophic conditions and could not grow well in low light. However, it showed high values in terms of ammonium and nitrate removal efficiency, especially in mixotrophic conditions with organic carbon added, even in low light. In these conditions it

attained only 50% of the biomass reached when cultures were grown autotrophically, however, the culture productivities showed similar values. A phytoremediation system based on this species could allow frequent collection of the biomass which then could be used for different purposes, such as anerobic digestor refeeding or conversion to fertilizers or animal food.

Last but not least, from a physiological point of view, a good photosynthetic efficiency was maintained even under low light, an aspect which is important considering the dark colour of many wastewaters, so that the survival and the activity of this species can be preserved.

References

Arumugam, M., Agarwal, A., Arya, M.C., Ahmed, Z., 2013. Influence of nitrogen sources on biomass productivity of microalgae *Scenedesmus bijugatus*. Bioresour. Technol. 131, 246–249.

Berges, J.A., Franklin, D.J., Harrison, P.J., 2001. Evolution of an Artificial Seawater Medium: Improvements in Enriched Seawater, Artificial Water Over the Last Two Decades. J. Phycol. 37, 1138–1145.

Chandra, R., Rohit, M.V., Swamy, Y.V., Venkata Mohan, S., 2014. Regulatory function of organic carbon supplementation on biodiesel production during growth and nutrient stress phases of mixotrophic microalgae cultivation. Bioresour. Technol.

Cheirsilp, B., Torpee, S., 2012. Enhanced growth and lipid production of microalgae under mixotrophic culture condition: effect of light intensity, glucose concentration and fed-batch cultivation. Bioresour. Technol. 110, 510–516. doi:10.1016/j.biortech.2012.01.125

Chisti, Y., 2007. Biodiesel from microalgae. Biotechnol. Adv. 25, 294–306.

Christenson, L., Sims, R., 2011. Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts. Biotechnol. Adv. 29, 686–702.

De la Noue, J., de Pauw, N., 1988. The potential of microalgal biotechnology: a review of production and uses of microalgae. Biotechnol. Adv. 6, 725–770.

Dortch, Q. (1990). The interaction between ammonium and nitrate uptake in phytoplankton.

Marine ecology progress series. Oldendorf, *61*(1), 183-201.

DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric Method for Determination of Sugars and Related Substances. Anal. Chem. 28, 350–356.

Fiori, E., Mazzotti, M., Guerrini, F., Pistocchi, R., 2013. Combined effects of the herbicide terbuthylazine and temperature on different flagellates from the Northern Adriatic Sea. Aquat. Toxicol. 128–129, 79–90.

r, R.J., Osborne, B.A., 1989. Respiration and microalgal growth: a review of the quantitative relationship between dark respiration and growth. New Phytol. 112, 327e341.

Genty, B., Briantais, J.-M., Baker, N.R., 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim. Biophys. Acta BBA - Gen. Subj. 990, 87–92.

González, L.E., A, R.B., 1997. Efficiency of Ammonia and Phosphorus Removal from a Colombian Agroindustrial Wastewater by the Microalgae *Chlorella vulgaris* and *Scenedesmus dimorphus*.

Heifetz, P.B., Förster, B., Osmond, C.B., Giles, L.J., Boynton, J.E., 2000. Effects of Acetate on Facultative Autotrophy in *Chlamydomonas reinhardtii* Assessed by Photosynthetic Measurements and Stable Isotope Analyses. Plant Physiol. 122, 1439–1446.

Marquez, F.J., Sasaki, K., Kakizono, T., Nishio, N., Nagai, S., 1993. Growth characteristics of *Spirulina platensis* in mixotrophic and heterotrophic conditions. J. Ferment. Bioeng. 76, 408–410.

Ogawa, T., Aiba, S., 1981. Bioenergetic analysis of mixotrophic growth in *Chlorella vulgaris* and *Scenedesmus acutus*. Biotechnol. Bioeng. 23, 1121–1132.

Oswald, W.J., 1992. Micro-algae and waste-water treatment. In: Borowitzka, M.A., Borowitzka, L.J. (Eds.), Microalgal Biotechnology. Cambridge University Press, Cambridge, pp. 305e328.

Perez-Garcia, O., Escalante, F.M.E., de-Bashan, L.E., Bashan, Y., 2011a. Heterotrophic cultures of microalgae: metabolism and potential products. Water Res. 45, 11–36.

Rodolfi, L., Chini Zittelli, G., Bassi, N., Padovani, G., Biondi, N., Bonini, G., Tredici, M.R., 2009. Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol. Bioeng. 102, 100–112.

Samorì, G., Samorì, C., Guerrini, F., Pistocchi, R., 2013. Growth and nitrogen removal capacity of *Desmodesmus communis* and of a natural microalgae consortium in a batch culture system in view of urban wastewater treatment: Part I. Water Res. 47, 791–801.

Shi, Zhang, Chen, 2000. Heterotrophic production of biomass and lutein by *Chlorella protothecoides* on various nitrogen sources. Enzyme Microb. Technol. 27, 312–318.

Tredici, M.R., 2004. Mass production of microalgae: photobioreactors. In: Richmond, A. (Ed.), Handbook of Microalgal Culture. Biotechnology and Applied Phycology. Blackwell Publishing, Oxford, UK, pp. 178e214

Voltolina, D., Cordero, B., Nieves, M., Soto, L.P., 1999. Growth of *Scenedesmus* sp. in artificial wastewater. Bioresour. Technol. 68, 265–268.

Xin, L., Hu, H., Ke, G., Sun, Y., 2010. Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus* sp. Bioresour. Technol. 101, 5494–5500.

Chapter 4

Effects of phytohormones on growth and photosynthetic efficiency of *Botryococcus braunii* and *Desmodesmus communis*

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Introduction

Metabolic engineering and synthetic biology are gaining attention due to their potential to enhance living systems especially microbes for medical, agricultural, industrial and environmental applications. However, genetic manipulations leads to inheritable changes in a species that might affect the ecosystem adversely when used for environmental and agricultural applications.

Many different species of microalgae are studied nowadays for industrial purpose, especially microalgae belonging to the Chlorophyta division. The freshwater algal genera *Desmodesmus* and *Scenedesmus* are commonly found in water bodies and different species of the latter have been studied worldwide *in vitro* due to their rapid growth, ease of handling and adaptation to the environmental conditions; in addition, *Scenedesmus* spp. are versatile organisms for the use in domestic and industrial wastewater treatment (Hodaifa et al., 2008; Lürling, 2003; Mayeli et al., 2005; Voltolina et al., 1999)*.* Another interesting microalgae in the field of biotechnology is *Botryococcus braunii,* which has a great potential because of the production of hydrocarbons, molecules that can be easily converted into fuels. In fact, up to 86% of the dry weight of *Botryococcus braunii* can be long chain hydrocarbons (Banerjee et al., 2002; Cheng et al., 2013; Hirose et al., 2013; Ranga Rao et al., 2007), on the other hand, this species is characterized by a very slow-growing rate (Banerjee et al., 2002; Metzger and Largeau, 2005).

At present, it is generally believed that phytohormones and the associated regulatory mechanisms emerged in an ancient organism prior to the split of seedless plants and seed plants (Rensing et al., 2008). Attempts to improve microalgal biomass productivity using alternative means such as phytohrmones and micronutrients, has been reported a few times since the 1930's. Although contemporary research remains almost completely focused on higher plants, there are few studies devoted to biochemical stimulants such as phytohormones, polyamines and chemicals offering significant potential to enhance microalgal productivity (Bajguz and Piotrowska-Niczyporuk, 2014; Czerpak et al., 1994; Czerpak and Bajguz, 1997; Hunt et al., 2011, 2010; Piotrowska and Bajguz, 2011). From early researches, several effects on microalgae have been observed. Exogenously applied phytohormones and plant growth regulator in microalgae cultures can modify the heavy metal biosorption (Piotrowska-Niczyporuk et al., 2012), can affect the growth and some molecular component content (Czerpak et al., 2006). For example Czerpark and Bajguz (1993) saw that protein excretion by *Chlorella pyrenoidosa* cells increased by 675-1050% with application of auxins (indolyl-3-acetic, indolyl-3-propionic, indolyl-3-lactic, and indolyl-3-butyric acids), by 250-275% with auxin precursors (anthranilic acid and tryptamine), and by 187-325% with chemical analogues of auxins (2,4-dichlorophenoxyacetic, phenylacetic, naphthyl-3-acetic, and naphthyl-3- sulphonic acids),

while Gonzalez and Bashan (2000) observed that the addition of exogenous indolyl-3-acetic acid (IAA) to *C. vulgaris* culture significantly increased cell multiplication. Moreover, it has been observed that the mechanism of auxins action in algal cells are associate with oxidative stress, having a precise control on H_2O_2 that allows cell division and metabolite production of the cells (Piotrowska-Niczyporuk and Bajguz, 2014).

Another cultivation method to enhance growth or change metabolic products in algae can take advantage from their ability to use organic carbon as an energy source and this aspect is important because it can minimize the inhibitory effects of seasonal and diurnal light limitation in outdoor cultures. Weetall (1985)

reported that a *B. braunii* strain did not grow heterotrophically in the dark, but its growth could be enhanced by the addition of carbon compounds including glucose, mannose, fructose, galactose, sucrose, lactic acid, or hydrolyzed cheese under light condition. However, there is still a lack of comprehension on how to optimize the growth of this species.

