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Ph.D. in Agriculture, Environment and Bioenergy – XXXI cycle

MICROALGAE PRODUCTION RECOVERING CARBON AND NUTRIENTS FROM AGRO-INDUSTRIAL WASTES

Supervisor: Prof. Fabrizio ADANI

Davide VERONESI

Matricola: R11199

Preface

This PhD thesis comprises the research carried out at the Department of Agricultural and Environmental Sciences - Production, Land, Agroenergy - at the University of Milan. Professor Fabrizio Adani was the supervisor of the whole work. The thesis is organized in four chapters, a first chapter that puts into context the findings of the PhD in an introductive review based on the book chapter "Microalgae mixotrophic growth: opportunity for stream depuration and carbon recovery" published in the book titled <u>Prospects and Challenges in Algal Biotechnology</u> by Springer. The second ad third chapters are presented here in two different main branches. The first part focuses on the cultivation of algae on agricultural wastewaters and livestock wastes in order to recover nutrients supporting biomass production. In the second part are analyzed the possibilities to growth microalgae on carbon-rich wastes coming from the agro-food sector through mixotrophic approach. The two main parts of this research work are a collection of the papers listed below and number written with the Roman numerals I-IV:

- **Chapter 2: Nutrients recovery from agricultural wastewaters**
    - **I** Veronesi D., D'Imporzano G., Salati S., Adani F., (2017) Pre-treated digestate as culture media for producing algal biomass. Ecological Engineering, 105, 335-340. http://dx.doi.org/10.1016/j.ecoleng.2017.05.007.

- **Chapter 3: Carbon recovery from agro-industrial wastes**

    - **II** Salati S., D'Imporzano G., Menin B., Veronesi D., Scaglia B., Abbruscato P., Mariani P., Adani F., (2017) Mixotrophic cultivation of Chlorella for local protein production using agro-food by-products. Bioresource Technology, 230, 82–89. http://dx.doi.org/10.1016/j.biortech.2017.01.030.

    - **III** D'Imporzano G., Veronesi D., Salati S., Adani F., (2018) Carbon and nutrient recovery in the cultivation of Chlorella vulgaris: A life cycle assessment approach to comparing environmental performance. Journal of Cleaner Production, 194, 685–694. https://doi.org/10.1016/j.jclepro.2018.05.174.

    - **IV** Veronesi D., D'Imporzano G., Menin B., Salati S., Adani F., (2018) Organic wastes as alternative to CO_2 for producing mixotrophic microalgae. Under revision on Process Biochemistry.

In Chapter 4 are discussed some conclusions on the possibility to produce microalgae biomass by mixotrophic approaches basing on the advances of the papers listed above.

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CHAPTER 2 - Nutrient recovery from agricultural wastewaters

2. Pre-treated digestate as culture media for producing algal biomass

CHAPTER 3 - Carbon recovery from agro-industrial wastes

3. Mixotrophic cultivation of Chlorella for local protein production using agro-food by-products

Abstract

In the last decades several companies worldwide pain significant interest on microalgae production biotechnology as these microorganisms are able to produce high added value compounds such as lipids and polyunsaturated fatty acids, proteins and essential amino acids, antioxidants and pigments that could be used in several sectors, i.e. food, feed, green chemistry, cosmetical, nutraceutical and pharmaceutical industries and bio-energy field. Microalgae have been established as unique biofactories which could play a role in the fields of energy independence and sustainability, carbon capture, conservation of arable land, water and other resources. Nowadays, autotrophic cultivation is the main modality in which microalgae are industrially produced, but its success is limited by factors such as light availability, carbon dioxide supply and high production costs. The main challenge to overcome however is the development of high efficiency strategies for the large-scale production of microalgae at low costs. One possibility to overcome these limits is to exploit the ability of some microalgae species to use organic substrates as a carbon source, i.e. Mixotrophy. Mixotrophy is a metabolic pattern in which microalgae drive both autotrophy and heterotrophy, thus utilizing organic carbon sources as substrates of growth, improving the productivity of the system. Agro-industrial wastes and wastewaters are rich in nutrients and have been widely considered as a potential nutrient source for the cultivation of microalgae. Integration with wastewater treatment is a possible synergy for algal production, where algae may grow on sewages as a water and nutrient source, reducing the total production costs. In Lombardy Region the Agro-food system is one of the most advanced in the whole European Union, and each year it produces huge amount of agro-industrial waste stream. This thesis was focused on the cultivation of some microalgal strains on nutrient

rich wastes, in particular in Chapter 2 are shown results of the cultivation of *Chlorella sp.* and *Phaedoctylum tricornutum* on livestock wastes, showing the ability to growth and the good depuration performances obtained. In Chapter 3, were analyzed the possibilities to growth *Chlorella sp.* and *Nannochloropsis salina* on carbon-rich wastes (cheese whey, wine lees, glycerol) sampled from different agri-food activities in Lombardy. Evaluation of the different mixotrophic performances were compared with autotrophy (control) and a macromolecular analysis of the obtained biomasses were performer showing very good results in terms of biomass quantity and quality produced under mixotrophic cultivation.

CHAPTER 1 – Introduction

1.1 Microalgae: aspects and biological role

The term 'algae' refers to a large variety of polyphyletic organisms, most of them able to perform photosynthesis, which have different evolutionary lines, origins and biochemistry (Domìnguez, 2013). Numerous aquatic organisms are classified under the common name of algae but algae do not constitute a natural group and are distributed in different systematic categories, phylogenetically distant (Perrone and Felicini, 2015). Since primeval periods, algae have been part of the pattern of life on earth: these organisms are the base of food webs in most aquatic ecosystems, drive biogeochemical cycles, produce the major fraction of world's oxygen and represent significant proportions of biodiversity (Minshall, 1978; Wetzel, 2001). Protecting natural levels of algal productivity in aquatic ecosystems is thus important to sustain these biological roles, in particular with regards of the threat of local and global change in environmental conditions (Cardinale et al., 2006; 2012). However, when the populations of phytoplankton become too large in response to high concentration of nutrients, i.e. nitrogen and phosphorus, algae can reduce the water transparency, oxygen availability for other organisms and through the production of secondary metabolite, e.g. toxins, could cause the death of other photosynthetic organisms, fishes and birds (Stevenson, 2014; Mata, et al., 2010). Algae and plants present many similarities: photosynthetic capacity, morphological correspondence and also algae produce the same storage compounds as well as they use similar defense strategies against predators and parasites (Barsanti and Gualtieri, 2014). However, distinguish algae from plants is easy since there are more differences than similarities between them (Barsanti and Gualtieri, 2006). The main difference is that plant show a high grade of differentiation

(with roots, leaves and stems) and presents a layer of sterile cells surround their reproductive organs. Plant have a digenetic life cycle with an alternation between a haploid gametophyte and a diploid sporophyte. Algae have not any of these features. Reproductive structures of algae consist of cells that are potentially fertile. (Barsanti and Gualtieri, 2006). Algae may reproduce in both sexual and asexual mode: simple cell division is the only pattern performed by some species, other use spores (mitospores) for the purpose (Bersanti and Gualtieri, 2014). Algae present a wide range of different features: cellular structure, size, morphology, ecology and habitats, photosynthetic pigments, reserve and structural compounds. For this reason, the term algae refer both to macroalgae and to a highly diversified group of microorganisms known as microalgae (Van den Hoek, et al., 1995 Barsanti and Gualtieri, 2006; Peltomaa & Ojala, 2010). Microalgae are an extremely differentiated collection of eukaryotic or prokaryotic photosynthetic microorganisms that can grow rapidly and are able to live in hard and adverse conditions due to their unicellular or simple multicellular structure (Mata, et al., 2010). Eukaryotic algae are for example green algae (*Chlorophyta*) or diatoms (*Bacillariophyta*) while prokaryotic algae are for example the photosynthetic bacteria (*Cyanobacteria*) (Richmond, 2004). It has been estimated that more than 50,000 species exist, but just over half have been studied, analyzed and classified (Richmond, 2008). The evolutionary and phylogenetic diversity also means a great diversity in chemical composition of these organisms, and therefore, this makes them extremely attractive for research and development efforts to use microalgae as commercial sources of a wide range of biomolecules with real applications in several sectors (Chisti, 2007; Rodolfi et al., 2009; Chojnacka et al.,. 2012; Borowitzka, 2013).

1.2 Microalgae application

The interest on microalgae biotechnology is becoming even more expanding due to the ability of these microorganisms to directly transform sunlight energy into high added value products with a real application in several sectors (Pulz and Gross, 2004; Markou and Nerantzis, 2013). Over the past 50 years, microalgal production technology has developed and diversified significantly. The potential of commercial application are very big and the related R&D sectors increase each year. Microalgae cultivation is being applied in the production of pharmaceuticals, biochemicals, health food, animal feed, biofertilizers and for the biofuels production. (Chisti, 2007; Costa et al., 2011). Biofuels from microalgae biomass appears to be a suitable solution towards the replacement of conventional fossil fuels. Microalgae biomass can be used to generate a range of renewable fuels such as biodiesel (Mata, et al., 2010), bioethanol (Ho, et al., 2012), biohydrogen (Liu, et al., 2013), methane (Alzate, et al., 2012) and syngas (Goyal, et al., 2008). Many studies have been focused to obtain biodiesel from microalgae since many species can accumulate huge amount of lipids (Sheehan, et al., 1998; Chisti, 2007; Christenson and Sims, 2011). The average lipid content varies between 1 and 70% but under certain conditions (e.g. nitrogen starvation) some species can reach until 90% of dry weight (Mata, et al., 2010; Li, et al., 2008). Even if at today it's proven the technical feasibility of the system, the main problem that limiting the possibility to lead this application process at industrial-scale is related to the high production costs (Wijffels, 2007; Chisti, 2007; Clarens et al., 2010), in particular the costs connected to the fertilizer and water input. For instance, biodiesel produced from palm oil has a market value of 0.52 \$ L^{-1} while biodiesel obtained from algae cost 2.80 \$ L^{-1} (Fernandez, et al., 2013; Chisti, 2007). The European Algae Biomass Association has

estimated that it may take another 10 to 15 years to move laboratory experiments into industrial-scale production of algal biofuel (Kovalyova, 2009). Although the commercial production of biofuels from microalgae until today is not feasible, it is generally claimed that microalgae have a great potential to produce a wide range of important compounds for health food, animal feed, pharmaceutical, biochemicals and other uses (Wijffels, 2007; Raja et al., 2008; Costa et al., 2011).

The main high-value molecules obtained from microalgae biomass and their uses are identified as follow:

- *Proteins*: Proteins can be found in different forms and locations such as components of the cell wall, as enzymes and bound to pigments or carbohydrates. A lot of works shown that both marine and freshwater strains can be an excellent reservoirs of proteins and derivatives having potent biological properties (Samarakoon and Jeon, 2012). In a study with 40 species of microalgae it was concluded that microalgae varied in their protein content (6-52 % of dry weight) but all species had a similar amino acid composition, and were rich in the essential amino acids (Brown ed al., 1997). Some studies show that *Arthrospira platensis* commonly known as Spirulina is able to accumulate proteins around 70% of dry weight (González López et al., 2010). The quality and the nutritional value of protein are determined by the content, availability and proportion of its amino acids (Schwenzfeier, et al., 2011; Waghmare, et al., 2016). Considering that the global population is estimated to increase by over a third (2.3 billion people) by 2050, requiring an increase in food production around 70 %, microalgae biomass became a good candidate to fulfill the word food demand (Bleakley and Hayes, 2017).

- *Polyunsaturated fatty acids (PUFAs):* Fish and fish oil are the common sources of long-chain PUFAs but safety issues have been raised because of the possible accumulation of toxins in fish (Rosenberg et al., 2008). Moreover, the application of fish oil as food additive is limited due to problems associated with its typical fishy smell, unpleasant taste and poor oxidative stability (Lee et al., 2001; Miao et al., 2006; Michiki et al., 1995). PUFAs content in fish comes from microalgae, which is the basis of their diet: following this is reasonable to consider microalgae as potential sources of PUFAs (Moheimani, 2005). As primary producers, many microalgae are rich in long chain polyunsaturated fatty acids, especially $\omega 3$ and $\omega 6$ series such as eicosapentaenoic (C20:5ω3, EPA), docosahexaenoic (C22:6ω3, DHA), and arachidonic (C20:4ω6, AA) are considered pharmacologically important for dietetics and therapeutics (Shahidi and Wanasundara, 1998; Horrocks and Yeo, 1999; Goldberg, et al., 2011; Rebolloso-Fuentes, et al., 2001; Adarme-Vega et al., 2012). Recent clinical and epidemiological studies have indicated that long-chain n-3 polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), are important in the treatment of atherosclerosis, cancer, rheumatoid arthritis, psoriasis, and diseases of old age, such as Alzheimer's disease and age-related macular degeneration (European Environmental Agency, 2007). Fatty acids content can be affected by nutritional and environmental factors, for example, nitrogen starvation is well known to improve the total amount of PUFAs in some algal species (Aslan et al., 2006; Li Y et al., 2008). PUFAs have been used for prophylactic and therapeutic treatment of chronic

inflammations (e.g. rheumatism, skin diseases, and inflammation of the mucosa of the gastrointestinal tract) (Mata, et al., 2010). Furthermore, they seem to have a positive effect on cardio circulatory diseases, coronary heart diseases, atherosclerosis, hypertension, cholesterol, and cancer treatment (Barrow & Shahidi, 2008). All these PUFAs can be found in animals, transgenic plants, fungi and many microorganisms but at low level so that they are typically extracted from fatty fish, putting additional pressures on global fish stocks (Borowitzka, 2013; Rosamond, et al., 2000). Many people believe that farmed fish relieves pressure on ocean fisheries, but the opposite is true for some types of aquaculture. Farming carnivorous species requires large inputs of wild fish for feed. Some aquaculture systems also reduce wild fish supplies through habitat modification, wild seedstock collection and other ecological impacts (Rosamond, et al., 2000). Aquaculture industry must reduce wild fish inputs in feed and adopt more ecologically management practices (Rosamond, et al., 2000).

- *Pigments*: Microalgae pigments have an important role in their photosynthetic metabolism and pigmentation. Pigments are widely studied thanks to their beneficial biological activities, such as antioxidant, anticancer, anti-inflammatory, anti-obesity, anti-angiogenic, and neuroprotective (D'Alessandro and Antoniosi Filho, 2016). The main classes of pigments in microalgae are carotenoids, phycobilins and chlorophylls.