It is also recognized that the research on the effect of plant growth regulators on microalgae is mainly focused on the species *Chlorella,* while few studies concern different microalgae, such as *Haematococcus pluvialis* or *Dunaliella salina* (Hunt et al. 2011). On the contrary, there are few or no information on the effect of phytohormons on *Scenedesmus* spp. (Mazur et al., 2001) and *B. braunii.*

Table 1 Experimental set up with biochemical stimulants and dosage added to *B. braunii* and *D.communis* batch cultures.

Organism	Culture	Stimulant	Type of stimulant	Concentration (mg L^{-1})	Price (€ g^{-1})
I experiment					
D. communis	**CTRL**	-	-	-	
	NO₃⁻	Nitrate	Macronutrient	28	
	PAA 15	Phenylacetic acid	Auxin	15	0.12
	PAA 30	Phenylacetic acid	Auxin	30	
	NAA 2.5	Napthalene acetic acid	Auxin	2.5	1.08
	NAA 5	Napthalene acetic acid	Auxin	5	
	GB 2.5	Gibberellic acid	Gibberellin	2.5	117.00
	GB 5	Gibberellic acid	Gibberellin	5	
	SPM 0.75	Spermidine	Polyamine	0.75	28.30
	SPM 1.5	Spermidine	Polyamine	1.5	
	PUT 0.05	Putrescine	Polyamine	0.05	6.46
	PUT 0.1	Putrescine	Polyamine	0.1	
II experiment					
B. braunii	**CTRL**	-	-	-	
	GLU	Glucose	Organic carbon	1000	
	PAA 15	Phenylacetic acid	Auxin	15	0.12
	PAA 30	Phenylacetic acid	Auxin	30	
	NAA 2.5	Napthalene acetic acid	Auxin	2.5	1.08
	NAA 5	Napthalene acetic acid	Auxin	5	
	GB 2.5	Gibberellic acid	Gibberellin	2.5	117.00
	GB 5	Gibberellic acid	Gibberellin	5	
	SPM 0.75	Spermidine	Polyamine	0.75	28.30
	SPM 1.5	Spermidine	Polyamine	1.5	
	PUT 0.05	Putrescine	Polyamine	0.05	6.46
	PUT 0.1	Putrescine	Polyamine	0.1	

The goal of this study was to optimize the growth capacity, through the addition of growth stimulators, of two microalgal species belonging to the Chlorophyta phylum: *Desmodesmus communis* and *Botryococcus braunii.* as a potential tool for phytoremediation applications and as source of renewable fuel, respectively. Additional aims were: to monitor an hypothetical stress through the photosynthetic efficiency measurement and to follow the fluctuation of pH, a parameter having a really important role on growth rate.

Materials and methods
Algal strains and inocula preparation
D. communis cultures were established following isolation from an artificial freshwater pond in the province of Forlì-Cesena (Emilia-Romagna, Italy)(Samorì et al., 2013) while *B. braunii* (strain 807.1) was purchased from SAG Culture Collections. Both strains were maintained in the aseptic autotrophic medium CHU 13 due to its superiority in terms of biomass productivity, over BG11 (data not shown). The medium, originally from Largeau et al. 1980, was modified and it contained (mg/l): KNO_3, 200; K_2HPO_4, 40; $MgSO_4 \cdot 7H_2O$, 100; $CaCl_2 \cdot 2H_2O$, 54; $C_6H_5FeO_7$, 10; Citric acid, 100; micro elements: B, 0.3; Mn, 0.2; Zn, 0.05; Cu, Co and Mo, 0.02. The same medium was used for *D. communis*, but in this case 107 mg/l of NH_4Cl was added instead on KNO_3 as nitrogen source. The cultures were pre-adapted to culture conditions in 250 ml flasks, illuminated by cold-white fluorescent lamps with light intensity of 100 µmol m^{-2} s^{-1}, and a photoperiod 12/12 h light/dark cycle. The temperature for algal broth was 20 ± 2 °C during the cultivation.

Biochemical stimulants selection and experimental set up
The selection of the phytohrmones and polyamines was done on the basis of the best performer from literature research (Hunt et al., 2010) and on the commercial availability and price of the stimulants. Phythormones and

polyamines were purchased from Sigma-Aldrich, St. Louis, MO, USA. Known quantity of each stimulant was dissolved in methanol and filtered at 0.22 µm to avoid bacterial contaminations. The final methanol concentration in the culture medium never exceeded 0.02%. The results presented in this study are a compilation of two experiments as described in table 1. The first experiment, with *Desmodesmus communis,* was conducted using CHU13 medium with ammonium as nitrogen source and by testing 11 different conditions: 5 individual biochemical stimulants under two different dosage, and one conditions with nitrate as nitrogen source to simulate the optimal growth (Mazzotti et al., this thesis). The control consisted of *D. communis* cultures in CHU13 medium with ammonium as nitrogen source. The second experiment was conducted to evaluate the effect of 5 different biochemical stimulants and 1 organic carbon source on *Botryococcus braunii,* grown in CHU 13 medium with NO_3^-. In this experiments, the control is represented by *B. braunii* grown with CHU13 medium with nitrate as nitrogen source. Both experiments were conducted in batch cultures performed in 45 ml glass tubes with 30 ml of medium, mixed with a vortex-mixer multi tube (VWR). All tubes were kept in a temperature controlled chamber at 20 ± 2 °C with light intensity of 100 µmol m^{-2} s^{-1}, and a photoperiod 12/12 h light/dark cycle. In all the experiments, the cultures were sampled on pre-determined days: 5, 10 and 14 for *D. communis* and 7 and 14 for *B. braunii.* Stationary phase samples from growing cultures of both microalgae were used as the inoculum, having an initial cell concentration of 0.04 and 0.05 g l^{-1} for *D. communis* and *B. braunii*, respectively. Each treatment was conducted in triplicates. In table 1 is also reported the cost of each substance, in order to explain the choice of the selected treatments based on previously reported performances and on the best price. All prices are from the Companies where the chemicals have been purchased.

Abbreviations
All the treatments are identified on the plot with the following abbreviations.

Desmodesmus communis with: nitrate, NO_3^-; 15 mg L^{-1} and 30 mg L^{-1} of Phenyl-acetic acid, PAA_{15} and PAA_{30}; 15 mg L^{-1} and 30 mg L^{-1} of Napthalene-acetic acid, NAA_{15} and NAA_{30}; 2,5 mg L^{-1} and 5 mg L^{-1} of Gibberellic acid, $GB_{2,5}$ and GB_5; 0.75 mg L^{-1} and 1.5 mg L^{-1} of Spermidine, $SPM_{0.75}$ and $SPM_{1.5}$; 0.05 mg L^{-1} and 0.1 mg L^{-1} of Putrescine, $PUT_{0.05}$ and $PUT_{0.1}$.

Botryococcus braunii with: glucose, GLU, 15 mg L^{-1} and 30 mg L^{-1} of Phenyl-acetic acid, PAA_{15} and PAA_{30}; 15 mg L^{-1} and 30 mg L^{-1} of Napthalene-acetic acid, NAA_{15} and NAA_{30}; 2,5 mg L^{-1} and 5 mg L^{-1} of Gibberellic acid, $GB_{2,5}$ and GB_5; 0.75 mg L^{-1} and 1.5 mg L^{-1} of Spermidine, $SPM_{0.75}$ and $SPM_{1.5}$; 0.05 mg L^{-1} and 0.1 mg L^{-1} of Putrescine, $PUT_{0.05}$ and $PUT_{0.1}$.

Analysis and calculations
Growth was followed by measuring the dry weight, obtained by filtering 7 ml of algal culture through a preweighted Whatman GF/C filter membrane (25 mm diameter). The membrane was then oven dried at 105°C for 1h and then cooled down in a desiccator and weighted.

In experiment 1 and 2 the effective quantum yield of PSII was measured through a Pulse Amplitude Modulation fluorometer (101-PAM connected to a PDA-100 data acquisition system, H. Walz, Effeltrich, Germany). 3 ml of algal samples were analyzed after 15 min dark adaptation. Continuous actinic light of about the same intensity as the algae were exposed to during the experiments, was applied for 5 min to permit the stabilization of the yield. The operational quantum yield Φ_M' was obtained by the ratio:

$$\Phi'_M = (F'_m - F)/F'_m = Fv'/F'_m$$

where F'_m is the maximum fluorescence after a saturation pulse (> 3000 µmol photons m^{-2} s^{-1} for 0.8 s) and F is the steady state fluorescence (Genty et al., 1989).

The pH was monitored using a pH glass electrode (PHM 92, Radiometer Copenaghen). Specific values of biomass productivity (P, g l^{-1} d^{-1}) were calculated using the following equation:

$$P = (X_1 - X_0)/(t_1 - t_0)$$

where X_1 and X_0 were the biomass concentration (g l^{-1}) on days t_1 and t_0 respectively.

Statistical analysis
Statistical analyses were performed with PAST 2.17 software. One-way ANOVA was conducted to test differences in dry weight measures, pH values, photosynthetic efficiency and biomass productivity among all treatments. Levene and Tukey test were performed to verify the homogeneity of the variance and to do pairwise comparison respectively. All the data are reported as means ± standard deviations of triplicates.

Results
Desmodesmus communis
The first experiment showed that in *D. communis* nine out of eleven treatments determined the increase in biomass values compared to the control (Fig. 1). Highest biomass values were observed for the ideal condition represented by growth in nitrate-added CHU 13 medium. On the other hand, if we consider the effect of phytohormones related to *D. communis* grown in NH_4^+- CHU 13 medium (control), both auxins had a significant positive effect ($p<0.05$) on biomass values particularly from the first days of growth. *D. communis* grown in presence of gibberellic acid mantained, during all the growth curve, biomass values lower than the control (ctrl), while the cultures added with spermidine (SPM) showed a slower increment of cell biomass respect to the control and the other treatments, with higher values, measured only in the last day of the experiment. On the contrary, the other polyamine tested
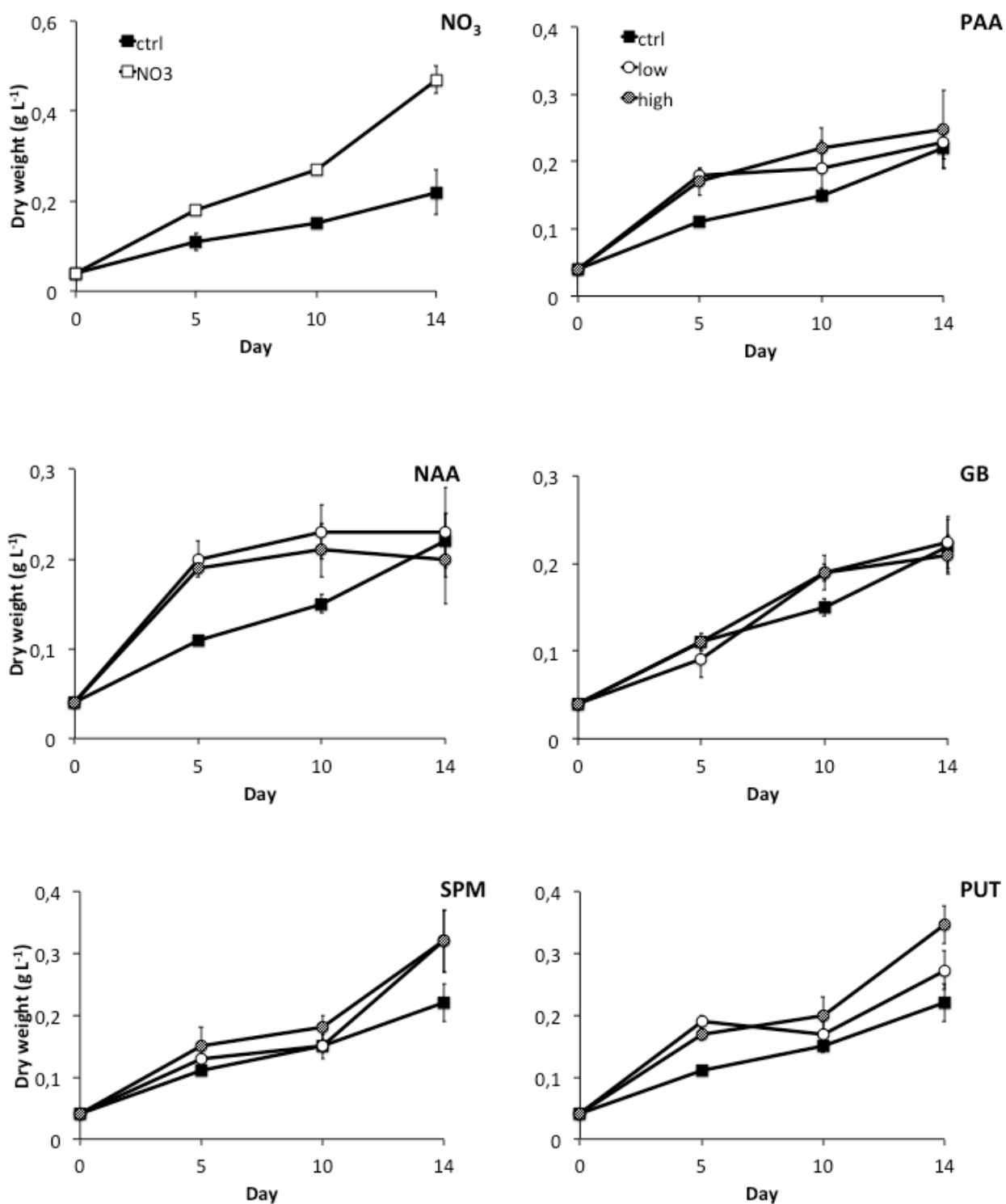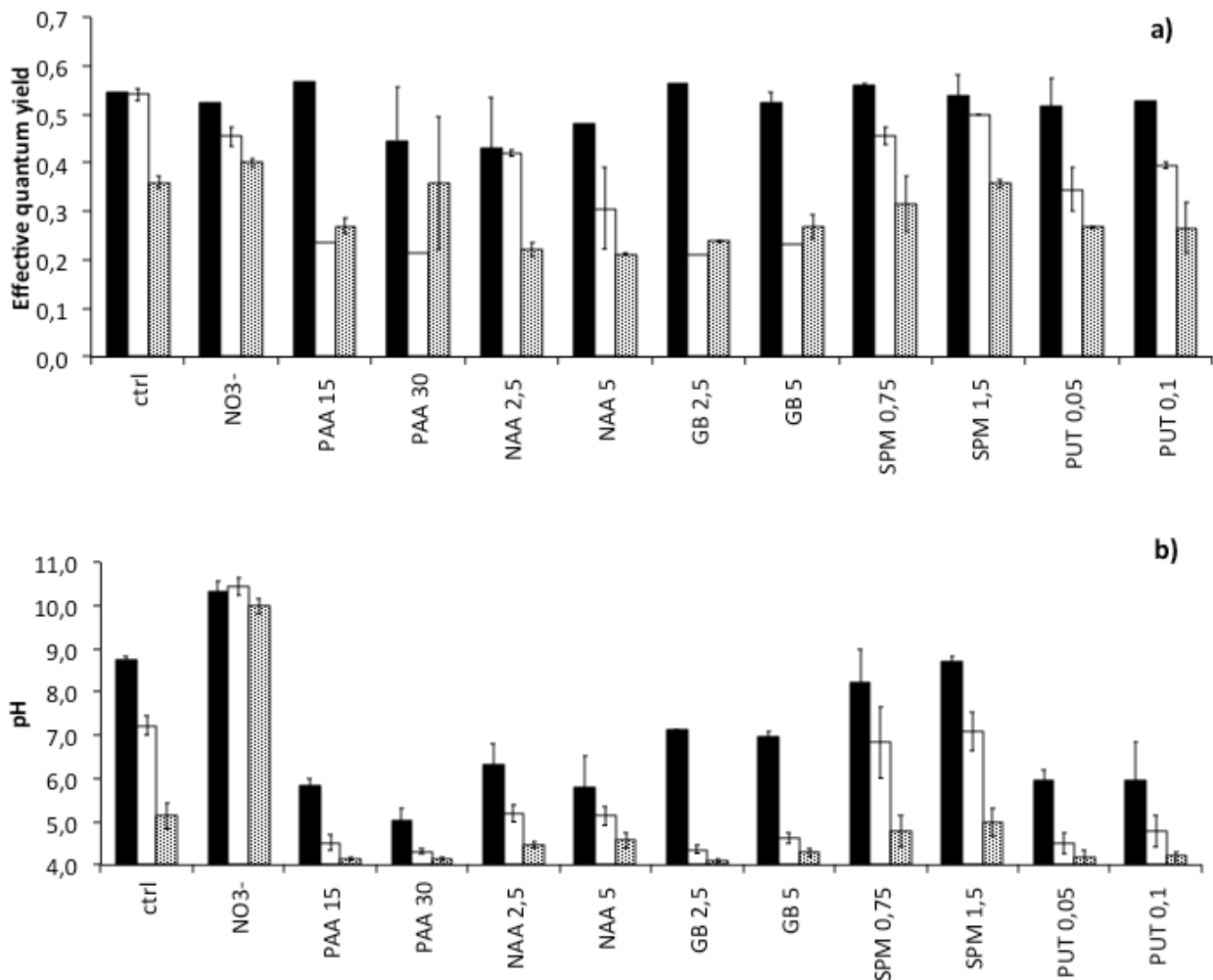
Figure 1 Growth curves of *D. communis* grown under different treatments. Data points are means ± standard deviation, n=3. Abbreviations are explained in the text.

Figure 2 Effect of the addition of phytohormones and polyammines on the photosynthetic efficiency (a) and external pH (b) of
D. communis **cultures. Measurements were performed at day 5 (black columns), 10 (white columns) and 14 (spotted columns) of growth and compared with a control culture where N source was ammonium, as in the treatments, and a culture where ammonium was replaced by nitrate. The double-sided bar represent the standard deviation (n=3).**

(putrescine, PUT), at both concentrations, determined an increase in the dry weight compared with the control, all along the growth curve.

Due to the reported effect of several phytohormones on growth which, in turn, is related to the photosynthetic activity, the efficiency of the effective quantum yield of PSII has been used to characterize the cell response to the added biochemicals (Fig. 2a). At day 5 yield measurements were the highest in all the conditions, with values around 0.5 and no significative ($p < 0.05$) differences between control and treatments. *D. communis* cultures treated with PAA_{15}, PAA_{30}, $GB_{2.5}$ and GB_5, showed a marked decrease of the photosynthetic efficiency on day 10 which

continued on day 14, while all the cultures with other biochemical stimulants, in particular polyammines, displayed only a low decrease of the PSII's yield. From fig. 1b it can be clearly observed that the control and the NO_3^-ccultures had a prolonged and more stable photosynthetic efficiency compared to all the conditions containing the bio-stimulants.