- *Carotenoids*: are fat-soluble substances with colors ranged from brown, red, orange to yellow. They have a fundamental role in photosynthesis protecting the photosyntetic system from high intensities of light and absorbing light in the region of visible in which chlorophyll does not absorb efficiently (D'Alessandro and Antoniosi Filho, 2016). The main carotenoids of microalgae are: β-carotene, lutein, astaxanthin, zeaxanthin, violaxanthin, and lycopene; of these the first three are the most studied ones (Del Campo et al., 2007). Literature report that these molecules could act as scavenger and deactivator of free radicals, acting thereby as antioxidants. Epidemiological evidence suggests that high carotenoid intake with daily diet and their tissue concentrations are associated with reduced cancer and cardiovascular disease risk (Agarwal, et al., 2012; Paiva & Russell, 1999). The carotenoids market in 2010 was about 1.2 billion $ with the bulk of the carotenoids generated by chemical synthesis (Borowitzka, 2013). Nevertheless, synthetic products often do not ensure the same biological activity of natural ones. β-carotene and astaxanthin from microalgae represent a major part of the natural production of carotenoids (Borowitzka, 2013). β-carotene was the first high-value product commercially produced from a microalga *Dunaliella salina* which produces β-carotene above 14% dry weight and it's used as colorant for food or nutritional supplement, because is a precursor of vitamin A (retinol) (Edge et al., 1997; García-González et al., 2005; Spolaore et al., 2006). The second carotenoid from algae to be commercialized was astaxanthin from the freshwater green alga

Haematococcus pluvialis (Joen et al., 2006), which contains the highest amount of astaxanthin of any natural source (Borowitzka, 2013). Synthetic compared to natural astaxanthin is different in isomerism and chemical structure. Furthermore, the fact that synthetic astaxanthin is derived from petrochemicals raises the issues of food safety, pollution and sustainability. Hence, the chemical astaxanthin is only allowed to be used in aquaculture (Li, et al., 2011). Astaxanthin is the strongest non-synthetic antioxidant existing in nature, that sequestering free radicals by removing reactive oxygen, it is used in disease treatment such as atherosclerosis and heart disease, chronic inflammatory diseases, diabetes and as cancers preventer (Uchiyama et al., 2002; Ciccone et al., 2013).

- *Phycobilins* (or phycobiliproteins) are accessory for collecting light during photosynthesis. They are widely used in molecular biology and immunology laboratories as fluorescent markers due to their absorption properties (D'Alessandro and Antoniosi Filho, 2016). These algae pigments are also used as natural colourants for food, cosmetics and pharmaceuticals purposes. For example, the algae Artrospira platensis produces the phycocyanin, a blue colourant used in chewing gum, ice slush, sweets, soft drinks, dairy products and wasabi (Spolaore et al., 2006; Raja et al., 2008).

- *Chlorophyll:* is the most famous and important pigment, involved in light energy capture during photosynthesis in plants and algae. Normally in microalgae is present only the chlorophyll a but some may have chlorophyll b and c (Bersanti and Gualtieri, 2006). Microalgae contain from 0.5 to 1.0% of chlorophyll per dry weight (Spolaore et al., 2006). Chlorophyll is used mainly as an additive in pharmaceutical and cosmetic products but also as a natural food colorant (Hosikian et al., 2010).

1.3 Trophic ways of microalgae

Microalgae species are mainly photoautotrophs and consequently depend completely upon their photosynthetic apparatus and light availability for their metabolic necessities (Morales-Sanchez, et al., 2015). The term "microalgae" is typically used in its narrowest sense as a synonym for photoautotrophic, unicellular algae utilizing CO_2 and gaining energy from light through photosynthesis. Although certain species are obligate photoautotrophs, numerous microorganisms currently classified as microalgae are in fact obligate heterotrophs (Droop 1974; Gladue and Maxey, 1994), and others are capable of both heterotrophic and photoautotrophic metabolism either sequentially or simultaneously, i.e. mixotrophy (Chojnacka and Marquez-Rocha, 2004; Gladue and Maxey, 1994; Lee, 2001). The latter have the flexibility to switch their nutritional mode from photo-autotrophic to heterotrophic metabolism depending on substrate availability and light conditions (Chandra et al., 2014) and it represents one of their evolutionary advantages (Barsanti and Gualtieri, 2006). Compared with photo-autotrophic and heterotrophic microalgal growth, mixotrophic approaches have the potential to provide

larger biomass and yield of valuable organic compounds (Garcia and Bashan, 2015). Four major modes of microalgae cultivation can be adopted namely photoautotrophic, heterotrophic, photoheterotrophic and mixotrophic (Wang et al., 2014).

1.3.1 Photoautotrophy

Photoautotrophic cultivation is the most commonly employed and most energy-saving mode of microalgae cultivation, generally carried out in open ponds or photobioreactors (Aslan et al., 2006; Voltolina et al., 2005). Photoautotrophic organisms produce chemical energy through photosynthesis. In this process microalgae utilize light as the sole energy source and inorganic carbon (CO_2) as the sole carbon source, converting them into carbohydrates. Carbohydrates will further form the base for the construction of all other carbon-containing biomolecules (Yoo et al., 2011). The main advantage related to photoautotrophic culture concerns the carbon dioxide consumption: microalgae cells use atmospheric CO_2 as carbon source, contributing to global CO_2 reduction. Furthermore, increasing artificially the level of CO_2 in the microalgae growth environment could enhance biomass productivity to a certain extent (Chiu et al., 2008). However, under autotrophic conditions, growth is limited by light availability and, during the night, productivity is further reduced because of respiration losses. Moreover, since light penetration decreases exponentially with the increase of broth turbidity (caused mainly by microalgal cells concentration), photoautotrophic cultivation has difficulty achieving high biomass concentration and biomass productivity (Markou and Georgakakis, 2011). Under photoautotrophic cultivation, the lipid content of microalgae varies widely, ranging from 5 to 68 % (Chen et al., 2011). Generally, higher lipid content could be obtained in a nutrient-limiting (in particular

nitrogen-limiting), but the biomass productivity achieved in this stressed condition is usually far lower than that in normal circumstances, which results in an unchanged or even lower microalgal lipid productivity (Mata et al., 2010; Liang et al., 2009).

1.3.2 Heterotrophy

Heterotrophic process is the mode in which microalgae are independent from light and are able to utilize organic substrates (e.g., glucose, acetate, glycerol) as both energy and carbon source (Mata et al., 2010). In this process, microalgae assimilate organic substrates and thus generate energy through oxidative phosphorylation accompanied by oxygen consumption as the final electron acceptor (Bashan, 2015). The heterotrophic condition present relevant advantages: heterotrophically it is possible to obtain high densities of microalgal biomass that provide an economically feasible method for large-scale mass production (Behrens, 2005; Perez-Gracia et al., 2011). In particular, thanks to its independence from light, heterotrophic cultivation could avoid the defects associated with photolimitation in photoautotrophy; thus, higher biomass productivity can be obtained (Liang et al., 2009). Under heterotrophic cultivation, lipid content is generally similar or higher than that obtained under photoautotrophic mode (Xu et al., 2006), which contributes to even higher lipid productivity, thus save the cost of downstream processing. According to Xiong et al., (2008), higher lipid productivity can be reported by using an improved fed-batch culture strategy in heterotrophic nutritional mode, where the lipid productivity is 20 times higher than that obtained under photoautotrophic cultivation. Another important advantage of heterotrophic growth condition is the possibility to perform a wastewater treatment. Moreover, the heterotrophic way allows cheaper and simpler bioreactor design, easier scaling-up

process and the possibility to manipulate biomass composition by changing the culture medium that stimulates specific metabolic and biosynthetic pathways (Brennan and Owende, 2010; Lu et al., 2010). However, heterotrophic process may present several problems: the large requirement of organic compounds make sometimes the cost of heterotrophic cultivation higher than photo-autotrophy (Zhang et al., 2013; Tabernero et al., 2012). Moreover, the presence of organic carbon in the heterotrophic culture could cause some contamination by other microorganisms (e.g. bacteria or fungi), which compete with microalgae and may reduce the quality and quantity of the algal biomass (Chen et al., 2011). Furthermore, only a limited number of microalgal species can grow heterotrophically; as a consequence, the heterotrophic cultivation is inappropriate for most microalgae because most species are obligate autotrophs (Behrens, 2005).

1.3.3 Photoheterotrophy

Photoheterotrophic algae use light as a source of energy, but they are not able to convert carbon dioxide into sugar; rather, as carbon source they use the organic compounds (Funke et al., 2008) that can be consumed only when there is light (Chen et al., 2011). The main difference between mixotrophy and photoheterotrophy is that photoheterotrophy requires light as energy source, while mixotrophy can use organic compounds to achieve that. Hence, because organic carbon and light are compulsory for photo-heterotrophic cultivation, it is rarely used as an approach to produce microalgal biomass to process valuable compounds (Wang et al., 2014).

1.3.4 Mixotrophy

Mixotrophic cultivation is a trophic way in which microalgae can drive photoautotrophy and heterotrophy and can utilize both inorganic and organic carbon sources (Kang et al., 2004). Inorganic carbon is fixed through photosynthesis, which is influenced by light conditions, while organic compounds are assimilated through aerobic respiration, which is affected by the availability of organic carbon (Hu et al., 2012a). Mixotrophy in ecosystem is a rule rather than an exception: it is wide spread among prokaryotes and protists (Matantseva, 2012). The main hypothesis is that the capability for mixotrophic growth might be the backup alternative of obtaining energy when photosynthesis is impossible, for instance, when illumination is insufficient, or other limiting factors occur in oligotrophic environment, providing significant competitive advantages to the organisms. Mixotrophic nutrition in protists is a prominent example of cellular mechanisms providing interaction of unicellular organisms with their environment and has a great ecological importance (Jones, 1994; Sanders, 1997; Esteban et al., 2010). Some scientists suggested that the specific growth rate of microalgae under mixotrophic cultivation is approximately the sum of those under photoautotrophic and heterotrophic modes (Marquez et al., 1993). Others suggested that the specific growth rate in mixotrophy is not the simple combination of those in photoautotrophy and heterotrophy, but some kind of synergetic mechanism is involved, and this data are consistent with the highlighted mechanism overcoming photoinhibition in mixotrophy (see section Mixotrophy and light). They consider that the two metabolic processes (i.e. photosynthesis for photoautotrophy and aerobic respiration for heterotrophy) affect each other under mixotrophic cultivation, contributing to synergistic effects and enhancing biomass productivity (Yu et al., 2009; Acién et al., 2013). Since organic compounds can

be utilized under mixotrophic cultivation, the growth of microalgae does not strictly depend on photosynthesis: light is no more an absolute limiting factor for microalgal growth. Complementing photoautotrophy with organic substrates, mixotrophic cultivation of microalgae can improve the growth rate, shorten the growth cycle, reduce biomass loss in dark hours due to pure respiration, and increase biomass productivity (Park et al., 2012). Sometimes lipid content can be augmented as percentage on dry weight, which leads to an even higher lipid productivity and it is of great importance for microalgal biodiesel production. Finally, the CO_2 released by microalgae via aerobic respiration can be trapped and reused for photosynthesis under mixotrophic cultivation, which enhance inorganic carbon availability for microalgae and thus further enhances biomass and lipid productivities (Mata et al., 2010).

1.4 What triggers mixotrophy

By now is not clear why, how and at which moment do autotrophs begin assimilating the dissolved organic substance. Simple approach would suggest that autotrophy is used when light and mineral nutrients (N and P) are no limiting factors, whereas light and nutrient limiting conditions combined with the presence of available organic substrates should promote heterotrophic nutrition (Hansen et al., 1997; Li et al., 2000). However, data have shown that regulation of mixotrophy is based on less linear mechanisms. Thus, it was found out that good illumination in protists can induce not only photosynthesis, but also phagocytosis, while the presence of organic substrates is able to accelerate the inorganic carbon fixation, thus supplying the organism with necessary biogenic elements (Moorthi et al., 2009; Burkholder et al., 2008). Many studies suggest that other factors such as temperature, CO_2 saturation, oxygen concentration, life cycle

stage, selection and growing media composition can play the trigger role. Currently regulation principles of mixotrophy in microorganisms have not been fully understood, and it can be merely stated that most likely there do not exist universal laws for all mixotrophic organisms (Matantseva and Skarlato 2013). Although it is not clearly understood how these factors affect mixotrophy metabolism, a comprehensive consideration and successful manipulation of these factors may lead to optimum cultivating conditions maximizing productivities (Wang et al., 2014). The main identified factors affecting mixotrophic responses are the carbon source and the illumination regime. Mixotrophy is triggered, first of all, by the presence of an organic carbon substrate in adequate amount and quality (Hu et al., 2012b). Illumination regime also plays an important role in mixotrophic cultivation. Although less sensitive toward light than photoautotrophy, illumination is still an important factor influencing productivities of mixotrophically-cultivated microalgae. It is generally considered that wavelengths of 600–700 nm (red light) are most efficient for photosynthesis, while wavelengths of 400–500 nm (blue light) may improve the overall growth rate of mixotrophic microalgae (Wang et al., 2007; Carvalho et al., 2011). Some authors also report that low light intensity encourages mixotrophic cultivation (Legrand et al., 1998; Graneli et al., 1999; Stoecker et al., 2006; Burkholder et al., 2008). Moreover the CO_2 supply seems to affect mixotrophic triggers, indeed carbon dioxide is the major limiting factor for algal growth and its excess strongly enhances photosynthetic productivity (Sforza et al., 2010); therefore, CO_2 supply is needed to achieve a high productivity even in mixotrophic conditions. However, it seems that the microalgae are not able to consume organic carbon with an excess of CO_2 concentration in the medium (Sforza et al., 2012), thus to pull mixotrophic growth a sharp control of CO_2 supply is required.