In figure 2b it is shown the effect of the phytohormones addition on the extracellular pH of *D. communis*. The cultures added with NAA, PAA, PUT and GB, showed a significant drop of the pH starting already on day 5, compared to the control. Only the cultures added with nitrate mantained a pH level stable, with values around 10. Overall these results evidence a direct correlation between the pH
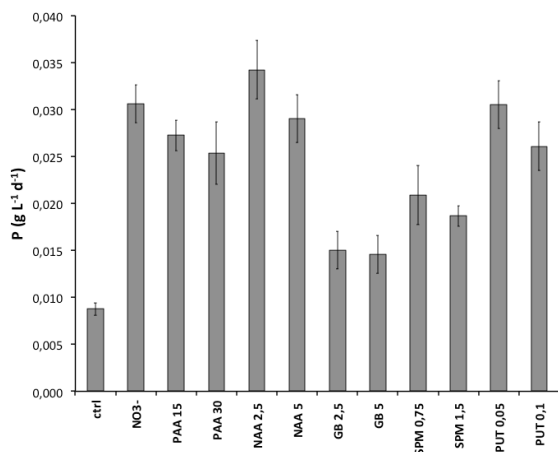
Figure 3 Productivity (P) (g L^{-1} d^{-1}) values of *D. communis* cultures added with biochemical stimulants calculated between day 0-5. The double sided bar represent the standard deviation (n=3).

drop and the decrease of the photosynthetic efficiency.

Cultures productivity (g L^{-1}d^{-1}) was also calculated during the exponential phase for each culture (Fig. 3). All treatments determined an increase of the productivity compared to the 0.009 g L^{-1}d^{-1} value of the control. In the first 5 days of growth *D. communis* added with PAA, NAA and PUT grew faster than the control, with a 200%, 278% and a 244% increase in biomass productivity. Specifically, in the presence of the above-mentioned stimulants the observed increment was similar or even higher than that obtained when nitrate was supplied to the medium. In contrast to the above pattern, we can mention the really low effect determined by the addition of gibberellic acid, which determined a productivity of only 0.015 g L^{-1} d^{-1} at both dosages.

We also observed that the use of different concentrations of the biochemical substance didn't produce any significative effect (p> 0.05): the treatments with the same stimulant at different dosage, presented the similar trend in terms of biomass, photosynthetic yield, pH level and productivity.

Botryococcus braunii

Figure 4 shows the effect biochemical stimulants on the biomass, the photosynthetic efficiency and external pH of *B. braunii*

cultures. The addition of PAA, NAA, GB and PUT caused only a slight increase in average biomass values between days 0 and 7 (fig. 3a); only the PAA$_{15}$ treatment, at day 14, induced a high increment in the biomass, with values of 0.36 g L^{-1} compared to 0.26 g L^{-1} of the control. The best performing substance, with the highest positive effect on growth, was glucose whose effect was observed all along the growth curve. Conversely, the treatment with the polyamine SPM$_{0.75}$ and SPM$_{1.5}$ did no cause statistically significant differences on biomass values, during all the experimental time, compared with the control (p<0.05).

In *B. braunii*, the presence of phytohormones, as PAA, NAA and GB, determined a decrease of the PSII photosynthetic efficiency from day 14, while in the cultures added with both polyamines the decrease was smaller. On the other hand, the control and the culture added with 1g L^{-1} of glucose had low values of yield, of 0.25 and 0.24 respectively, only at the end of the growth (fig 4a).

The response to the treatments in terms of pH (fig. 4b) did no show statistically significant differences with the control, except for the condition with glucose, in which the pH slightly increased from 8.3 to 8.5. In all the other treatments as well as in the control, a similar trend was found: the pH value was 8.6 on day 7, and after seven days it slightly decreased to around 8.3, reaching a stabilization till the end.

From the productivity calculations (fig 5) it was found that 9 treatments caused an increase compared to the control value, which was of 0.015 g L^{-1}d^{-1}. The best performing conditions was represented by the organic carbon supply in fact glucose induced a 76% increase of the productivity which reached the value of 0.030 g L^{-1}d^{-1}. The least effective treatment was represented by the addition of SPM, at both dosages, as the cultures had the same productivity values of the control. All the other substances improved *B. braunii* productivity no more than 47%.

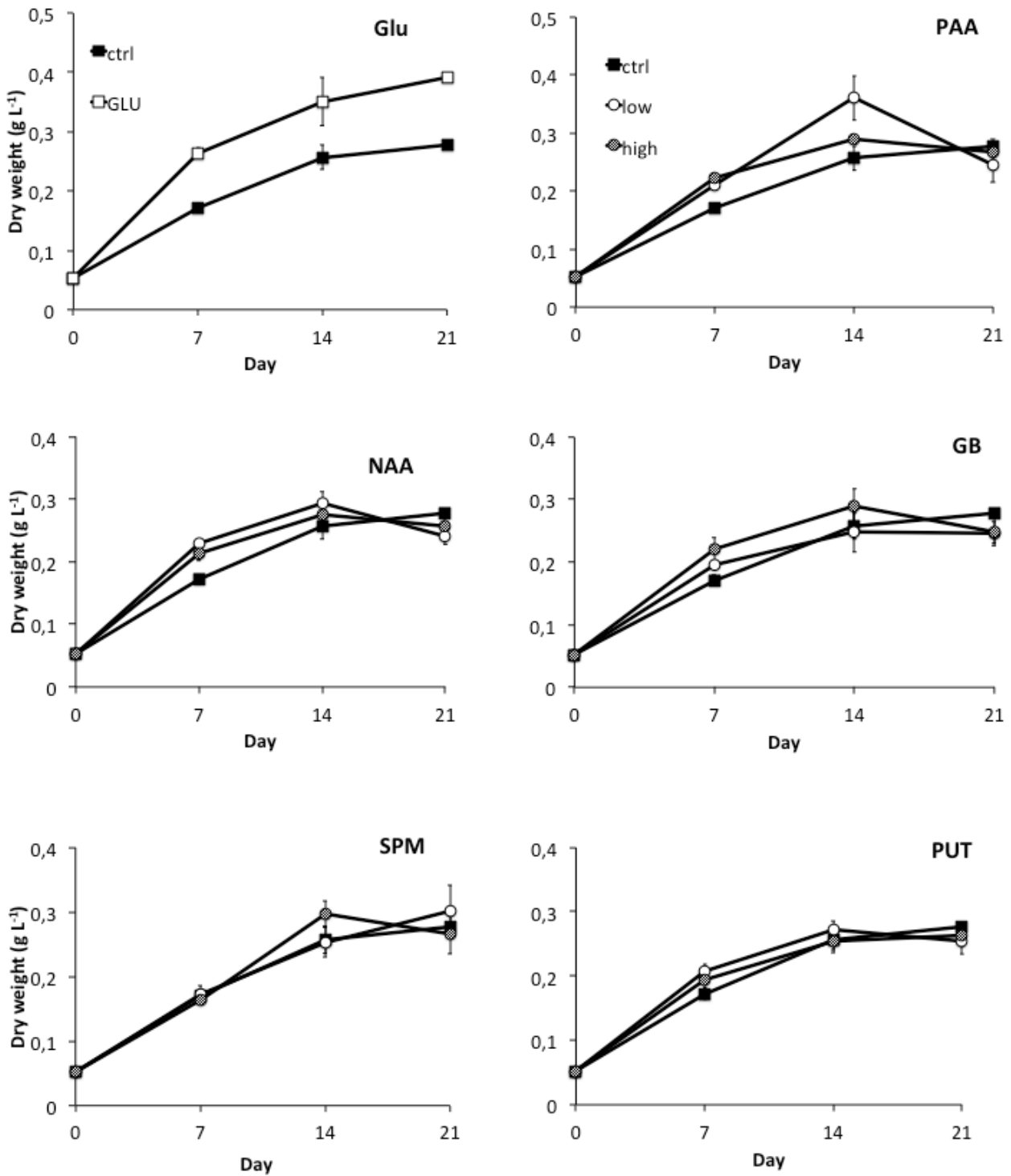No significant differences were observed between controls and the cultures added with

Figure 4 Growth curves of *B. braunii* grown under different treatments. Data points are means ± standard deviation (n=3).
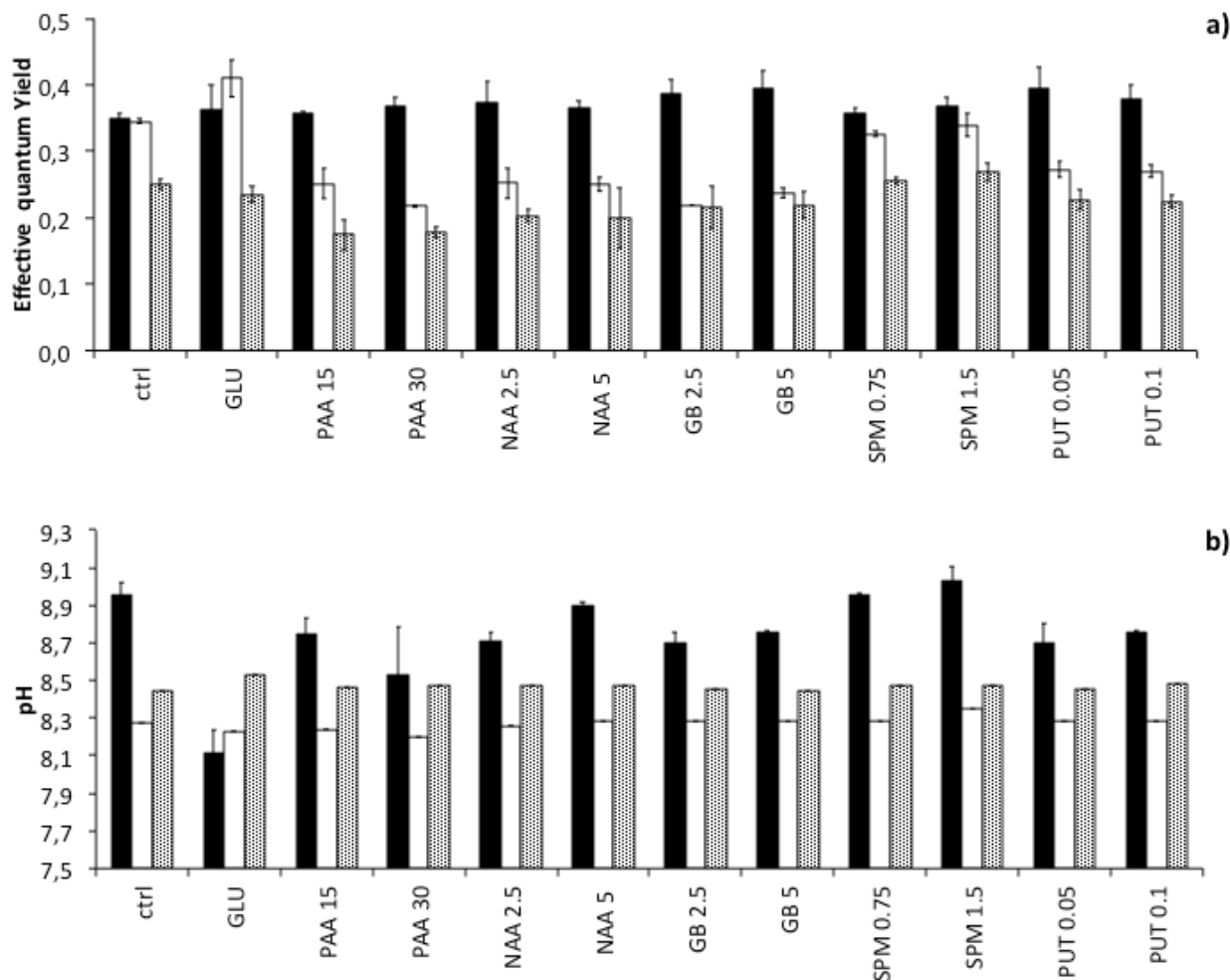
Figure 5 Effect of the addition of glucose, phytohormones and polyammines on the photosynthetic efficiency (a) and external pH (b) of *B. braunii* cultures measured at day 7 (black columns), 14 (white columns) and 21 (spotted columns) of growth. The double-sided bar represent the standard deviation (n=3).

0.02% methanol for both *D. communis and B. braunii* (data not shown).

Discussions

The two auxins, PAA and NAA, determined a similar pattern of increasing biomass, in both microalgae. The increase in biomass productivity was higher in the first part of the growth curve and then drastically reduced between day 5 and 10 and day 14 and 21 for *D.communis* and *B. braunii*, respectively. This result could be indicative of the role of auxins in shortening the lag period thus enhancing the biomass productivity within a short cultivation period. The same phenomenon has already observed by Hunt et al. (2010).

A positive effect of phytohormones of the auxin group on other microalgae, e.g. *Chlorella,* had been previously studied. Czerpak et al. (1999) found a strong stimulation by IAA on protein, aldohexoses, and chlorophyll content in *Chlorella pyrenoidosa.* The stimulating activity of the auxins studied on the molecular level of cells in green algae is similar to vascular plants (Czerpak et al. 1999).

Polyamines had a positive effect on the photosynthetic efficiency of both species: higher yield values in the first period of growth followed by a graduate decrease have been observed compared with all the tested phytohormones. SPM and PUT had also the positive effect of causing a marginal increase of productivity in *D. communis* . These results confirmed the stimulation of the photosynthetic process, previously found in *Chlorella* sp. by Czerpak et al. (2003) through
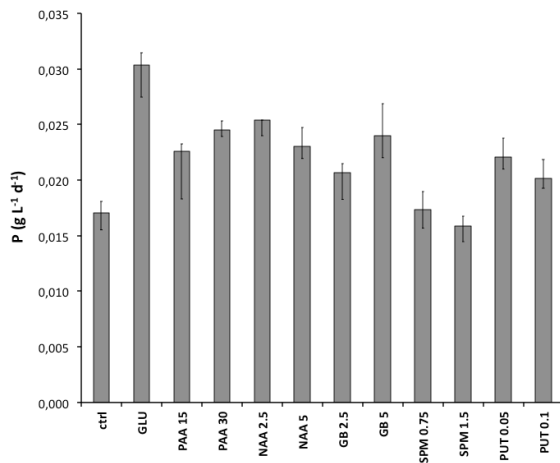
Figure 6 Productivity values (P) (g L^{-1} d^{-1}) of *B. braunii* cultures added with biochemical stimulants calculated between day 0-7. The double sided bar represent the standard deviation (n=3).

the addition of similar concetrations of spermidine and putrescine. It is although well known that Chlorophyta can uptake great amounts of polyamines passively (Badini et al., 1994) but slightly increase their biomass productivity if the culture is growing with low CO_2 (Logothetis et al., 2004).

Higher pH variations were observed in *D. communis* cultures supplied with auxins and gibberellins, compared to *B. braunii* grown with the same treatments, probably due to the concurrence of ammonium presence in the medium causing the release of protons (Xin et al., 2010). The higher biomass values and the medium acidification in presence of auxins can be also explained with the "cell wall acidification" model, that has been proposed to explain the effect of IAA in plant cell elongation (Salisbury and Ross, 1994). This model suggests that the auxins make the receptor cells situated in sections of the stem or the coleoptiles to excrete protons in the surrounding primary cell walls, thereby reducing the pH. Lower pH allows the activity of cell wall-degrading enzymes that are inactivated at higher pH. These enzymes are thought to break the bonds between polysaccharides (cellulose, hemicelluloses) in the cell wall, allowing it to expand easier. This model is limited by the facts that acidification

of the cell wall happens even in the absence of the auxin and that there is growth promotion even without acidification of the cell wall (Salisbury and Ross, 1994). From the biomass and the photosynthetic efficiency results on both microalgae, this study seems to suggest a mechanism of hormone-induced cell division or proliferation rather than growth stimulation due to enhancedphotosynthetic efficiency (Hunt et al. 2011).

The gibberellic acid addition slightly increased the productivity of both species, however the best productivity of *B. braunii* was measured in glucose-added cultures. Padhy and Pattanaik, (1976) found that the growth of *Westiellopsis prolifica*, a blue-green alga, in presence of exogenous gibberellin, was accelerated if organic compounds were also present. This result indicates the possibility that gibberellins can improve heterotrophic culture growth because these substances are involved in the control of the assimilation of the exogenous sources of organic carbon by cells. For this reason a mixture of glucose and gibberellins could be tested in the future.

As the different dosages used didn't show differences in results, this is an indicator that such small concentrations of phytohormones are necessary to induce positive effects on microalgae and also that high dosages can became toxic for the microorganism (Lau et al., 2009).

Conclusions

In conclusion, considering these preliminary data, we can assume that the best-case scenario for biomass production with *D. communis* is represented by PAA and NAA addition at the lowest concentration of 15 mg/l and 2.5 mg/l respectively. These two substances recorded a 200 % increase in productivity of cultures performed in a media containing ammonium instead of nitrate, as it could happen in a phytoremediation process; an additional positive aspect is that their actual cost is the lowest compared with all the other chemicals tested in this study. Conversely, despite the 50% increase of productivity due to the

treatment with auxins, the condition that caused a more rapid growth, with high photosynthetic performance, in *B. braunii* was glucose supply.

However, more detailed researches to test the effective role of phythormones on microalgae productivity is necessary. Future studies can examine, for example, the effect of NAA and PAA on the inorganic nutrient removal and molecular content of *Desmodesmus communis* and the response, in terms of growth rate, of *Botryococcus braunii* by adding phythormones under different environmental conditions, such as oganic carbon supply, temperature and light.

References

Badini, L., Pistocchi, R., Bagni, N., 1994. Polyamine Transport in the Seaweed *Ulva Rigida* (chlorophyta) 1. J. Phycol. 30, 599–605.

Bajguz, A., Piotrowska-Niczyporuk, A., 2014. Interactive effect of brassinosteroids and cytokinins on growth, chlorophyll, monosaccharide and protein content in the green alga *Chlorella vulgaris* (Trebouxiophyceae). Plant Physiol. Biochem. 80, 176–183.

Banerjee, A., Sharma, R., Chisti, Y., Banerjee, U.C., 2002. *Botryococcus braunii*: A Renewable Source of Hydrocarbons and Other Chemicals. Crit. Rev. Biotechnol. 22, 245 – 279.

Cheng, P., Ji, B., Gao, L., Zhang, W., Wang, J., Liu, T., 2013. The growth, lipid and hydrocarbon production of *Botryococcus braunii* with attached cultivation. Bioresour. Technol. 138, 95–100.

Czerpak, R., Bajguz, A., 1997. Stimulatory effect of auxins and cytokinins on carotenes, with differential effects on xanthophylls in the green alga *Chlorella pyrenoidosa* Chick. Acta Soc. Bot. Pol. 66, 41–46.

Czerpak, R., Bajguz, A., Białecka, B., Wierzchołowska, L.E., Wolańska, M.M., 1994. Effect of auxin precursors and chemical analogues on the growth and chemical composition in *Chlorella pyrenoidosa* Chick. Acta Soc. Bot. Pol. 63, 279–286.

Czerpak, R., Piotrowska, A., Szulecka, K., 2006. Jasmonic acid affects changes in the growth and some components content in alga *Chlorella vulgaris*. Acta Physiol. Plant. 28, 195–203.

Genty, B., Briantais, J.-M., Baker, N.R., 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim. Biophys. Acta BBA - Gen. Subj. 990, 87–92.