1.5 Mixotrophy and light

Algal growth is related to light intensity and the generalized Light Response Curve (LRC) relating algal growth (P) to light intensity (I) (P-I) has the shape shown in Figure 1.1. LRC can be divided into three phases: I) photolimitation phase, in which growth rate increases with the augment of light intensity, II) photosaturation phase in which growth rate is relatively independent from light intensity, and III) photo-inhibition phase in which growth rate declines with the increase of light intensity (Ogbonna et al., 2000). Because most algal species become light saturated at a low fraction of peak solar-light intensity, much potentially useful solar energy is essentially wasted for photosynthesis.
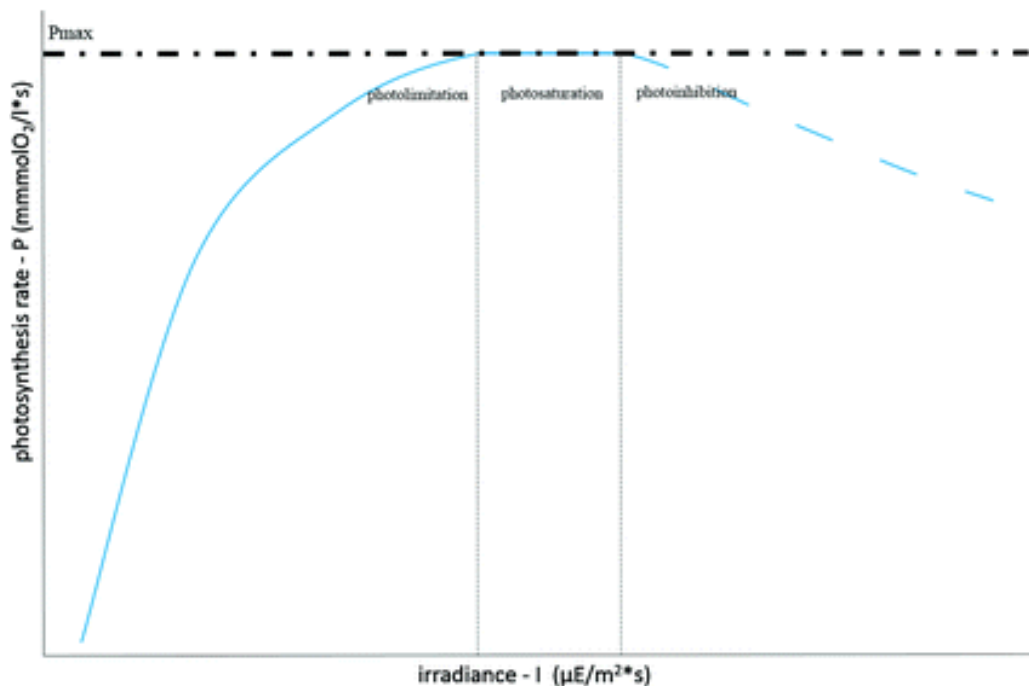
Fig. 1.1. Light response curve for microalgae

For example, the light saturation constants for microalgae *Phaeodactylum tricornutum* and *Porphyridium cruentum* are 185 and 200 µE m^{-2} s^{-1}, respectively (Mann and Myers 1968; Molina Grima et al., 2000). Veirazka (2011 and 2012) reported as photosaturation

range a light intensity ranged from 100 to 500 µE m^{-2} s^{-1}. The typical midday outdoor light intensity in equatorial regions is about 2000 µE m^{-2} s^{-1}, and value around 1000-1500 µE m^{-2} s^{-1} can be reached in sunny days at higher latitude location. Above a certain value of light intensity, beyond light saturation, a further increase in light level, not only does not increase photosynthesis, but also reduces the biomass growth rate (Figure 1.1). This phenomenon is known as photoinhibition. Microalgae become photoinhibited at light intensities only slightly greater than the light level at which the specific growth rate peaks. Elimination of photoinhibition or its postponement to higher light intensities can greatly increase the average daily growth rate of algal biomass. Because of light saturation, and subsequent photoinhibition the biomass growth rate and thus total yield, is much lower than theoretically possible.

1.5.1 Sensitivity to photoinhibition

Mixotrophically cultivated microalgae are less sensitive to light inhibition than those cultivated under photoautotrophy, no matter to which phase the intensity of illumination belongs. Photo-inhibition of *Spirulina sp.* was observed at light intensities above 50 W m^{-2} under photoautotrophic cultivation, whereas under mixotrophic cultivation inhibition was not observed up to light intensities of 65 W m^{-2} (Chojnacka and Noworyta 2004). Moreover, after photoinhibition occurred, microalgae under mixotrophic cultivation recovered faster and to a higher extent.

It is generally accepted that photoinhibition results from:

1. the inability of the photosynthetic apparatus to use excess light energy absorbed by the photosynthetic antenna: there is a mismatch between the fast rate of photon

capture by the light harvesting apparatus and the slower downstream rate of photosynthetic electron transfer (Perrine et al., 2012);

2. the production of reactive oxygen species (ROS): algae absorb light energy in order to oxidize water, exchanging electrons in proximity of molecules such as singlet oxygen or triplet chlorophyll a, thus producing harmful reactive oxygen species (ROS). When ROS accumulate and cause more damage than that can be reconciled, algae experience oxidative stress.

1.5.2 Protection mechanism against photoinhibition

The reduced sensibility of mixotrophic cells to photoinhibition has been attributed to five main mechanisms:

1. Higher Cell concentration: the mixotrophic culture allows to obtain higher biomass production and thus higher cell density, since radiant energy is not the only promoting factor for the growth, but the carbon provides an additional energy input. The higher cell density of microalgae grown in mixotrophy determines a greater shadowing and thus a lower average exposure of each cell to light radiation, i.e. the same radiant energy can be distributed to a higher number of cells in mixotrophic condition, thus limiting possible damage. Mixotrophic cultures have a 20-40 % higher growth rate at any given light intensity in comparison with photoautotrophic cultures (Vonshak et al., 2000).

2. Re-balance of light dependent and enzymatic dependent reaction. The light-capture reaction is faster than the subsequent enzyme-mediated reactions, thus the maximum rate of photosynthesis must be controlled by the concentration of one of the enzymes of the Calvin cycle (Sukenik et al., 1987). A lack of electron sinks downstream of

photosystem I (e.g. carbon fixation) can result in accumulation electrons in the electron transport chain and subsequently an increased risk of photoinhibition and ROS production (Niyogi 2000). An increase in Calvin cycle activity, due to the abundance of organic carbon, can lead to increase in the consumption of reduction power (Vonshak et al., 2000).

3. Rapid repair of damage to photosystem II: damages from photoinhibition are promptly repaired, depending on the environmental conditions and the physiological conditions of the cell, through the action of D1 protein. PSII is susceptible to be damaged by high irradiation. Ohad et al., (1984) suggested that the turnover of D1 protein is part of a repair system to replace the damaged function centers with newly synthesized protein D1 thus restore the normal PS II activity. The recovery from the photoinhibition is not just stress reaction process but it requires an active anabolic process to re-synthetize D1 protein. The faster recovery rate observed in mixotrophic cultures is attributed to higher metabolic activity (Vonshak et al., 2000), and this mechanism had been already highlighted by Chueng et al., (1984) that obtained greater recovery from photoinhibition in mixotrophy. Anyway the increased recovery was cancelled when chloramphenicol (a protein inhibitor) was applied to culture: in this case mixotrophic and autotrophic recovery was the same.

4. Reduction in the size of the light-harvesting antenna and reduction in chlorophyll content (Beckmann et al., 2009; Liu et al., 2009). This mechanism reduces the light adsorbing capacity of individual cell, increasing light penetrance in deep layers of photobioreactors and reducing heat dissipation of absorbed light energy, thereby increasing photosynthetic efficiency in high light and high cell density culture (Eriksen 2008). This mechanism is highly effective considering not the individual

efficiency, but the whole production system (high densities PBR). The less effective performance of individual cell allows to protect the single cell and to distribute light thus achieving a better performance of the whole system.

5. Oxygen decrease in the culture medium: high dissolved oxygen concentration in close photobioreactors might accelerate oxidative reactions.

   – Increased oxygen consumption. The oxygen produced by photosynthesis is released in the culture medium. In algae culture exposed to high Photon Flux Densities (PFD) the dissolved oxygen concentration in the medium can reach above 200% of air saturation, limiting condition for algae growth and chlorophyll synthesis (Ugwu et al., 2007). Many algae strains cannot survive in significantly O^{2-} oversaturated milieu longer than 2–3 h (Pulz 2001). Cells growing mixotrophically, thanks to the respiration reactions promoted by carbon abundance, consume oxygen and allow a considerable decrease of the concentration of dissolved oxygen in the culture medium and the entire photobioreactor, thus reducing photoxidative damage (Chojnacka and Marquez-Rocha 2004).

   – Decreased oxygen production: Roach et al., (2013) showed that thylakoids from mixotrophic *C. reinhardtii* produced less O_2 than those from photoautotrophic cultures due to destabilization in secondary quinone acceptor favouring direct non-radiative charge recombination events that do not lead to oxygen production.

1.5.3 Light limiting condition

Taking into consideration light limiting condition, i.e. the left-most part of the Light Response Curve, mixotrophically cultivated microalgae are less sensitive toward various levels of light intensities, and they can better acclimate to and recover from diurnal light changes, which would alleviate the burden of artificial illumination cost. The lower light sensitivity of mixotrophic cultivation is especially advantageous for cultivating microalgae at high cell densities or with dark colored (opaque) growth medium such as wastewater, in which occasions light penetration often becomes a limiting factor (Li et al., 2012).

1.6 Productivity and energy balance

1.6.1 Biomass productivity

With complementary organic substrates, the productivity of microalgal biomass in mixotrophy is generally much higher than that in photoautotrophy and higher than in heterotrophy (Wan et al., 2011; Xiong et al., 2010; Ogbonna et al., 2000). The highest biomass productivities of *Nannochloropsis oculata, Dunaliella salina, Chlorella sorokiniana, Spirulina platensis*, and *Scenedesmus obliquus* under mixotrophic cultivation with glucose supply were 1.4 times, 2.2 times, 2.4–4.2 times, 3.8 times, and up to 8.7 times that of photoautotrophic cultivation (Chen et al., 1997; Mandal et al., 2009; Wan et al., 2011). The addition of glucose, acetate, and glycerol under mixotrophic cultivation, respectively, improved the biomass productivity of *Phaeodactylum tricornutum* of 1.5-, 1.7-, and 2.5-fold of that obtained in photoautotrophy (Liu et al., 2009). Some strains of microalgae can achieve synergistic effect under strictly controlled mixotrophic mode and can reach higher biomass

productivities than in heterotrophic culture (Yu et al., 2009). Bhatnagar et al., (2011) investigated the biomass productivities of *Chlamydomonas globosa, Chlorella minutissima*, and *Scenedesmus bijuga* under three major cultivation modes. Experimental results indicated that with 1 % (w/v) glucose addition, biomass productivities of *Chlamydomonas globosa, Chlorella minutissima*, and *Scenedesmus bijuga* under mixotrophic cultivation were 9.4 times, 6.7 times, and 5.8 times of those under photoautotrophic cultivation and were 3.0 times, 2.0 times, and 4.4 times of those under heterotrophic cultivation. Compared with other cultivation modes, the relatively high microalgal biomass productivity in mixotrophy contributes to a higher biomass production, allowing to reaching a better economic viability for large-scale microalgal production plants.

1.6.2 Lipid productivity

Lipid productivity is determined by both biomass productivity and lipid content, which can be expressed as follows:

Lipid productivity = biomass productivity × lipid content

It is evident that, to achieve the highest possible lipid productivity, integrated effects of biomass productivity and lipid content should be taken into consideration. Since the highest levels of the two parameters can seldom be simultaneously achieved, mixotrophy is considered to be of great advantage due to higher biomass productivity obtained with limited lipid content reduction. Compared with photoautotrophic cultivation, the lipid productivity of mixotrophically cultivated *Nannochloropsis sp.* with glycerol as organic carbon source was improved by 40-100 % (Probir et al., 2011).

Supplemented with glucose, the lipid productivities of *Nannochloropsis oculata,* *Dunaliella salina,* and *Chlorella sorokiniana* under mixotrophic cultivation were 1.1 - 1.6 times, 1.8 - 2.4 times, and 4.1 - 8.0 times of those under photoautotrophic cultivation (Chojnacka and Marquez-Rocha 2004). According to Mandal and Mallick (2009), the lipid productivity of *Scenedesmus obliquus* under mixotrophic cultivation with 1.5 % (weight/volume) glucose supply could be as high as 270 mg l^{-1} day^{-1}, which was approximately 50 times of that achieved in the photoautotrophic culture as control. Compared with heterotrophic cultivation, mixotrophically cultivated *Chlorella protothecoides* on glucose was reported to achieve 69 % higher lipid productivity (Xiong et al., 2010). Liang et al., (2009) investigated the lipid production of *Chlorella vulgaris* under photoautotrophic, mixotrophic, and heterotrophic cultivation conditions. Experimental results indicated that with 1 % (w/v) glucose addition, the lipid productivity of *Chlorella vulgaris* under mixotrophic cultivation was, respectively, 1.5 times and 13.5 times of that under heterotrophic and photoautotrophic cultivation.

1.6.3 Energy efficiency

The performance of microalgae culture can be evaluated and compared through the efficiency of conversion (E) by which all the energy supplied to the culture is utilized for biomass production. To do that the inlet energy supplied to the system (radiant energy and chemical energy) is compared with the energy content in the microalgae biomass (chemical energy) i.e.

Energy in biomass / (energy from light+ energy from organic carbon)

Yang et al., (2000) reported biomass yields on the supplied energy (Y_{SE}) equal to 0.00924 g kJ^{-1} for mixotrophy, 4 times higher than that recorded for autotrophy finally

finding that Y_{SE} was the lowest in the autotrophic cultivation; opposite the mixotrophy gave the most efficient utilization of energy for biomass production. The efficiency of conversion from light energy to biomass in autotrophy was around 3.5% (Table 1.1), opposite in mixotrophy the total efficiency of conversion into biomass was equal to 18%. This data are confirmed by Ren et al., (2014), that founded a total efficiency of conversion of energy in autotrophy equal to 1.2 % and 14.6 for mixotrophy (Table 1.1). Not surprisingly, the efficiency of conversion in the autotrophic culture was the lowest due to the inefficient conversion of light energy into biomass. Average data recorded for light conversion efficiency into chemical energy range from 1 to 8 % at lower photon flux densities (Molina Grima et al., 1997). If we consider only the amount of energy provided as organic carbon to algae the conversion efficiency is quite variable, from 18% (Yang et al., 2000) to 45% (Ren et al., 2014) to be compared with 58% recorded for unicellular microorganism, e.g. *Candida utilis* (Trinh et al., 2009). It is interesting to underline (Table 1.1) that conversion efficiency of organic carbon in algae is boosted with light in mixotrophy; in fact the total carbon conversion efficiency is 34.4 in heterotrophy but is 45.7 for mixotrophy, being that consistent with synergistic effects previous reported for mixotrophy.