Gonzalez, L.E., Bashan, Y., 2000. Increased Growth of the Microalga *Chlorella vulgaris* when Coimmobilized and Cocultured in Alginate Beads with the Plant-Growth-Promoting Bacterium *Azospirillum brasilense*. Appl. Environ. Microbiol. 66, 1527–1531.

Hirose, M., Mukaida, F., Okada, S., Noguchi, T., 2013. Active Hydrocarbon Biosynthesis and Accumulation in a Green Alga, *Botryococcus braunii* (Race A). Eukaryot. Cell 12, 1132–1141.

Hodaifa, G., Martínez, M.E., Sánchez, S., 2008. Use of industrial wastewater from olive-oil extraction for biomass production of *Scenedesmus obliquus*. Bioresour. Technol. 99, 1111–1117.

Hunt, R.W., Chinnasamy, S., Bhatnagar, A., Das, K.C., 2010. Effect of Biochemical Stimulants on Biomass Productivity and Metabolite Content of the Microalga, *Chlorella sorokiniana*. Appl. Biochem. Biotechnol. 162, 2400–2414.

Hunt, R.W., Chinnasamy, S., Das, K.C., 2011. The Effect of Naphthalene-Acetic Acid on Biomass Productivity and Chlorophyll Content of Green Algae, Coccolithophore, Diatom, and Cyanobacterium Cultures. Appl. Biochem. Biotechnol. 164, 1350–1365.

Largeau, C., Casadevall, E., Berkaloff, C., Dhamelincourt, P., 1980. Sites of accumulation and composition of hydrocarbons in *Botryococcus braunii*. Phytochemistry 19, 1043–1051.

Lau, S., Shao, N., Bock, R., Jürgens, G., De Smet, I., 2009. Auxin signaling in algal lineages: fact or myth? Trends Plant Sci. 14, 182–188.

Logothetis, K., Dakanali, S., Ioannidis, N., Kotzabasis, K., 2004. The impact of high CO2 concentrations on the structure and function of the photosynthetic apparatus and the role of polyamines. J. Plant Physiol. 161, 715–724.

Lürling, M., 2003. Phenotypic plasticity in the green algae *Desmodesmus* and *Scenedesmus* with special reference to the induction of defensive morphology. Ann. Limnol. - Int. J. Limnol. 39, 85–101.

Mayeli, S.M., Nandini, S., Sarma, S.S.S., 2005. The efficacy of Scenedesmus morphology as a defense mechanism against grazing by selected species of rotifers and cladocerans. Aquat. Ecol. 38, 515–524.

Mazur, H., Konop, A., Synak, R., 2001. Indole-3-acetic acid in the culture medium of two axenic green microalgae. J. Appl. Phycol. 13, 35–42.

Metzger, P., Largeau, C., 2005. *Botryococcus braunii*: a rich source for hydrocarbons and related ether lipids. Appl. Microbiol. Biotechnol. 66, 486–496.

Padhy, S.N., Pattanaik, H., 1976. The effect of hormones on heterotrophic growth of a blue-greed alga *Westiellopsis prolifica*. Hydrobiologia 50, 99–100.

Piotrowska, A., Bajguz, A., 2011. Conjugates of abscisic acid, brassinosteroids, ethylene, gibberellins, and jasmonates. Phytochemistry 72, 2097–2112.

Piotrowska-Niczyporuk, A., Bajguz, A., 2014. The effect of natural and synthetic auxins on the growth, metabolite content and antioxidant response of green alga *Chlorella vulgaris* (Trebouxiophyceae). Plant Growth Regul. 73, 57–66.

Piotrowska-Niczyporuk, A., Bajguz, A., Zambrzycka, E., Godlewska-Żyłkiewicz, B., 2012. Phytohormones as regulators of heavy metal biosorption and toxicity in green alga *Chlorella vulgaris* (Chlorophyceae). Plant Physiol. Biochem. 52, 52–65.

Ranga Rao, A., Sarada, R., Ravishankar, G.A., 2007. Influence of CO2 on growth and hydrocarbon production in *Botryococcus braunii*. J. Microbiol. Biotechnol. 17, 414–419.

Rensing, S.A., Lang, D., Zimmer, A.D., Terry, A., Salamov, A., Shapiro, H., Nishiyama, T., Perroud, P.-F., Lindquist, E.A., Kamisugi, Y., Tanahashi, T., Sakakibara, K., Fujita, T., Oishi, K., Shin-I, T., Kuroki, Y., Toyoda, A., Suzuki, Y., Hashimoto, S., Yamaguchi, K., Sugano, S., Kohara, Y., Fujiyama, A., Anterola, A., Aoki, S., Ashton, N., Barbazuk, W.B., Barker, E., Bennetzen, J.L., Blankenship, R., Cho, S.H., Dutcher, S.K., Estelle, M., Fawcett, J.A., Gundlach, H., Hanada, K., Heyl, A., Hicks, K.A., Hughes, J., Lohr, M., Mayer, K., Melkozernov, A., Murata, T., Nelson, D.R., Pils, B., Prigge, M., Reiss, B., Renner, T., Rombauts, S., Rushton, P.J., Sanderfoot, A., Schween, G., Shiu, S.-H., Stueber, K., Theodoulou, F.L., Tu, H., Peer, Y.V. de, Verrier, P.J., Waters, E., Wood, A., Yang, L., Cove, D., Cuming, A.C., Hasebe, M., Lucas, S., Mishler, B.D., Reski, R., Grigoriev, I.V., Quatrano, R.S., Boore, J.L., 2008. The *Physcomitrella* Genome Reveals Evolutionary Insights into the Conquest of Land by Plants. Science 319, 64–69.

Samorì, G., Samorì, C., Guerrini, F., Pistocchi, R., 2013. Growth and nitrogen removal capacity of *Desmodesmus communis* and of a natural microalgae consortium in a batch culture system in view of urban wastewater treatment: Part I. Water Res. 47, 791–801.

Voltolina, D., Cordero, B., Nieves, M., Soto, L.P., 1999. Growth of *Scenedesmus* sp. in artificial wastewater. Bioresour. Technol. 68, 265–268.

Xin, L., Hong-ying, H., Ke, G., Jia, Y., 2010. Growth and nutrient removal properties of a freshwater microalga *Scenedesmus* sp. LX1 under different kinds of nitrogen sources. Ecol. Eng. 36, 379–381.

Chapter 5

Applicative study on a digestate from anaerobic digestion

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Introduction

Combining wastewater remediation with algal biomass production is likely to be one of the most economically and environmentally sustainable ways to produce bio-energy and bio-products since wastewaters provide not only a water source but also most of the necessary nutrients for algal growth. Thus significant reductions in production costs associated with credits for wastewater treatment, as well as mitigation of the greenhouse gas emissions can be achieved (Andres F Clarens, 2010; Pittman et al., 2011; Zhou et al., 2011).

Devising new biological wastewater treatments depends more on the needs of human communities than on the availability of organisms and microorganisms where the demand occurs. The usefulness of any strain of microalgae for wastewater treatment depends on two major parameters: (i) the capacity of the strain to grow under the environmental conditions prevailing in the area and (ii) its capacity to remove the pollutants efficiently. Searching for local microorganism capable of growing in the local wastewater treatment facility requires an isolation strategy for candidate microorganisms. What must be determined is whether they pass the scrutiny of adaptability to the local wastewater scheme (de-Bashan et al., 2008). A wastewater of particular interest is the liquid fraction obtained through the anaerobic digestion process, which is a primary waste treatment used to reduce organic loading and related noises in agricultural and zoo-technical effluents. The use of anaerobic digestion has been spread in Europe many years ago, thanks to the support of specific legislative tools aimed at increasing the production of biogas in different economic sectors. Nowadays biogas production from agricultural biomass is of growing importance as it offers considerable environmental benefits (Chynoweth, 2005) and is an additional source of income for farmers.

On the other hand one, limitation of anaerobic digestion is that it does not significantly reduce the amount of nutrients in the digestate. In fact, it favours more bioavailable nitrogen forms such as ammonium, and so several studies have tested algal strains for the treatment of the digestate (Franchino et al., 2013).

Nutrient removal can be performed through high rate ponds. Raceways typically consist of independent closed-loop recirculation channels in which paddle wheel-generated flow is guided around bends by baffles placed in the flow channel; such systems can yield algal productivities of greater than 10 g ash-free dry weight m^{-2} d^{-1} (Sherman and Funkhouser, 1989). Engineering designs and operating procedures for cultivating these organisms in unmixed ponds and stirred raceways have been intensively studied (Borowitzka, 1999). Shallow water depths of 0.2–0.3 m are typically used, while areal dimensions range from 0.5 to 1 ha for raceway or central pivot ponds (circular ponds incorporating centrally pivoted rotating agitator), to greater than 200 ha for extensive

ponds used in Australia for *Dunaliella salina* production. Water management procedures vary according to the intensity of operation and may include direct CO_2 addition under automated pH-stat control in shallow raceways. The microalgal biomass may be harvested by flocculation or centrifugation (Campo et al., 2007).

Several microalgae species are widely studied for phytoremediation processes: *Scenedesmus* spp. (Shi et al., 2007), *Chlorella* spp. (Cho et al., 2011), *Arthrospira* spp. (Olguín et al., 2003), *Botryococcus braunii* (An et al., 2003) and cyanobacters belonging to *Phormidium* order (Dumas et al., 1998). In particular the green microalga *Scenedesmus* has shown extraordinary vitality in urban wastewaters, registering growth rates similar to those reported for a complete synthetic medium. This freshwater alga tolerates a wide range of temperature and pH, making it versatile for sewage purification (Kessler, 1991; Samorì et al., 2013).

The present study is part of a major project focused on nitrogen removal from the liquid fraction obtained through an anaerobic digestion process. In particular with the experiment described below, focusing on the batch cultivation of a *Desmodesmus communis* strain, the growth ability of this species in the above mentioned digestate, at different dilutions, was tested.

Materials and methods

Algal strain, culture conditions and inoculum preparation

The microalga *Desmodesmus communis* was selected for this study based on its short doubling time and high biomass productivity, tolerance to wastewater toxicity (such as municipal, agro-industrial and urban wastewater) and capacity to grow in open systems where it is able to outcompete other species (Pistocchi, personal communication).

Batch experiments were conducted to determine the growth rate and the $N-NH_4^+$ removal of a microalgal strain using 50 mL flasks. Cells for the inoculum were grown in a

250 mL flask. An inoculum of 10^5 cells harvested during the stationary phase, when cells where under starvation, was used for each treatment. Both inoculum and experimental flasks were incubated under white fluorescent light illumination at a light intensity of 100-120 µmol photons m^{-2} s^{-1} and a light/dark cycle of 16/8h at 22-25°C for 7 days. During the incubation, each flask was mixed with a magnetic stirrer.

Table 1 Characterization of wastewater from the anaerobic digester

Parameters	Unclarifed digestate
TN (mg L^{-1})	1078.0
$N-NH_4^+$ (mg L^{-1})	1075.0
$N-NO_3^-$ (mg L^{-1})	3.0
PO_4^{3-} (mg L^{-1})	15.84
Cr (mg L^{-1})	0.655
Cu (mg L^{-1})	3.543
Pb (mg L^{-1})	0.548
Ni (mg L^{-1})	0.550
Zn (mg L^{-1})	15.51
Mn (mg L^{-1})	13.87
Fe (mg L^{-1})	150.1
Ca (mg L^{-1})	982.6
Mg (mg L^{-1})	531.2
K (mg L^{-1})	6598
Na (mg L^{-1})	1037

Growth medium

The liquid fraction of the digestate was collected from the effluent of an anaerobic digestion plant situated in Fusignano (Ravenna, Italy) working mainly with maize biomass. Physico-chemical characterization of the effluent was performed (Tab. 1), and due to the dark colour of the medium, a clarification process was carried out by the ICL Water Solution (Milano, Italy). To further improve clarification of the digestate it was diluted 1:3. Substantial clarification was achieved by pH fluctuations with anionic and cationic polymers to provoke the flocculation of the solid fraction. The dark component was completely removed and the concentration of $N-NH_4^+$ was 705 mg L^{-1}. The clarified liquid was then diluted with distilled water to 6 different concentrations (Tab. 2). Finally, 8 test solutions including undiluted clarified digestate and 100%

synthetic medium were prepared. The culture medium used for control samples was CHU 13 mod. (Largeau et al., 1980) with NH_4Cl instead of KNO_3 as nitrogen source, as it offers the optimal conditions for the growth of *D. communis* in terms of nutrient concentrations and absence of turbidity, therefore being a good reference to evaluate the algal growth in the digestate.

Table 2 Experimental conditions and nitrogen concentration added in each treatment

Culture condition	medium	$N-NH_4^+$ (mg L^{-1})
C	Synthetic medium CHU 13 mod.	30
U	Undiluted waste water	705
2	Diluted waste water	353
5	Diluted waste water	141
10	Diluted waste water	71
20	Diluted waste water	35
50	Diluted waste water	14
100	Diluted waste water	7

Measurement of cell growth
Due to the high turbidity and the presence of small particulates in the digestate, algal growth was monitored by cell counting which was carried out using an Optical Microscope (Axiovert S 100) at 32 x following the Uthermöl protocol (Hasle et al. 1978). The morphology of the cell was also observed.

Ammonium removal and pH analysis
Ammonium absorption was analysed using the Ammonia – Selective Electrode Method (APHA 4500-NH$_3$ D). After bringing samples to room temperature, the pH of standards and samples was monitored with a pH glass electrode (PHM 92, Radiometer Copenaghen) and then raised to over 11 with NaOH solution (10 N). 0.1 mg L^{-1}, 1 mg L^{-1}, 10 mg L^{-1}, 100 mg l^{-1} and 1000 mg L^{-1} stock solutions were used to develop calibration curves. Ammonia concentrations were then measured.

Calculation
The growth rate μ (d^{-1}) was calculated following the formula:

$$\mu = \frac{\ln(X_t / X_0)}{t_t - t_0}$$

where X_t and X_0 were the number of cell on days t_t and t_0.
The nutrient elimination capacity EC (mg L^{-1} d^{-1}) and the removal efficiency RE (%) were calculated as follow:

$$EC = \frac{C_i - C_f}{\Delta t}$$

$$RE = \frac{C_i - C_f}{C_i} \cdot 100$$

where C_i and C_f were the initial and final nutrient concentration respectively (Franchino et al., 2013).

Statistical analysis
Statistical analysis was performed with PAST 2.17. Levene's test was used to check the homogeneity of variance, while Tukey test was used for a pairwise comparison. Each treatment was performed in triplicates, and the parameters measured were given as the mean with respective standard deviations for each set of triplicates shown in the figures.

Results
In order to examine the possibility of growing the microalgae *D. communis* in a digestate, and its capacity to remove high ammonium concentrations, a short experiment with many dilutions was run. The algae survived and grew at all treatments, and Fig. 1 shows the number of cells reached in each condition after 7 days. The graph shows that the control culture displayed the highest number of cells, with 1.4 10^6 cell mL^{-1}, and this number was nearly double than the maximum cell number attained by the conditions with the digestate. Four out of the 7 treatments had similar numbers of cells independent of the dilution and were not significantly (p>0.05) different from the undiluted condition. The lowest number of
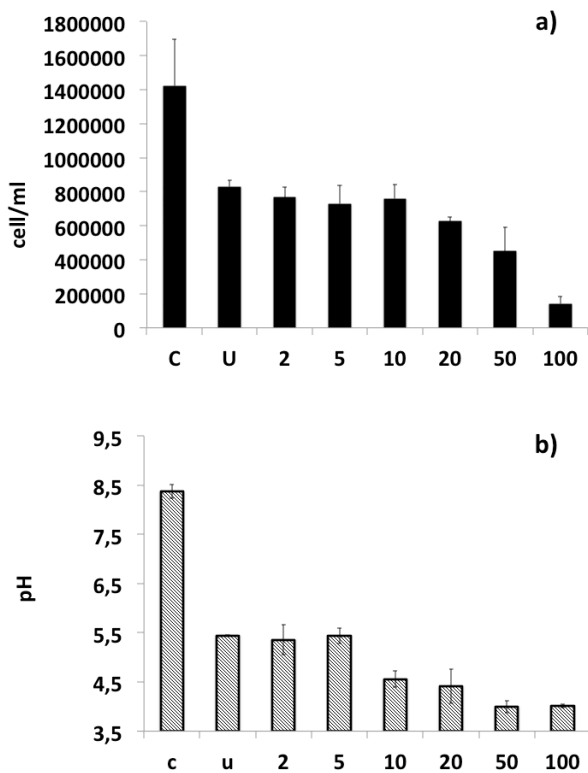
Figure 11 Influence of undiluted and diluted digestate on *D. communis* growth and the pH of the medium. Results are shown in terms of number of cell (a) and pH (b) measured after 7 days. On the horizontal axes there are displayed the experimental conditions.

cells was obtained in the 1:100 dilution, with only 1.4 10^5 cell mL^{-1}. This decrease was presumably due to the low nitrogen concentration present that was insufficient to support a prolonged growth, indeed the ammonium concentration was only one fourth of the N concentration in CHU 13 medium. For the same reason, 1:50 dilution treatment, having 14 mg L^{-1} of N, could not sustain a high number of cells. The highest cell density reached in the control could due to the nutritional complexity and a better uptake of nutrients from the growth medium. This could be expected however it is remarkable that the best performing treatments were the lower dilutions and the undiluted digestate, which recorded around between 43 and 58% of the number of cells of the control. Park et al., (2010) reported that the growth rate of microalgae was consistent in the presence of

moderate NH_4-N concentrations, ranging between 200 and 800 mg L^{-1}, but significantly (p < 0.05) decreased at ammonium concentrations over 1000 mg L^{-1}. However, microalgal growth is also strongly dependent upon the strain and other growth conditions (Klausmeier et al., 2004) and Martin et al., (1985) suggested that a desirable N:P ratio for cultivating freshwater microalgae should be in the range 6.8 to 10.

For these reasons, treatments with high dilutions could have had too low phosphate concentrations to support the algal growth.