Table 1.1. Energy conversion efficiency

Cultivation mode	Ec (%)	Et (%)	References
Autotrophy	0	1.2	Ren et al., 2014
Mixotrophy	45.7	14.6	
Heterotrophy	34.1	34.1	

	Ec	Et	
Autotrophy	0	3.5	Yang et al., 2000
Mixotrophy	40.5	18.5	
Autotrophy	0	3-8	Molina Grima et al., 1997

Ec: conversion efficiency of chemical energy
Et: conversion efficiency of the total supplied energy (light and organic carbon)

Considering all the energy balance, i.e. energy from light and from organic carbon, mixotrofically grown microalgae show the highest energy conversion efficiency. Due to rapid light attenuation by the suspending cells, shadowing and light distribution heterogeneity occurs inside the photobioreactor: i.e. light energy limitation is the most commonly encountered problem in practical cultures of photosynthetic cells. For maximum energy use efficiency, the light intensity should be homogeneously distributed in the entire photobioreactor, keeping the light intensity between the critical and the saturation ranges. In a practical photobioreactor, simultaneous existence of complete dark, light limitation, light saturation and light inhibition zones inside the same photobioreactor is a common phenomenon. Light energy supply and its efficient utilization is the greatest scientific and technological challenge in research and development on cultivation of photosynthetic microorganisms. In mixotrophic cultures, the energy source form organic carbon is homogeneously distributed inside the bioreactor so it is possible to exploit the heterotrophic metabolism occurring in some photosynthetic cells that are in light limiting condition. This carbon supply can counterbalance the very heterogeneous light distribution in photobioreactors and rebalance the energy flux within the microalgae cells thus gaining a better energy efficicency.

1.6.4 Advantages of mixotrophic cultivation

In addition to the ecological significance mixotrophy is an interesting productive opportunity due to the possibility: I) to depurate organic downstream, and II) to increase the production of valuable compounds using organic carbon so overcoming light limitation or eventually softening light inhibition, in any case increasing production. Compared with heterotrophy that relies merely on organic carbon sources, mixotrophic cultivation of microalgae yields higher productivities with identical organic carbon supply.

1.7 Mixotrophy exploiting wastewaters

Great volumes of wastewaters from industries processing agricultural raw materials, livestock, industries and wastewaters from domestic treatment plants, are annually dumped to aquatic ecosystems worldwide (Dareioti et al., 2009; Bhatnagar and Sillanpää 2010). These effluents are characterized mainly by a high concentration of organic matter, nitrogen and phosphorous, and a variable pH (Drogui et al., 2008). Both the flow rate and characteristics of these wastewaters are industry specific and can vary throughout the year (Dareioti et al., 2009). Uncontrolled disposal of such effluents into natural water bodies often results in surface and groundwater contamination and other environmental problems such as eutrophication and ecosystem imbalance (Drogui et al., 2008; Posadas et al., 2014). Therefore, it becomes necessary to develop low cost and environmentally friendly methods for the treatment of wastewaters. The initial purpose of introducing microalgae to wastewater treatment process was to realize tertiary treatment focusing on nutrients removal, it was further observed that microalgae could also remove efficiently organic pollutants from sewage (Wang et al., 2010a) and

increase productivity of biomass thanks to organic carbon, thus improving productivity and depuration. While the ability of algae to remove N and P from wastewater has been extensively studied (Woertz et al., 2009; Liang et al., 2013; Gentili 2014; Zhang et al., 2014), how algae growth relates to the organic carbon content in wastewater medium had less research attention (He et al., 2013; Tian–Yuan et al., 2013). The coupling of microalgae with wastewater is an effective way of waste remediation and a cost-effective microalgal biomass production (i.e. for biofuel, see paragraph 1.7.5). Combination of mixotrophic microalgal biomass production with wastewater treatment has been tested on various wastewater streams, including concentrated and un-concentrated municipal wastewater, digested and/or undigested animal manure, and agricultural raw material. Microalgae from the *Chlorella* and *Scenedesmus* families display excellent adaptation in wastewater and can achieve high biomass productivity, thus are the most commonly used strains for simultaneous algae biomass production and wastewater treatment (Li et al., 2012; Craggs et al., 2013). Li et al., (2011a) also found that algae strain *Chlorella sp.* cultivated in centrate wastewater stream, provided comparable biomass accumulation and lipid productivity with those grown in standard cultivation medium showing excellent adaptation in wastewater and great potential to accumulate valuable compounds (Li et al., 2012).

1.7.1 Urban wastewater

Human beings generate every year billions tons of domestic wastewater (FAO Aqua-stat), containing average carbon nitrogen and phosphorus amount as indicated in Table 1.2. Municipal wastewater can be generally divided in: I) primary wastewaters (PW), i.e. wastewaters after primary settling; II) secondary wastewaters (SW), which is wastewater after secondary treatment by activated sludge; III) tertiary wastewaters

(TW) after tertiary treatment (N and P uptake) has been performed; IV) centrate wastewater (CW), generated after dewatering, sludge by centrifuge.

Table 1.2. Chemical composition of urban wastewaters (range)

Type of wastewaters	COD	N	P	References
	(mg L^{-1})	(mg L^{-1})	(mg L^{-1})	
Primary wastewater	150- 500	33- 100	4-25	Zhou 2014; Henze 2008; Samorì et al., 2014
Secondary wastewater	24-34	8-15	0.5-50	Zhou 2014; Bunani et al., 2015
Centrate	2250	131	200	Zhou 2014

Primary treatment of wastewater aims at removing large particles in the sewage by means of grids or sedimentation. Secondary treatment reduces the biochemical oxygen demand (BOD) in the wastewater by oxidizing organic compounds and ammonium. This process, which is often carried out in aerated tanks with so-called activated sludge, involves both heterotrophic bacteria and protozoa. The bacteria degrade the organic material and the protozoa graze the bacteria, and in both cases organic material is converted to carbon dioxide and water. Tertiary wastewater treatment mainly aims at removing the plant nutrients nitrogen and phosphorus. Algal systems have traditionally been employed as a tertiary process (Lavoie and De la Noüe 1985; Martin et al., 1985a; Oswald 1988b). The ability of microalgae to uptake organic carbon justify and support the attempt to use microalgae also for secondary treatment of wastewaters and the treatment of centrate. Three quarters of organic carbon in sewage are present as carbohydrates, fats, proteins, amino acids and volatile acids thus they are readily

available carbon sources, suitable for microalgae uptake. The inorganic constituents include large concentrations of sodium, calcium, potassium, magnesium, chlorine, sulphur, phosphate, bicarbonate, ammonium salts and heavy metals (Lim et al., 2010). Centrate is high in carbon content (around 1000 mg L^{-1}) and proved to be favourable to selected mixotrophic genus such as *Chlorella sp., Heynigia sp., Hindakia sp., Micractinium* (Zhou et al., 2011). In the case of primary wastewater and centrate the mechanisms for nutrients removal in wastewater by microalgae include typical photosynthetic assimilation and/or chemosynthetic assimilation by heterotrophic/mixotrophic metabolic pathway.

Table 1.3. Phycodepuration of urban wastewaters

Microalgae strain	Wastewaters	Removal			References
		COD (%)	N (%)	P (%)	
Auxenochlorella prototecoides UMN280	Concentrated municipal wastewater	88	59	81	Zhou et al., 2012
Euglena sp.	Sewage treatment plant	-	93	66	Mahapatra 2013
Chlorella vulgaris	Synthetic sanitary sewage	78.7	74.6	72.8	Xu 2013
Botryococcus braunii	Domestic wastewater	-	79.6	100	Sydney et al., 2011
Auxenochlorella prototecoides	Concentrated municipal wastewater	81.4	73.6	75.1	Hu et al., 2012
Chlorella sp.	Centrate wastewater	70	61	61	Min et al., 2011
Chlorella sp. and *Scenedesmus sp.*	Domastic wastewater	90	100	-	Hammouda 1995

Auxenochlorella prototecoides UMN280 isolated from municipal wastewater plant showed high nutrient removal efficiency as well as its high growth rate and lipid productivity. Batch cultivation showed maximal removal efficiencies for total nitrogen,

total phosphorus and chemical oxygen demand (COD) over 59%, 81% and 88% respectively, with high growth rate (0.490 d^{-1}), high biomass productivity 269 mg L^{-1} d^{-1}, and high lipid productivity (78mg L^{-1} d^{-1}) (Zhou et al., 2012). The presence of organic carbon may counter balance for the shortage of CO_2 dissolved in the growing medium, which is often the limiting factor for the growth of microalgae in low cost growing systems (e.g. open ponds). The presence of organic carbon replaces the presence of CO_2, both by heterotrophic metabolism (organic carbon assimilated as such) that by autotrophic (carbon employed in the form of CO_2 produced after mineralization by microorganisms present in the culture medium or produced by enhanced respiration of microalgae heterotrophic metabolism). The increased algal growth due to organic carbon finally allows for greater purification of the wastewater. Ledda et al., (2015) proved that *N. gaditana* could be produced using centrate as the only nutrient source at percentages below 30%, while higher percentage resulted in ammonia inhibition. Nitrogen depuration decreased from 85% to 63% with the increase of centrate percentage in the culture medium and the decrease in biomass productivity, Phosphorus depuration from the culture medium was 85% whatever the centrate percentage in the culture medium indicating a phosphorus limitation into the cultures. The use of centrate was confirmed as a useful method for reducing microalgae production costs and for increasing process performance.

1.7.2. Livestock wastewaters

In the last few decades global agriculture and livestock activities have increased rapidly in conjunction with the growing food demand of the global population (FAO 2014). Technological innovations have led to profound structural changes and improvements in

the agro-zootechnical sector, increasing the productivity efficiency, but at the same time raising negative environmental implications associated with the expansion of this sector. Agriculture and livestock sectors produce large amounts of effluents especially animal manure wastewaters that are widely available all over the world and can cause severe pollution issue if not properly managed (Zhou 2014). In the United States approximately 35 million dry Mg of livestock wastes are produced every year, while in the EU-27 more than 1500 million fresh Mg of livestock wastes are generated annually (Choi et al., 2014). Nowadays, the management of livestock wastes mainly includes conversion of livestock wastes to bioenergy through biological (i.e. anaerobic digestion) or thermo-chemical processes, composting for agricultural applications, and combustion for heat and electricity generation (Zhu and Hiltunen 2016). The challenge is the developing sustainable approaches to manage, recycle and give value to agriculture wastewaters minimizing the impacts on the environment. In this context, microalgae-based processes constitute a cost effective technology for the degradation of livestock wastewaters (de Godos et al., 2009; Mulbry et al., 2008). Effluents from poultry, piggery and dairy farms contain high concentrations of nitrogen, phosphorus, and organic carbon (Table 1.4) in both soluble and particulate forms; the composition mainly depending on animal nutrition and farming practices (Bernet and Bèline 2009). Another interesting stream able to support microalgae growth is the digestate produced by the anaerobic digestion process (see paragraph 1.7.2.1). Some authors sustain the possibility to recovery and reuse nutrients from digestate through the cultivation of microalgae. (Franchino et al., 2013; Ledda et al., 2015a, Ledda et al 2015b). The high nutrients concentration, high turbidity and possible contamination by microorganism are the main challenges to overcome for the microalgae agro-wastewaters treatment

feasibility. Many works reported toxic effects for ammonia concentrations higher than 100 mg L^{-1} (Collos and Harrison 2014) although a wide range of tolerance has been reported for several microalgae species. For example, *Chlorella sorokiniana* was completely inhibited at an ammonia concentration above 210 mg L^{-1} (Munoz et al., 2005) whereas *Spirulina platensis* was inhibited at 150 mg L^{-1} (Ogbonna et al., 2000). Sepúlveda et al., (2015) reported the absence of inhibition for *Nannochloropsis gaditana* cultures at an ammonia concentration of up to 334 mg L^{-1}. As livestock wastewater contains amount of ammonia (Table 1.4) at least one order of magnitude higher, the commonly adopted strategy is to dilute the stream to reach the proper nutrients level requirement for algae growth (Zhou et al., 2012) contemporary reducing the shading effect due to the dark colour of the effluents.

Table 1.4. Chemical composition of livestock wastewaters

Effluents	pH	TS	TN	TP	COD	References
		(mg L^{-1})	(mg L^{-1})	(mg L^{-1})	(mg L^{-1})	
Liquid pig slurry	6.3	26000	2880	710	3189	Misselbrook et al., 2013
Anaerobic Digestate	7.97	80000	2940	50	9906	Ledda et al., 2013 Ledda et al., 2015
Pig manure	8.37	211100	6295	3194	54498	Li et al., 2012
Chicken manure	6.95	550000	24035	10120	49045	Ho et al., 2013
Dairy manure	7.5	117000	1884	551	13161	Liu et al., 2011

Trials shown in Table 1.5 demonstrate that different microalgae species are able to grow on livestock wastewaters determining high nutrient removal efficiency. *Chlorella* is the most renowned genus used for nutrient removal in wastewaters, thanks to the excellent adaptation of these microalgal species on this substrate (Li et al., 2012). Franchino et

al., (2013), reports that *Chlorella vulgaris* presented the highest removal capacity of ammonium in a diluted 1:10 digestate sample (derived from a mix of cattle manure and raw cheese way), with a 96% removal efficiency, and it was also observed that only the 4% of ammonia was removed by stripping. This author sustains that *C. vulgaris* has higher growth rate than the other two strains used *Scenedesmus obliquus* and *Neochloris oleoabundans,* with a μ (day^{-1}) of 0.64, 0.49 and 0.27 days^{-1}, respectively. Similar results were observed by Wang et al (2010b) that assessed that *Chlorella vulgaris* grown in anaerobic digestate dairy manure removed ammonia, total nitrogen, total phosphorus, and COD by 100%, 75.7–82.5%, 62.5–74.7%, and 27.4–38.4%, respectively. As regards to carbon removal, Kim et al., (2000), showed that *Spirulina platensis* grown on different concentration of swine waste for 12 days in batch culture, was able to reduce 80-90 % of COD, the highest detected. De Godos et al., (2009) reports that a microalgal-bacteria consortium have reached a total Kjeldahl nitrogen and COD removals of 88 ± 6% and 76 ± 11% in a high rate algal ponds (HRAPs) cultured on diluted swine manure for 245 days with an hydraulic residence time of 10 days. In considering the use of wastewater to cultivate microalgae, another key issue is the negative effect of bacteria on microalgal biomass survival and quality leading to an important constraint in the scale-up of cultivation of microalgae using wastewaters. This issue could be overcome by using and/or combining different strategies e.g. isolating wild microalgae strains that tolerate substrates, such as livestock slurries. Ledda et al (2015) isolated a wild microalgae strain from digested pig slurry to evaluate differences in growth and remediation performances in sequential digestate liquid fractions sampled from a full-scale digestate treatment plant. The isolated *Chlorella* proved to be a strong

strain, capable of reducing about 95%–98% of N-NH$_4$$^+$ and 61–73% of COD, while micronutrients were almost completely removed.

Table 1.5. Phytodepuration of livestock wastewaters

Microalgae strain	Wastewaters	Removal			References
		COD (%)	N (%)	P (%)	
Neochloris oleoabundans	Agro-zootechnical digestate	-	99.9[a]	96.9[c]	Franchino et al., 2013
Chlorella vulgaris		-	99.9[a]	96[c]	
Scenedesmus obliquus		-	83.7 - 92.4[a]	96.1[c]	
Chlorella sp.	Digested manure	27.9 - 38.4	100[a]; 75.7 - 82.5[b]	62.5 - 74.7[d]	Wang et al., 2010
Chlorella pyrenoidosa	Primary piggery wastewater	36.5 - 55.4	91.2 - 95.1[a] 54.7 - 74.6[b]	31 - 77.7[d]	Li et al., 2012
Chlorella sp. UMN271	Fermented liquid swine manure	62.45 - 72.58	26.7 - 99.9[a] 12.9 - 55.8[b]	79.08 - 88.56[c]	Hu et al., 2012
Chlorella sorokiniana and aerobic bacteria	Liquid fraction of pig manure	62.3	82.7[a]	58[c]	Hernández et al., 2013
Ourococcus multisporus		-	19[b]	-	
Nitzschia cf. pusilla		-	17[b]	-	
Chlamydomonas mexicana	Piggery wastewater	-	62[b]	28[d]	Abou-Shanab et al., 2013
Scenedesmus obliquus		-	60[b]	-	
Chlorella vulgaris		-	51[b]	-	
Spirulina platensis	Swine waste	80 - 90	67 - 93	70 - 93[c]	Kim et al., 2000
Microalgal-bacterial consortium	Diluted swine manure	76	88	10[c]	de Godos et al., 2009
Scenedesmus obliquus	Piggery wastewater	42	36[a]	27 - 65[d]	de Godos et al., 2010
Chlorella sorokiniana		42 - 47	21 - 25[a]	20 - 54[d]	
Euglena viridis		51 - 55	34 - 39[a]	28 - 60[d]	

Neochloris oleoabundans	Anaerobic effluents from pig waste	-	98[a]	98[c]	Olguin et al., 2015
Chlorella sp.	digested swine manure	61-63	95-98	85-99%	Ledda et al., 2015

[a] Ammonium (NH_4^+)
[b] Total nitrogen
[c] Phosphates (PO_4^{3-})
[d] Total phosphorous

1.7.2.1 Anaerobic digestion plant and microalgae: a perfect model for exploiting downstream

Anaerobic digestion (AD) is used to stabilize organic waste streams (mainly livestock slurries, but also by-products and waste) producing biogas (50-75% CH_4 and 25-50% CO_2) that can be used to produce renewable energy in substitution for fossil fuel-derived energy. The downstream of biogas production are: digestate rich in N P and residual COD, heat, CO_2. Recently new paradigm for AD has been developed in order to overcome problem related to the AD cost vs. subside (Manenti and Adani 2015) and develop an exemplary model of circular economy. In this paradigm the biogas plants has been indicated as the facility unit to build a diffused bio-refinery model (Manenti et al., 2016), producing different goods: bio-methane, and nutrient (N and P), organic nutrients (COD), CO_2 and heat useful to produce 3rd generation biomass (microalgae). This approach allows diversifying biogas products, reducing biogas cost and increasing circular economy implementation. Ledda et al., (2015b) investigated the possibility of integrating microalgae production with anaerobic digestion of dairy cattle manure and subsequent digestate treatment, thus helping to reduce the cost of slurry treatment and improving the energy balance of the process. Real biogas and digestate-treatment units were monitored for energy, mass and nutrient balances. Microalgae production was integrated with this system by using untreated ultra-filtered digestate as the growth medium for the production of *Scenedesmus sp.*. The tolerance of this strain to digestate

was evaluated and results demonstrated that a percentage of digestate of over 10% inhibited the growth of this microalga, but below this value productivity of up to 124 mg L^{-1} d^{-1} was obtained. The composition of the culture medium influenced the biomass composition, with protein content being positive correlated with ammonia concentration. Finally, it was demonstrated that integrating microalgae production with anaerobic digestion, it is possible to produce 166-190 Mg y^{-1} of valuable microalgal biomass (Figure 1.2).
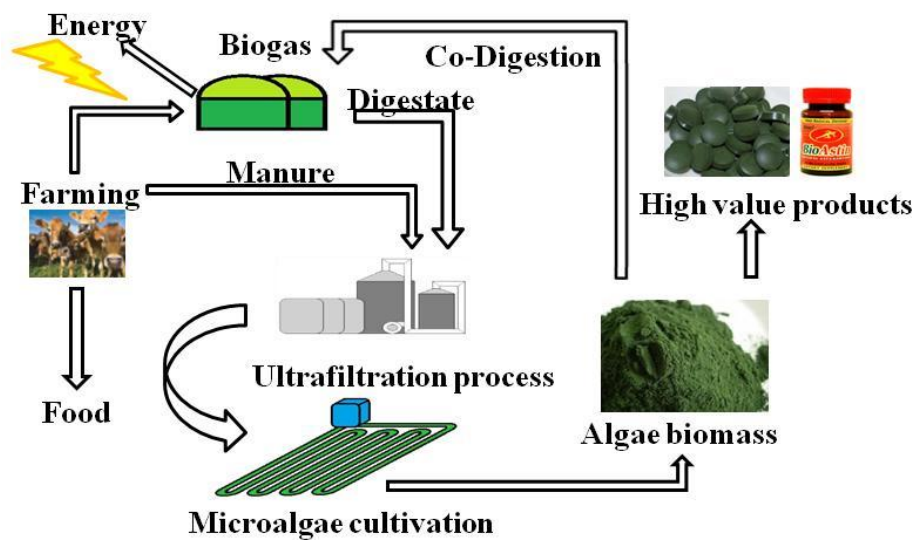
Fig. 1.2. Anaerobic digestion plant as model for exploiting downstream

1.7.3 Agro-industrial wastewaters

The compositions of agro-industrial wastewaters are industry specific and can vary significantly during the year considering the seasonal variation of the processed materials.

Table 1.6. Chemical composition of agro-industrial wastewaters

Type of Wastewater	COD	N	P	References
	(mg O_2 l^{-1})	(mg l^{-1})	(mg l^{-1})	
Cheese factory anaerobic effluent	1500	125	80	Blier et al., 1995
Fish farm wastewater	152	-	-	Dumas et al., 1998
Soybean processing	13215	267.1	56.3	Hongyang et al., 2011
Potato processing	1536	33.7	4.2	Hernandez et al., 2013
Potato processing	872	69	6	
Fish processing	1016	82	6	
Animal feed production	2557	197	27	Posadas et al., 2014
Coffee manufacturing	22752	766	59	
Yeast production	3163	703	7	
Digested palm starch processing	1340	40	21	Phang et al., 2000
Dairy industry wastewaters	6000	18.45	5.58	Kothari et al., 2013

Despite their relevance, little attention has been given to the treatment of agro-industrial wastewaters (Posadas et al., 2014). Nutrient reduction in agro-industrial wastewaters varies greatly depending on their composition; in the literature are reported reductions of COD, N and P ranging from 30-40% to nearly 100% (Table 1.7). Dumas et al., (1998) investigated the use of cyanobacterium *Phormidium bohneri,* to remove dissolved inorganic nutrients from fish farm effluents. Average efficiencies of ammonia nitrogen and orthophosphate removal was 82% and 85% respectively. Blier et al., (1995) investigated the growth and nutrient removal capacity of the cyanobacterium

Phormidium bohneri and of the endogenous microalga *Micractinium pusillum* for the bio-treatment of a cheese factory anaerobic effluent. In the presence of this cyanobacterium or this microalga, ammonia was completely removed after four days, although the kinetics of removal were different for both species. Removal of phosphorus after four days of culture was only 33% for *Micractinium,* and 69% with *P. bohneri*. Phang et al., (2000) grew *Spirulina* on anaerobically digested palm starch factory wastewaters: the percentage of reductions in COD, ammonia and phosphate reached 98.0%, 99.9% and 99.4% respectively. More recently (Hongyang et al., 2011) *Chlorella pyrenoidosa* was cultivated in soybean processing wastewater. The alga was able to remove about 78% of soluble chemical oxygen demand (COD), 89% of ammonium nitrogen and 70% of total phosphate. Hernandez et al., (2013), treated potato processing wastewaters with a microalgae-bacteria consortium of *Chlorella pyrenoidosa* and aerobic sludge. The removal efficiency was very high, indeed ammonium was almost exhausted (decrease >95%), phosphorous removal efficiency was 80.7%, while total COD was utilized for 85% if its initial content.

Table 1.7. Phycodepuration of agro-industrial wastewaters

Microalgae strain	Wastewaters	Removal			References
		COD (%)	N (%)	P (%)	
Phormidium bohneri	Fish farm wastewater	66	82	85	Dumas et al., 1998
Chlorella pyrenoidosa	Soybean processing	78	89	70	Hongyang et al., 2011
Chlorella sorokiana + aerobic sludge	Potato processing	85	>95	81	Hernandez et al., 2013
Phormidium (71 %), Oocystis (20 %) and	Potato processing	54	60	-	

Microspora (9 %)	Fish processing	64	74	-	Posadas et al., 2014
	Animal feed production	49	80	-	
	Yeast production	33	50	-	
	Coffee manufacturing	56	80	-	
Phormidium bohneri	Cheese factory anaerobic effluent	-	98	69	Blier et al., 1995
Micractinum pusillum		-	97	33	
Spirulina platensis	Palm starch processing	98	99.9	99.4	Phang et al., 2000
Chlamydomonas polypyrenoideum	Dairy industry wastewaters	64	90	70	Kothari et al., 2013
Chlorella vulgaris	Textile wastewater	38-62	44	33	Lim et al., 2010

Kothari et al., (2013), performed a process of phyco-remediation of dairy industry wastewater by *Chlamydomonas polypyrenoideum*. Results obtained indicate that dairy industry wastewater was good nutrient for algal growth in comparison with BG-11 growth medium. Algae grown on dairy industry wastewater demonstrated to use and carbon for biomass generation (64% of uptake) reduced the pollution load of nitrogen (90%) and phosphate (70%) in 10 days of treatment. Posadas et al., (2014) tested the potential of algal–bacterial symbiosis for the removal of carbon, nitrogen and phosphorus from five agroindustrial wastewaters: potato processing, fish processing, animal feed production, coffee manufacturing and yeast production. The highest removals of nitrogen (85%) and total organic carbon (64%) were observed for fish processing wastewaters while the maximum P-PO$_4$ removal achieved was 89 % in undiluted potato processing wastewaters. Authors moreover observed that the biodegradable TOC was, in most cases, the limiting component in the treatment of the wastewaters evaluated. Dumas et al., (1998) observed a maximum growth rate of

Phormidium bohneri cultivated on fish farm effluents of 0.06 mg d.m. day^{-1}. These values were expected because the concentration of inorganic nutrient were very low respect to Blier et al., (1995) that investigated the growth and nutrient removal capacity of the cyanobacterium *Phormidium bohneri* and of the endogenous microalga *Micractinium pusillum* for the biotreatment of a cheese factory anaerobic effluent. *Phormidium bohneri* demonstrated higher growth rate (μ_{max} = 0.62 d^{-1}) and biomass yield (329 mg dm l^{-1}) than that of *M. pusillum* (0.35 d^{-1} and 137 mg dm l^{-1}) over four days. Phang et al., (2000) used wastewater from the production of palm starch to cultivate *S. platensis*. The specific growth rate was 0.51 1/d and the biomass productivity was 14.4 g m^2 d^{-1}. The highest protein, carbohydrate and lipid content of the biomass were 68%, 23% and 11% respectively. Hongyang et al., 2011 reported an average biomass productivity of 0.64 g L^{-1} d^{-1} with a lipid productivity of 0.40 g L^{-1} d^{-1} using fed-batch culture. Hernandez et al., (2013) treated potato processing wastewaters using microalgae-bacteria consortium, biomass production achieved 18.8 mg DW l^{-1} d^{-1}, and the microalgae lipid content was 30.2%. Kothari et al., (2013) involved a process of phyco-remediation of dairy industry wastewater by *Chlamydomonas polypyrenoideum* for biodiesel production. The lipid content of algal biomass grown on dairy wastewater on 10th day (1.6 g) and 15th day (1.2 g) of batch experiment was found to be higher than the lipid content of algal biomass grown in BG-11 growth medium on 10th day (1.27 g) and 15th day (1.0 g) of batch experiment.

1.7.4 Full scale reactors for phytoremediation

Algal high-rate ponds (HRPs) were developed beginning in the 1950s as an alternative to oxidation ponds for BOD, suspended solids, and pathogen removal. HRPs are

raceway shape ponds 30 100 cm deep, equipped with a pump to mix wastewater. Hydraulic retention time (HRT) is very short (4–10 days) depending on climatic conditions (Rawat et al 2011). HRP can be used as a combined secondary/tertiary system for wastewater treatment. Microalgae in these ponds can produce high protein biomass at a rate of 10 to 20 g m^{-1} d^{-1}, productivities an order of magnitude greater than land crops (Oswald 1995). In these systems, productivities of up to 50 t ha^{-1} y^{-1} are feasible and consume approximately 0.57 kWh kg^{-1} BOD removed. In contrast, mechanical aerated ponds consume a much higher amount of energy in the range of 0.80–6.41 kW h kg^{-1} BOD removed. HRPs are actually used to treat urban wastewater and waste from pig farms and digestate (Olguín et al., 2003; Fallowfield et al., 1999) and for the treatment of the effluent from aquaculture system (Pagand et al., 2002). Wastewater treatment plants consume half of their total energy use in supplying oxygen to the bacteria consortium so as to oxidise the organic carbon and nitrogen to CO_2 and N_2, which are then released to the atmosphere. Alternatively, microalgae can produce O_2 by taking up the CO_2 released by the bacteria thus reducing both the energy consumption and the CO_2 released to the atmosphere (Acièn et al., 2013). The utilization of microalgae-bacteria consortiums requires large surface areas and favourable environmental conditions, thus this technology cannot universally replace current processes based on activated sludge (Gómez-Serrano 2015).