The pH values during the growth are shown in fig. 2. During seven days the control maintained the same pH value of 7.5, while all treatments showed a trend of pH values which parallel the growth pattern in Fig. 1. The more acidic pH values in low ammonium conditions, compared with U, 1:2 and 1:5 samples, could be due to the fact that the calcium carbonate that had been added in the effluent during the clarification process, was also diluted and it could not counteract the H^+ released by the ammonium absorption process. In addition, a lower Ca_{2^+} concentration could have limited *D. communis* growth. Calcium is a very important micronutrients for algae growth, and it has been already observed that calcium deficiency not only decreased the nitrogen uptake but also their grown rate (Kuffner and Paul, 2001). Most of the nitrogen in the digestate was in the form of ammonium. The elimination capacity EC (Fig. 2a) showed a different trend compared with the RE. It varied among the different dilution conditions, being related to the initial concentrations. For this reason U and 2, with high ammonium concentrations had higher values of removal capacity, with 13.2 ± 2.7 and 8.9 ± 0.2 mg L^{-1} d^{-1} respectively, compared with the control and the others treatments. The control, with the initial concentration of N-NH_4^+ 28 mg L^{-1}, resulted in EC 3.0 ± 0.2 mg L^{-1} d^{-1}. Therefore *D. communis* was able to remove the ammonium in all the treatments and the amount of nutrient removed was greater in samples with higher initial nitrogen

concentrations. The elimination capacity of *D. communis* was high compared with the values
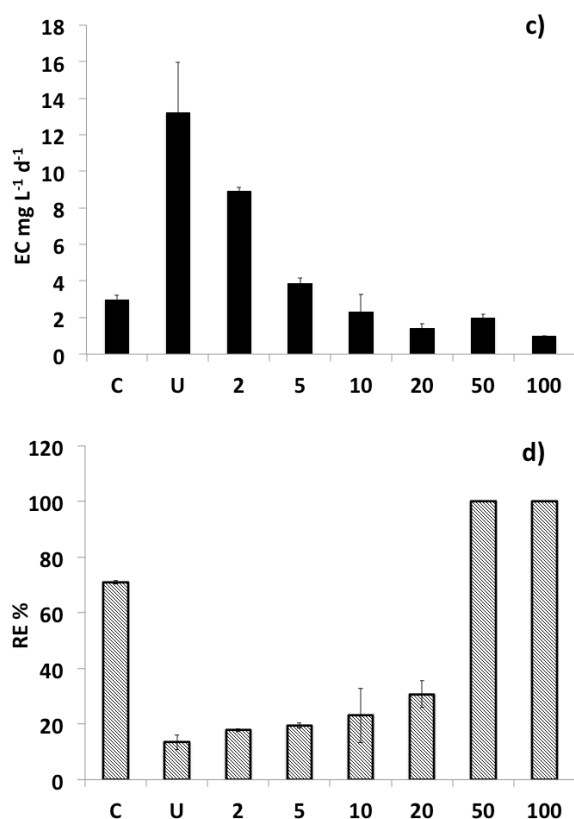
Figure 2 Ammonium elimination capacity (a) and removal efficiency (b) in all treatments tested. On the horizontal axes there are displayed the experimental conditions

found by Franchino et al., (2013) for *Scenedesmus obliquus* grown in digestate effluent with 109 mg/L of NH_4^+-N. From Fig. 1b and 2 it is confirmed that ammonium is one of the preferred N-source for microorganism and the assimilation of either NO_3^- and NH_4^+ by algae is related to the pH of the culture medium (Richmond, 2004).

The removal efficiency calculated through the measurements of N-NH_4^+ levels in the medium at the beginning and at the end of the experiment is shown in Fig. 2b. The 100% RE was found for cells in the digestate diluted 1:50 and 1:100, where the amount of ammonium was low with 14 and 7 mg L^{-1} of ammonium respectively. No significant differences (p> 0.05) were found in terms of RE between U, 1:2 and 1:5 conditions. In general the percentage removal efficiency of ammonium showed an

increase correlated with the effluent dilution: high dilutions corresponded to high RE percentages. A high removal rate of ammonium was observed in other anaerobic digestates of dairy manure by several authors with different microrganisms (Levine et al., 2011; Wang et al., 2010; Yang et al., 2011).

Moreover *D. communis* was able to remove high amounts of ammonium from the undiluted effluent and in 5 out of the 10 treatments, after 7 days, the nutrient reached a final concentration well below the 50 mg L^{-1} (data not shown) limit imposed by European Nitrate Directive.

Conclusions

The microalgae *D. communis* was able to grow in ammonium-rich clarified digestate, even without a dilution, and its growth appeared more influenced by the pH value than by the ammonium concentration of the effluent. Nitrogen was actively removed by the microalgae, with different elimination capacity according to the initial nitrogen concentrations. From this preliminary study *D. communis* offers real opportunity to grow and remove nitrogen from waste-water rich in ammonium.

References

Andres F Clarens, E.P.R., 2010. Environmental life cycle comparison of algae to other bioenergy feedstocks. Environ. Sci. Amp Technol. 44, 1813–9.

An, J.-Y., Sim, S.-J., Lee, J.S., Kim, B.W., 2003. Hydrocarbon production from secondarily treated piggery wastewater by the green alga *Botryococcus braunii.* J. Appl. Phycol. 15, 185–191.

Borowitzka, M.A., 1999. Commercial production of microalgae: ponds, tanks, and fermenters, in: R. Osinga, J.T., J. G. Burgess and R. H. Wijffels (Ed.), Progress in Industrial Microbiology, Marine Bioprocess Engineering Proceedings of an International Symposium Organized under Auspices of The Working Party on Applied Biocatalysis of the Eurpean Federation of Biotechnology and The European Society for Marine Biotechnology. Elsevier, pp. 313–321.

Campo, J.A.D., García-González, M., Guerrero, M.G., 2007. Outdoor cultivation of microalgae for

carotenoid production: current state and perspectives. Appl. Microbiol. Biotechnol. 74, 1163–1174.

Cho, S., Luong, T.T., Lee, D., Oh, Y.-K., Lee, T., 2011. Reuse of effluent water from a municipal wastewater treatment plant in microalgae cultivation for biofuel production. Bioresour. Technol., Special Issue : Biofuels-III: Biohydrogen 102, 8639–8645.

Chynoweth, D.P., 2005. Renewable Biomethane From Land and Ocean Energy Crops and Organic Wastes. HortScience 40, 283–286.

de-Bashan, L.E., Trejo, A., Huss, V.A.R., Hernandez, J.-P., Bashan, Y., 2008. *Chlorella sorokiniana* UTEX 2805, a heat and intense, sunlight-tolerant microalga with potential for removing ammonium from wastewater. Bioresour. Technol., Exploring Horizons in Biotechnology: A Global Venture 99, 4980–4989.

Dumas, A., Laliberté, G., Lessard, P., de la Noüe, J., 1998. Biotreatment of fish farm effluents using the cyanobacterium *Phormidium bohneri*. Aquac. Eng. 17, 57–68.

Franchino, M., Comino, E., Bona, F., Riggio, V.A., 2013. Growth of three microalgae strains and nutrient removal from an agro-zootechnical digestate. Chemosphere 92, 738–744.

Kessler, E., 1991. *Scenedesmus*: Problems of a Highly Variable Genus of Green Algae. Bot. Acta 104, 169–171.

Klausmeier, C.A., Litchman, E., Daufresne, T., Levin, S.A., 2004. Optimal nitrogen-to-phosphorus stoichiometry of phytoplankton. Nature 429, 171–174.

Kuffner, I.B., Paul, V.J., 2001. Effects of nitrate, phosphate and iron on the growth of macroalgae and benthic cyanobacteria from Cocos Lagoon, Guam. Mar. Ecol. Prog. Ser. 222, 63–72.

Largeau, C., Casadevall, E., Berkaloff, C., Dhamelincourt, P., 1980. Sites of accumulation and composition of hydrocarbons in *Botryococcus braunii*. Phytochemistry 19, 1043–1051.

Levine, R.B., Costanza-Robinson, M.S., Spatafora, G.A., 2011. *Neochloris oleoabundans* grown on anaerobically digested dairy manure for concomitant nutrient removal and biodiesel feedstock production. Biomass Bioenergy 35, 40–49.

Martin, C., de la Noüe, J., Picard, G., 1985. Intensive cultivation of freshwater microalgae on aerated pig manure. Biomass 7, 245–259.

Olguín, E.J., Galicia, S., Mercado, G., Pérez, T., 2003. Annual productivity of *Spirulina (Arthrospira)* and nutrient removal in a pig wastewater recycling process under tropical conditions. J. Appl. Phycol. 15, 249–257.

Park, J., Jin, H.-F., Lim, B.-R., Park, K.-Y., Lee, K., 2010. Ammonia removal from anaerobic digestion effluent of livestock waste using green alga *Scenedesmus* sp. Bioresour. Technol. 101, 8649–8657.

Pittman, J.K., Dean, A.P., Osundeko, O., 2011. The potential of sustainable algal biofuel production using wastewater resources. Bioresour. Technol., Special Issue: Biofuels - II: Algal Biofuels and Microbial Fuel Cells 102, 17–25.

Richmond, A., 2004. Handbook of Microalgal Culture: Biotechnoly and Appl. Phycology. Blackwell Publishing, Oxford, OK.

Sherman, T.D., Funkhouser, E.A., 1989. Induction and synthesis of nitrate reductase in *Chlorella vulgaris.* Arch. Biochem. Biophys. 274, 525–531.

Shi, J., Podola, B., Melkonian, M., 2007. Removal of nitrogen and phosphorus from wastewater using microalgae immobilized on twin layers: an experimental study. J. Appl. Phycol. 19, 417–423.

Wang, L., Li, Y., Chen, P., Min, M., Chen, Y., Zhu, J., Ruan, R.R., 2010. Anaerobic digested dairy manure as a nutrient supplement for cultivation of oil-rich green microalgae *Chlorella* sp. Bioresour. Technol. 101, 2623–2628.

Yang, Y., Xu, J., Vail, D., Weathers, P., 2011. Ettlia oleoabundans growth and oil production on agricultural anaerobic waste effluents. Bioresour. Technol. 102, 5076–5082.

Zhou, W., Li, Y., Min, M., Hu, B., Chen, P., Ruan, R., 2011. Local bioprospecting for high-lipid producing microalgal strains to be grown on concentrated municipal wastewater for biofuel production. Bioresour. Technol. 102, 6909–6919.

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