Table 1.8. Comparison of energy input for wastewater depuration

Treatment	Energy consumption (kWh m^{-3})	References
Standard secondary + tertiary treatment	0.2-1.6	Rawat et al., 2011
	1.05	Singh et al., 2012

Microalgae secondary + tertiary treatment (HRP)	0.14	Rawat et al., 2011
Standard tertiary treatment	0.23-0.96	Acién Fernández et al., 2013
	0.5-1.0	Gómez-Serrano et al., 2015
Microalgae tertiary treatment	0.05-0.11	Acién Fernández et al., 2013
	0.1-0.2	Gómez-Serrano et al., 2015

1.7.5 Possible products recovery from wastewater depuration: biodiesel

The use of organic wastewaters to produce microalgal biomass present some disadvantages such as high organic and inorganic pollutants (e.g. urban and industrial wastewaters,) and biological contaminants like bacteria or fungi (Pittman et al., 2011; Abinandan and Shanthakumar 2015) which may reduce the quality and quantity of microalgae biomass (Chen et al., 2011). For this reasons the biomass produced by wastewaters depuration should be better addressed to the production of no- food products, such as biofuel. Indeed the depleting resource of petroleum fuels and the environmental concerns associated to them have created urgent needs for alternative fuels. Microalgae family includes species that can accumulate large amounts of lipids in the form of triglycerides (TAGs) that can be turned into biofuel (Collet et al., 2014). Among these, microalgae biodiesel is a well known option due to its high energy density, better environmental performance compared to diesel and suitable for use in diesel vehicles with small modifications to their engines (Tan et al., 2015). However, the main problem related to the real feasibility of this application process at industrial-scale is related to the high production costs (Chisti 2007), in particular the cost related to the fertilizer and water input. For instance, microalgae cultivation shows an N-fertilizer consumption in the range of 0.29 to 0.37 kg/kg oil, which is nearly ten times

higher than that for oil palm (0.048 kg kg^{-1} oil) (Lam and Lee 2012) and two fold higher that of other land plant producing oil. One promising way to make algal biofuel production more cost effective is to integrate wastewater treatment with algae biomass production (Clarens et al., 2010; Olguin 2012; Li et al., 2014). Yang et al., (2011) reports that the use of wastewaters could reduce the need for additional nitrogen and phosphorous sources by approximately 55%. Therefore, the possibility to use wastewaters derived from municipal, agricultural, and industrial activities like source of nutrients for microalgae cultivation could significantly reduce the operational costs of algal production systems (Lardon et al., 2009) performing an environmental service (depuration) at the same time. As described before (see paragraph 1.6.2), lipid's productivity is be determined by the product between the biomass productivity and the lipid content. The lipid productivity of a mixotrophically-cultivated microalgae could increase up to 8-times more than photoautotrophic cultivation (Probir et al., 2011; Chojnacka et al., 2004) (see data reported in paragraph 1.6.2). Recent studies involving the use of Life Cycle Analysis have indicated the necessity of decreasing the energy and fertilizer consumption in biodiesel process (Lardon et al., 2009; Yang et al., 2011). In conclusion the use of microalgae for bioenergy purposes (e.g. biodiesel), it's technically feasible, but still needs more considerable R&D efforts to achieve the high productivities required at low cost, could so competing with fossil diesel.

1.8 Mixotrophy exploiting agro-industrial by products

1.8.1 Type of by-products used in microalgae cultivation and biomass production

Various organic compounds can be utilized by microalgae under mixotrophic cultivation. Glucose is the most efficient and most frequently adopted source. Glucose

was employed as carbon source in mixotrophic culture of several microalgal species reaching high production of both biomass and lipids (Liu et al., 2009; Yang et al., 2000; Santos et al., 2011; Wan et al., 2011; Bhatnagar et al., 2011; Herredia-Arroyo et al., 2010). However the cost of the organic carbon substrate for mixotrophic cultivation of microalgae is estimated to be about 80% of the total cost of the cultivation medium (Bhatnagar et al., 2011) and the high cost of glucose does not make it the best candidate substrate for cost effective production. For this reason alternative carbon sources such as by-product from industrial processes are to be explored as microalgae feedstock (Liang et al., 2009). Less expensive organic substrates like, crude glycerol from biodiesel production, acetate from anaerobic digestion, carbohydrates from agricultural wastes (Lee 2004; Heredia-Arroyo et al., 2010; Bhatnagar et al., 2011; Heredia-Arroyo et al., 2011; Sforza et al., 2012) and cheese whey, a liquid by-product remaining from the cheese manufacturing process (Dragone et al., 2009) offer great promise as organic substrates for the cultivation of microalgae under mixotrophic condition. The use of these bio-products from agro-industrial processes, higher in quality with respect to wastewaters, allows to obtain high quality algae biomass, suitable for food and fine chemicals production.

Table 1.9. Microalgae grown on by-products

Microalgae strain	By-products	C-source	Biomass	Lipid	Carotenoid	References
		($g\,L^{-1}$)	($g\,L^{-1}$)	(% TS)	(% TS)	
Chlorella vulgaris	Glucose	30	10	13	-	Heredia-Arroyo et al., 2011
Chlorella vulgaris	Cheese whey	0	1.22	40	-	Abreu et al., 2012
		10	1.98	40	-	

Species	Substrate					Reference
		10	3.58	40	-	
	Glucose + galactose	10	2.24	40	-	
Chlorella vulgaris	Glycerol + glucose	5+2	2.60	-	0.4	Kong et al., 2013
	Glycerol	1	0.62	-	0.1	
Cholorella zofingiensis	Glucose	30	9	-	0.14 (Astaxanthin)	Ip et al., 2004
	Glucose	15	8.5	-	-	
	Glucose	17.1	8.5	29.4	-	
Chlorella protothecoides	Glucose	16.5	8.6	39.9	-	Wang et al., 2013
	Glucose	17.1	7.6	57.3	-	
	Glucose	15.4	7.7	38.4	-	
Scenedesmus obliquus	Cheese whey	0	1.9	-	-	Girard et al., 2014
		40	4.9	10 (PUFA)	-	
Parietochloris incisa	Glucose	0.9	1.2	-	-	
Chlorella protothecoides	Glycerol	4	2.67	-	-	Sforza et al., 2012
Nannochloropsis salina		4	0.43	-	-	
		0	0.22	-	-	
Parietochloris incisa	Glucose	0.9	0.71	8.6	-	Tababa et al., 2012
		0.9	1.1	7.4	-	
Nannochloropsis salina	Glucose	5.4	0.51	4.6 (EPA)	-	Xu et al., 2004a
	Ethanol	1.4	0.45	-	-	
Chlorella marine	Glucose	2	1.45	25.4	-	Cheirsilp et al., 2012
Nannochloropsis sp.		2	1.2	25.5	-	
Nannochloropsis sp.	Glucose	5	1.2	31	-	Xu et al., 2004b

Phaeodactylum tricornutum	Glycerol	0.1	7.04	2.4 (EPA)	0.45	
	Fructose	0.02	3.5	1.59 (EPA)	0.5	
	Glucose	0.05	2.2	-	-	Cerón-García et al., 2006
	Mannose	0.01	1.05	-	-	
	Lactose	0.005	0.77	-	-	
Phaeodactylum tricornutum	Glycerol	9.2	13.8	-	-	
	Fructose	3.6	8.2	-	-	Cerón-García et al., 2013
	Glycerol	11.9	12	5.4 (PUFA)	-	
Phaeodactylum tricornutum	Glycerol	0.1	2.4	16.8 (PUFA) 2.2 (EPA)	-	Cerón-García et al., 2000
Spirulina sp.	Glucose	1	10.2	-	10.7 (Phycocianin)	Chen et al., 1997
Spirulina sp.	Acetate	-	0.91	-	-	Lodi et al., 2005

Data in table 1.9 outline interesting production, where final biomass concentration is in the range of 8-10 g L^{-1}, which is, as outlined in paragraph 4.1 more than twice the magnitude of production which can be achieved in favorable autotrophic condition.

1.8.2 Fine chemicals from mixotrophic culture of selected by-products

Some microalgal species can produce valuable compounds; the production and storage of this compounds is related to the growing condition of microalgae, to the light intensities (i.e. saturating condition) and nutrient availability. In some cases algae cultivated under stress conditions (sub- or supra-optimal conditions) change their metabolic strategies affecting the biomass composition and the relative content of the biomass compounds (Hu 2004), i.e. under stress conditions microalgae are able to

synthesize and produce various secondary metabolites that act as antioxidants, pigments, hormones, antibiotics or dietary supplements (Markou et al., 2011; Skjånes et al., 2013). Some of these secondary metabolites are fine chemicals particularly interesting for food, nutraceutical pharmaceutical or cosmetic sector (Skjånes et al., 2013). Some of these fine chemicals can be effectively produced by mixotrophic culture.

1.8.2.1 PUFA

Lipids produced by microalgae generally include neutral lipids, polar lipids, wax esters, sterols and hydrocarbons, as well as prenyl derivatives such as tocopherols, carotenoids, terpenes, quinines and pyrrole derivatives such as the chlorophylls. Lipids produced by microalgae can be grouped into two categories, storage lipids (neutral lipids) and structural lipids (polar lipids). Storage lipids are mainly in the form of triacyglycerols made of predominately saturated Fatty Acids (FAs) and some unsaturated FAs which can be transesterified to produce biodiesel. Structural lipids typically have a high content of Long Chain Poly Unsaturated Fatty Acids (LC-PUFAs), which are also essential nutrients for aquatic animals and humans. Numerous studies have demonstrated that dietary omega 3 LC- PUFAs have a protective effect against atherosclerotic heart disease. The two principal omega 3 LC-PUFAs in marine oils, eicosapentaenoic acid (EPA; 20:5 omega 3) and docosahexaenoic acid (DHA; 22:6 3), have a wide range of biological effects. Both EPA and DHA are known to influence lipoprotein metabolism, coagulation, and blood pressure. More specifically, EPA performs many vital functions in biological membranes, and is a precursor of several lipid regulators involved in the cellular metabolism. DHA is a major component of

brain, eye retina and heart muscle, it has been considered as important for brain and eye development and also good cardiovascular health.

1.8.2.2 Carothenoids and Astaxantin

The potential of microalgae as a commercial source of carotenoids is widely recognized (Borowitzka 1988 and 1992; Del Campo et al., 2000). In the microalgae, carotenoids function as accessory pigments in the photosystems, as structural components of light harvesting complexes, as well as photoprotective agents (Taylor, 1996; Eskling et al., 1997; Del Campo et al., 2000). A few number of carotenoids like β-carotene, lycopene, astaxanthin and lutein have commercial application as food dyes, as feed additives in aquaculture, to enhance the pigmentation of chicken and egg yolks and in cosmetic industries (Borowitzka 1988 and 1992; Johnson and Schroeder 1995). Carotenoids are also proposed as preventive agents for a variety of human diseases, for exemple β-carotene lutein and zeaxanthin are claimed to display cancer-preventing properties (Richmond 1990; Le Marchand et al., 1993; Ziegler et al., 1996). The two main microalgae recognized as commercial sources of carotenoids are the freshwater green alga *Haematococcus pluvialis*, which accumulates astaxanthin (Boussiba and Vonshak 1991) and the halophilic green flagellate *Dunaliella salina*, which accumulates β-carotene (Avron and Ben-Amotz 1992). Astaxanthin is an abundant carotenoid pigment responsible for the color of the bodies of many aquatic animals (Gu et al., 1997) such as salmonids and crustaceans. Astaxanthin is widely used as feed additive in aquaculture as pigment source for crustacean and fish because it gives an attractive pigmentation to their eggs, flesh and skin (Cordero et al., 1996). In addition, the strong anti-oxidative activity of astaxanthin over other carotenoids such as β-carotene, zeaxanthin and lutein

has attracted remarkable commercial interest for medicinal and nutraceutical uses (Miki et al., 1991). Several other microalgal genera such as *Chlorella sp., Chlorococcum sp or Scenedesmus sp.* are reported as potential producers of astaxanthin (Del Campo et al., 2004). *Chlorella zofingiensis* as well represent a promising producers of natural astaxanthin, as it grows fast phototrophically, heterotrophically and mixtrophically, (Del Campo et al., 2004; Feng et al., 2011; Liu et al., 2012) is easy to be cultured and scaled up both indoors and outdoors, and can achieve ultrahigh cell densities (Liu et al., 2014b). Astaxanthin accumulation in *Haematococcus* was observed only in encysted cells and encystment was reported to be induced under unfavourable growth conditions such as nutrient starvation, salt stress, elevated temperature and high light intensity (Boussiba et al., 1991; Fábregas et al., 2003; Del Campo et al., 2004; He et al., 2007).

1.8.3 Light and fine chemical biosynthesis

It has been proposed that increased neutral lipids synthesis is perhaps the "default pathway" to defend against photo-oxidative stress that can occur as a result of excess of reducing energy (Hu et al., 2008). The same does not seem to apply to LC-PUFAs. Light intensity seems to affect the cellular composition of algae LC-PUFAs as EPA as it plays a role in the functioning of the thylakoid membrane and photosynthesis (Kates and Volcani 1966; Cohen et al., 1988). At higher irradiance algae become less photosynthetically efficient and thus less thylakoid membranes are required. As a result, LC-PUFAs content could be lower in high light-acclimated algae (Harwood and Jones 1989). Consistent with this hypothesis in some studies light intensity proved to be negatively correlated with PUFA content, i.e in *Nannochloropsis sp.*, grown under low light conditions (35 $\mu E \cdot m^{-2} \cdot s^{-1}$), 40% of the total lipids were found to be galactolipids

with high LC-PUFAs content and 26% were found to be triacylglycerols. In the same system, high light (550 $\mu E \cdot m^{-2} \cdot s^{-1}$) conditions resulted in an increased synthesis of triacylglycerol with a reduction in galactolipid synthesis and LC-PUFAs (Sukenik et al., 1989). *Nannochloropsis sp.* grown in saturating light conditions was characterized by a high content of lipids and fatty acids, as compared to cells grown in light limiting conditions (Sukenik et al., 1989), but at subsaturating light conditions preferentially synthesizes galactolipids enriched with EPA (Sukenik et al., 1993) up to 40 % of total cellular fatty acids. Similar results were reported by Renaud et al., (1991), they found a significant decrease in the relative abundance of EPA when cultures of *Nannochloropsis oculata* were grown at high photon densities. DHA production processes with *Crypthecodinium cohnii* using glucose as carbon source in heterotrophy (Kyle et al., 1996) has resulted in overall productivity of DHA on glucose equal to 19 mg L^{-1} h^{-1} (de Swaaf et al., 1999) and production up to 45 mg L^{-1} h^{-1} in cultivations with acetic acid as carbon source (Ratledge et al., 2001; de Swaaf et al., 2003). *Phaeodactylum tricornutum* (Sukenik et al., 1989) and *Nannochloropsis sp.* (Thompson et al., 1996) demonstrated an EPA content of up to 39% of total fatty acids, while strains such as *Thraustochytrium* (Burja et al., 2006) and *Schizochytrium limacinum* (Zhu et al., 2007) contained a DHA percentage between 30–40% of the total fatty acids when grown heterotrophically. High oil production, including DHA from *Schizochytrium* (50% w/w), can be obtained as a result of high growth rate by controlling nutrients such as glucose, nitrogen, sodium and some other environmental factors, such as oxygen concentrations as well as temperature and pH. According to Cerón-García et al., (2013), mixotrophic cultures had elevated levels of chlorophylls, carotenoids, and the major fatty acids, relative to controls. Kitano et al., (1997) found that mixotrophic growth in acetic acid effectively promotes the

productivity of EPA with a high growth rate and high EPA content of the biomass in *Navicula saprophila*. Ceron Garcìa et al., (2000) reported EPA productivity of 33.5 mg L^{-1} d^{-1} was obtained in *P. tricornutum* culture carried out in 9.2 g L^{-1} glycerol. This yield was 10-fold greater than the maximum EPA productivity obtained in the photoauthotrophically grown control culture. Finally production of long-chain unsaturated fatty acid (EPA and DHA) is the most widely investigated by heterotrophic culture of many species, *Crypthecodinium, Schizochytrium*, *Ukenia* in the last decade. EPA and DHA production have been already successfully commercialized at large scale by fermentation (Chen and Chen 2006; Wen and Chen 2003). According to up to date references LC-PUFAs content is inhibited by high photon flux density and stimulated by low irradiance, thus the mixotrophic culture mode can be the optimal condition for LC-PUFAs production: mixotrophic culture are more dense and less exposed to light over saturation regimen and at the same time total growth and productivity is positively affected by organic carbon.

1.8.4 Other ways to trigger light induced products

Some chemicals capable of inducing oxidative response for enhancing accumulation of high-value bioproducts were also investigated in place of light (e.g. for the accumulation of astaxanthin). An early study showed that Fe^{2+}, superoxide anion radical (from methylene blue and methyl viologen), H_2O_2, were capable of triggering astaxanthin biosynthesis in *H. pluvialis*, (Kobayashi et al., 1993). HO or other active oxygen species might then enhance carotenoid formation in algal cyst cells by participating directly in the carotenogenic enzyme reactions as an oxidizer or an H acceptor (Beyer and Kleinig 1989). In a recent study Ip and Chen (2005) proposed

sodium hypochlorite (NaClO) as another oxygen species to enhance astaxanthin production of *C. zofingiensis* in the heterotrophic cultivation medium. Also the addition of 100 mM pyruvate into the culture medium of *C. zofingiensis* enhanced the yield of astaxanthin from 8.36 to 10.72 mg L^{-1}. In addition, citrate and malic acid also had the similar stimulatory effects on the formation of astaxanthin (Chen et al., 2009). For DHA accumulation in *Schizochytrium sp. HX-308*, an addition of 4 g L^{-1} malic acid to the culture medium at the rapid lipid accumulation stage can increase DHA content of total fatty acids from 35 to 60%. In addition to functioning as a possible carbon precursor, it was speculated that malic acid added at rapid lipid accumulation stage could activate malic enzyme activity and enhance NADPH generating reaction from malic acid to pyruvate (Ren et al., 2009). Again the enhancement in metabolic activity due to mixotrophy is a driving element to increased valuable compounds production.

CHAPTER 2 - Nutrient recovery from agricultural wastewaters

2. Pre-treated digestate as culture media for producing algal biomass.

In this work an agro-zootechnical ultrafiltered digestate (UF) coming from an anaerobic digester plant was used to grow two strains of microalgae: Chlorella sp. and Phaeodactylum tricornutum, in a comparison with standard substrates. Chlorella sp. and P. tricornutum were able to grow on UF with similar growth rates (μ) to those obtained using standard substrates, i.e. μ of 0.216 d^{-1} and of 0.200 d^{-1} for Chlorella sp., and of 0.128 d^{-1} and 0.126 d^{-1} for P. tricornutum, on synthetic media and UF, respectively. Algae grown on UF showed similar final biomass composition to those obtained by using synthetic media. Algae were able to remove nitrogen from UF, i.e. 92% and 71%, for Chlorella sp. and P. tricornutum respectively. Microalgae can grow on UF producing good quality final biomass.

2.1. Introduction

Microalgae production involves huge consumptions of water and fertilizers (Sandefur et al., 2016), representing more than the 20% of the total production costs (Lam and Lee, 2012). In recent years, the price of nitrogen and phosphorus fertilizers have considerably increased, translating into higher costs of algal biomass yield: for economic feasibility, the production costs should be reduced by 20-25 folds (Bhatnagar

et al., 2011). As consequence of that, the reuse of wastewaters and other liquid streams rich in nutrients could represent good substrates to support algal biomass production (Ji et al., 2013; Hammed et al., 2016; Ledda et al., 2016). Large amounts of wastewaters from industries processing agricultural raw materials, livestock and wastewaters from domestic treatment plants are annually discharged to aquatic ecosystems worldwide. Uncontrolled discharging of such liquid sewage into the environment often causes water's pollution damaging the ecosystems and causing severe environmental problems such as eutrophication (Posadas et al., 2014). Some authors (Franchino et al., 2013; Ledda et al., 2015) have already shown the high efficiency of algae in removing nutrients from anaerobic digestate so that algae can be indicated as an appropriate system for nutrient removal and recovery. However, high nutrients concentration, high turbidity and bacterial contamination of wastewaters could negatively affect microalgae biomass survival and quality, leading to important limitations in the scale-up of microalgae cultivation of using wastewaters (Collos and Harrison, 2014). This problem could be overcome by using resilient microalgae strains (e.g. *Chlorella* sp., *Scenedesmus* sp.) and/or reducing the wastewaters' carbon load in order to prevent bacterial contamination. Recently, Ledda et al., (2013) demonstrated an innovative process for livestock waste treatment coupled with AD allowing both water and nutrient recovery from digestate. In particular, a diluted pre-treated digestate, i.e. ultrafiltered (UF), was used as a substrate to support microalgae growth proposing a biogas-algae production biorefinery (Ledda et al., 2015). On that occasion only one resistant algal species was tested (*Scenedesmus* sp.), giving very good results. In this work, two different microalgal species were used to test their ability to grow on pre-treated digestate (UF) in comparison with standard substrates. In particular, an algal strain well

known for its ability to grow on wastewaters (*Chlorella* sp.*)* was tested as well as more sensitive microalgal strains, i.e. *Phaeodactylum tricornutum*, able to accumulate huge amount of lipids and polyunsaturated fatty acids. In particular, very few works have examined the possibility to grow this algal strain on wastewaters (Libralato et al., 2016) and no data are available about the real feasibility of cultivating *P. tricornutum* on wastewaters.

2.2. Materials and methods

2.2.1 Microalgae strains and preparation of inocula

An indigenous strain of *Chlorella* sp. previously isolated from a digestate storage tank (Ledda et al., 2015) and *Phaeodactylum tricornutum* strain SAG1090-1a, acquired from Sammlung von Algenkulturen, Pflanzenphysiologisches Institut (Universität Göttingen, Germany) were used in this work. The inocula were prepared batch-wise and maintained at a controlled temperature of $22 \pm 1°C$ in Erlenmeyer flasks of 500 ml with synthetic media i.e. sterilized modified Bold's Basal Medium for *Chlorella* sp., and sterilized modified f/2 medium for *P. tricornutum*. Inocula were constantly aerated and mixed by using filtered air (filter of 0.2 µm), under an illumination flow of 60 µmol m^{-2} s^{-1}, continuously provided by cool fluorescent tubes.

2.2.2 Experimental set-up, media and culture condition

All strains were cultivated in triplicate in Erlenmeyer flasks with 3,000 ml of working volume. During all experiments, pH was constantly maintained at the optimal value reported in the literature for each strain (i.e. 7 ± 0.3 and 8.2 ± 0.3 for *Chlorella sp* and *P. tricornutum*, respectively) by a pure CO_2 injection on demand. The cultures were

grown on synthetic medium mentioned above, and maintained under an artificial light of 90 µmol m^{-2} s^{-1} provided by cool fluorescent tubes (36W/6400K), with a constant airflow supply and at a steady temperature of 22 ± 1°C. After the reaching of the stationary phase, a part of the cultures was collected to perform a biochemical characterization of the algae biomass grown under optimal conditions, while the remaining share was transferred to nitrogen-depleted medium, i.e. N-starvation phase, to stimulate the production of high-value compounds. The same procedure was carried out when growing the microalgae strains on the UF medium (Ledda et al., 2013). The UF effluent was sampled from an AD plant located in northern Italy (Lombardy Region) that produces 1 MW of electrical power by co-digesting a mixture of energy crops and pig slurries and is equipped with a full-scale digestate treatment unit, as reported by Ledda et al., (2013). Samples were stored in 10 L tank at 4°C for subsequent analyses. Chemical characterization consisted in the determination of pH, total solids (TS), total Kjeldahl nitrogen (TKN), ammonium nitrogen (N-NH$_4^+$), total phosphorus (TP) and chemical oxygen demand (COD) (IRSA CNR, 1994) (EPA, 2007).

2.2.3 Microalgae growth evaluation

Algal growth was evaluated by optical density (OD) at 560 nm using a Jeneway 7305 UV-visible spectrophotometer (Bibby Scientific Limited, Staffordshire, ST15 OSA, UK). Dry weight (DW) was determined three times at week sampling 5 ml of algae suspension. The samples were centrifuged at 4,300 rpm for 10 min and then washed twice with an equivalent volume of distilled water. Culture samples were then filtered by pre-weighed Whatman GFC filter 1.2 µm, dried at 80°C overnight and subsequently

weighed (Ledda et al., 2015). The specific growth rate μ (day $^{-1}$) was calculated from the Equation (1):

$$\mu = \frac{1}{t} \times ln\left(\frac{X_f}{X_0}\right)$$

in which X_0 and X_f are the dry weight values (g L^{-1}) at the beginning and at the end of the run, respectively, and t (days) is time of the run.

Daily biomass productivity (Dp as mg L^{-1} d^{-1}) was calculated by the Equation (2):

$$Dp = \frac{X_f - X_0}{t}$$

Nitrogen removal (N_{re} %) was calculated according to Equation (3):

$$Nre = \left(\frac{N_0 - N_0}{N_0\%}\right)$$

in which N_0 is the nitrogen concentration at the beginning and N_f is the nitrogen concentration at the end of the experiment.

2.2.4 Biochemical analysis

Total lipids content in lyophilized biomass was evaluated by gravimetric assay by using slightly modified version of the method proposed by Kochert et al., (1978). Protein content was calculated by multiplying the total Kjeldahl nitrogen by nitrogen-to-protein conversion factors of 5.95 for *Chlorella* sp. (González-López et al., 2010) and 4.68 for *P. tricornutum* (Templeton and Laurens, 2015). Carbohydrate content was determined by the slightly modified phenol-sulfuric acid method of DuBois et al., (1956).

2.2.5. Statistical analysis

All statistical analyses were performed using analysis of variance (ANOVA) with the Tukey test used to compare means (SPSS statistical software, SPSS Chicago IL).

2.3. Results and Discussion

2.3.1 Substrates chemical characterization

UF contains nitrogen as ammonium form at high concentration, therefore it was diluted ten times (UF 1:10) with deionized water. *Chlorella* sp. was grown using only 1:10 (v/v) diluted UF as medium, whereas for *P.tricornutum* salt was added to the diluted UF in order to achieve seawater salinity. All characteristics of substrate used for algae growth are reported in Table 2.1. The three media used in this study contain similar N content (i.e. 123.5 mg kg^{-1} of N for both BBN-3N and f/2-10N, and 120 ± 5 mg kg^{-1} of N in form of ammonia for diluted UF, respectively) which were suitable to support algal growth (Richmond, 2008). On the other hand, chemical data revealed different P content in the three media: 53.24 mg kg^{-1} in BBM-3N, 1.2 mg kg^{-1} in f/2-10N and 3 ± 0.2 mg kg^{-1} in the UF 1:10, respectively (Table 2.1). Phosphorus deficiency could cause lower growth capacity (Richmond, 2008) as it is indispensable for algal growth, development and reproduction. Nonetheless, Yin-Hu et al., (2012) reported that *Scenedesmus sp.* grown in batch mode with a N:P ratio of 45:1 (phosphorous starvation), has similar growth rates to those obtained in rich-phosphorus conditions. Following this concept, some authors (Ledda et al., 2015; Schmidt et al., 2016) advised that a luxury phosphorous uptake did not trigger more biomass production, so that, to prevent any phosphorous pollution, its dosage should be kept low.

Table 2.1. Chemical characterization of different media used to growth algae.

Parameters	BBM-3N	f/2-10N	UF	UF 1:10
pH	6.6	7.3	8.40	8.25
TS (g kg^{-1})	u.d.l.[a]	u.d.l.	8.9 ± 0.5	0.88 ± 0.1

TKN (mg kg^{-1})	123.5	123.5	1377 ± 33	138 ± 2
N-NH$_4^+$ (mg kg^{-1})	-	-	1155 ± 49	120 ± 5
N-org (mg kg^{-1})	-	-	27 ± 5	2.2 ± 0.6
TP (mg kg^{-1})	53.24	1.2	28 ± 0.5	3 ± 0.2
COD (mg O$_2$ L^{-1})	u.d.l.	u.d.l.	1487 ± 1.8	148 ± 0.02

[a]u.d.l.: under detection limit

2.3.2 Chlorella sp. growth

Results (Figure 2.1a) showed that for the first 10-12 days there was a similar algal growth for the two substrates used. On the other hand, after the 12th day trends appeared different, i.e. *Chlorella* sp. grown on BBM-3N reached a final biomass concentration of 1.52 ± 0.1 g L^{-1} whereas algae growing on UF medium supported a final biomass concentration of 1.16 ± 0.1 g L^{-1} (Table 2.1.). This difference could be ascribed (Figure 2.1a) to the higher self-shading effect in the UF than BBM-3N because of its dark-brown color which reduced light penetration (Ledda et al., 2015). Moreover, the different phosphorus content in the two media (Table 2.1.), could have affected the growth, even if, as already mentioned, some studies demonstrate that extra-uptake P availability does not influence the biomass production. Results of this work seem to confirm this fact as algae showed similar specific growth rates during the logarithmic phase, i.e. 0.216 ± 0.01 d^{-1} and 0.200 ± 0.01 d^{-1}, growing on different substrates, i.e. BBM-3N and UF 1:10 respectively, similar to those reported in the literature (Frumento et al., 2016; Li et al., 2014; Tam and Wong, 1996; Cho et al., 2013) (Table 2.2). After 21 days of cultivation, cultures were collected, washed and placed in a N-depleted medium. Surprisingly, after a few days of lag, during the 10 days following the

replacement of N-replete to N-depleted medium, an increase in the dry weight was observed in both media trials. This event could be explained by the capacity of some microalgae to still perform photosynthesis even if there was not available nitrogen in the medium, in parallel with the accumulation of reserve compounds (Chia et al., 2015). Results obtained suggest that the cultures grown under conditions of nitrogen deprivation have sufficient N-reserve for elongated periods of growth with continuing photosynthesis; after 10 days of N-starvation, final biomass concentrations were of 2.41 $\pm$ 0.22 g L^{-1} and 1.62 $\pm$ 0.08 g L^{-1} for f/2-10N and UF media, respectively.

2.3.3 *Phaeodactylum tricornutum growth*

P. tricornutum grew on both f/2-10N and UF media showed similar growth trends (Figure 2.1b) giving a final biomass concentration of 0.47 $\pm$ 0.01 g L^{-1} and of 0.40 $\pm$ 0.03 g L^{-1} for f/2-10N and UF media respectively (Table 2.2). These results were obtained by very similar growth rates i.e. 0.128 $\pm$ 0.01 d^{-1} and 0.126 $\pm$ 0.01 d^{-1}, for f/2-10N and UF media, respectively, being these data not so far from literature data (Table 2.2). From the data reported in Table 2.2, it is clear that there was a strict correlation between the maximum dry weight obtained and the light intensity used, i.e. higher light intensity led to obtaining better growth performance (Liang et al., 2001). Our results seem to indicate that UF was able to provide similar conditions to f/2-10N media, as indicated, also, by the similar N and P contents (Table 2.1). Such as reported also for *Chlorella* sp., throughout the 10 days of N-starvation phase, *P. tricornutum* increased in biomass concentration, with a similar trend for both media (Figure 2.1b) reaching final dry weights of 0.64 $\pm$ 0.05 g L^{-1} and of 0.60 $\pm$ 0.03 g L^{-1} for f/2-10N and UF media, respectively.

2.3.4 Nitrogen removal

Chlorella sp. and *P. tricornutum* demonstrated a remarkable ability to remove nitrogen (Figure 2.1c). *Chlorella* sp. trials showed the highest removal rate ($92 \pm 3\%$ of total starting N); in detail starting from an initial N concentration of 138 ± 12 mg L^{-1}, after 21 days of the growth phase, inside the reactors only 12 ± 0.5 mg L^{-1} of N were present, while for *P. tricornutum* at the end of the 21 days, liquid media still contained 32 ± 5 mg L^{-1} of nitrogen, which means that $71 \pm 3\%$ of the total nitrogen was removed. Data obtained agree with data of both Franchino et al., (2013) and Wang et al., (2010) who looked at the growth of *Chlorella vulgaris* on digestate. As regards the nutrient removal capacity of *Phaeodactylum tricornutum*, there are few studies in the literature: however, data obtained in this work are consistent with the results of Craggs et al., (1995) that were able removing 80 % of N growing on diluted wastewaters (primary sewage effluent).

Table 2.2

Culture conditions and growth results of *Chlorella sp.* and *P. tricornutum* obtained in this work , in comparison with literature data.

Strains	Culture medium/ wastewaters	Nitrogen (mg L^{-1})	Light intensity (μmol m^{-2} s^{-1})	Max DW (g L^{-1})	μ (d^{-1})	Productivity (mg L^{-1} d^{-1})	References
Chlorella vulgaris	Bold's Basal Medium	Nitrate (41)	70	0.34	0.136 ± 0.01	24.6 ± 1.1	(Frumento et al., 2016)
Chlorella sorokiniana	Kuhl medium	Nitrate (140)	100	0.47	0.35	-	(Li et al., 2014)
Chlorella vulgaris	Bristol medium	Ammonia (125)	65	-	0.213	-	(Tam and Wong, 1996)
Chlorella sp.	AD effluent	Ammonia (130)	200	1.25	-	-	(Cho et al., 2013)
Chlorella sp.	Bold's Basal Medium 3N	Nitrate (123)	90	1.52 ± 0.1b	0.216 ± 0.01b	68.6 ± 4.8b	This work

Chlorella sp.	Ultrafiltered digestate	Ammonia (120 ± 5)	90	1.16 ± 0.1a	0.200 ± 0.01a	49.2 ± 5a	This work
Phaeodactylum tricornutum	f/2 medium	Nitrate (12.3)	50	0.46 ± 0.03	0.11 ± 0.02	-	(Liu et al., 2009)
Phaeodactylum tricornutum	Modified f/2 medium	Nitrate (-)	150	0.88	-	-	(Morais et al., 2009)
Phaeodactylum tricornutum	-	Ammonia (-)	115	0.58 ± 0.02	0.68	-	(Fidalgo et al., 1995)
Phaeodactylum tricornutum	f/2 medium 10N	Nitrate (123)	90	0.47 ± 0.01b	0.128 ± 0.01a	18.6 ± 2a	This work
Phaeodactylum tricornutum	Ultrafiltered digestate	Ammonia (120 ± 5)	90	0.40 ± 0.03a	0.126 ± 0.01a	15.2 ± 1.5b	This work

Values in the same column followed by the same letter, are not statistically different at $p < 0.05$ according to to Tukey test.

(a)

(b)

(c)
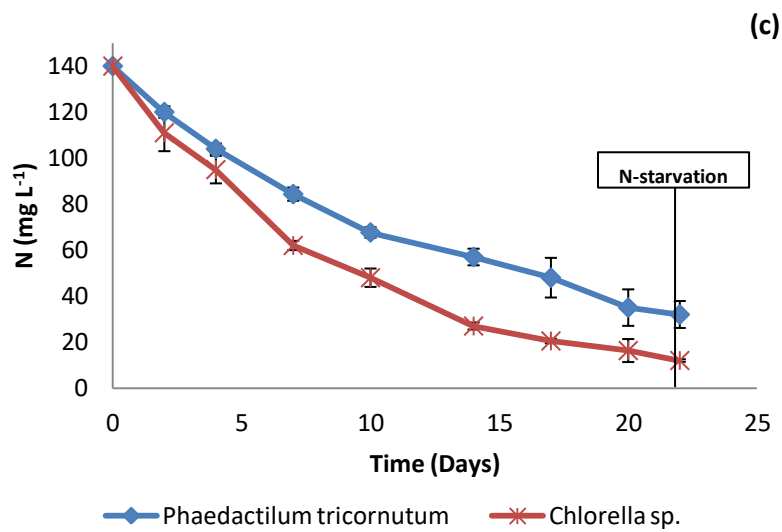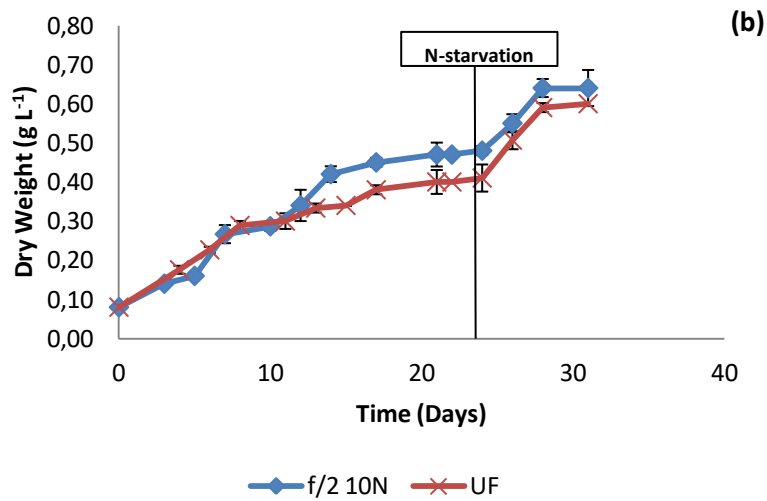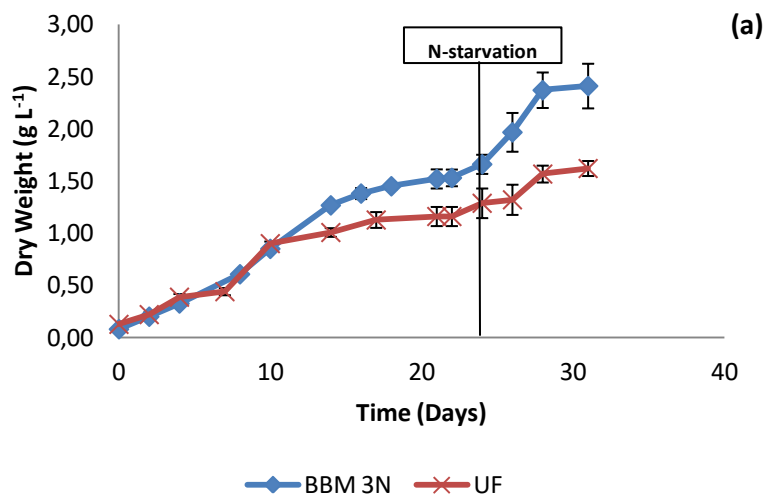
Figure 2.1. Microalgae growth and nutrient removal: **(a)** growth curve of *Chlorella sp.* on UF and BBM-3N; **(b)** growth curve of *Phaedactylum tricornutum* on UF and f/2-10N; **(c)** nitrogen removal determined for *Chlorella sp.* and *P. tricornutum* grown on diluted (1:10) UF digestate.

2.3.5 Biomass composition

For all the trials it was possible to observe (Table 2.3) that during the N-starvation period the total protein content decreased, evidently becoming incorporated in storage molecules (i.e. lipids or carbohydrates). Protein reductions were in the range 4.3 to 7.4% for *Chlorella sp.* and 5.6% to 11.5% for *P. tricornutum*. Literature reported studies indicating a remarkable increase of the lipid fraction content because of nitrogen deprivation in *Chlorella* species (Negi et al., 2015). However these data are in contrast with the findings of this work: *Chlorella* sp. cultivated here on different media did not present any lipid increase during the N-starvation phase. In all conditions adopted, lipids content remained constant and around 13-14% TS, while there was an increase of carbohydrate concentration, i.e. 8.8% TS and 5.9% TS on BBM-3N and UF media, respectively. This was in agreement with the findings of Bono et al., (2013) who showed that nitrogen limitation triggers the synthesis of more carbohydrates. The improvement of carbohydrates accumulation under nitrogen limitation occurred because the biomolecules served as a sink for the surplus fixed carbon produced from an unbalanced carbon and nitrogen metabolism (Otero and Vincenzini, 2004). Ho et al., (2013) obtained similar data in three different *Chlorella* species, i.e. the increase of carbohydrate content was counterbalanced by protein content reduction. Results of this work seem to confirm these findings as *Chlorella* sp. cultured with different nitrogen

sources (nitrate for BBM-3N and ammonia for UF) did not show substantial differences in chemical biomass composition. Biochemical composition of *P. tricornutum* can be deeply modified under particular growing conditions, i.e. nitrogen starvation (Fidalgo et al., 1995). In particular, results of this work attested that for this strain both lipids and carbohydrates increased their content during the N-starvation phase, meanwhile proteins decreased. Maximum total proteins content was obtained during the growth on UF medium (296 ± 19 g kg^{-1} TS), whereas on the synthetic medium it reached only 184 ± 3 g kg^{-1} TS. This difference could be explained by taking into consideration the ammonia concentrations in the media. Some authors have reported that good ammonia content in the growth medium, can stimulate protein accumulation in some diatoms. In general, during the nitrogen limitation phase, there was a reduction in microalgal protein content, in agreement with other work (Chia et al., 2015). Nutrient availability is a crucial factor that enhances lipid content in microalgae cells (Markou and Nerantzis, 2013). During the cultivation on N-depleted media, *P. tricornutum* accumulated lipids both on synthetic medium (from 326 ± 6 to 389 ± 8 g kg^{-1} TS) and UF (from 266 ± 13 to 353 ± 15 g kg^{-1} TS). Accumulated lipids are often used as alternative sources of energy needed to survive during difficult environmental conditions (Hu et al., 2008). Sugars serve as important organic carbon source in order to synthesize important biomolecules such as proteins, lipids and nucleic acids (Afkar et al., 2010). During the growth phase, there was a slight difference between carbohydrate contents in algae grown on f/2-10N (336 ± 2 g kg^{-1} TS) and UF (289 ± 8 g kg^{-1} TS), while there was an identical accumulation i.e. 36 ± 6 g kg^{-1} TS, on both media through the nitrogen depletion step.

Table 2.3. Biochemical characterization of microalgal biomass obtained on synthetic medium and UF digestate.

Strains	Medium	Lipids (g kg^{-1})	Proteins (g kg^{-1})	Carbohydrates (g kg^{-1})
Chlorella sp.	BBM-3N	132 ± 5a	405 ± 5b	362 ± 5b
	UF 1:10	143 ± 7b	306 ± 3a	342 ± 8a
	BBM-3N N-starvation	131 ± 1a	331 ± 2b	470 ± 3b
	UF 1:10 N-starvation	143 ± 6b	263 ± 8a	421 ± 5a
Phaeodactylum tricornutum	f/2-10N	326 ± 6b	184 ± 3a	336 ± 2b
	UF 1:10	266 ± 13a	296 ± 19b	289 ± 8a
	f/2-10N N-starvation	389 ± 8b	128 ± 1a	372 ± 6b
	UF 1:10 N-starvation	353 ± 15a	181 ± 1b	325 ± 3a

Values in the same column followed by the same letter, are not statistically different at $p < 0.05$ according to to Tukey test.

2.4. Conclusions

This work confirms that nutrient rich UF digestate can be successfully utilized as a substrate to support the growth of microalgae (*Chlorella* sp. and *P. tricornutum*) just as well as standard substrates. Moreover, because of its low organic load, the use of the UF digestate limits any potential contamination by microorganisms that could damage the quality of the final biomass product. The present work confirmed previous data about the possibility to develop a biogas-digestate treatment-algae production biorefinery approach.

Acknowledgements

CHAPTER 3 - Carbon recovery from agro-industrial wastes

3. Mixotrophic cultivation of Chlorella for local protein production using agro-food by-products.

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A local strain of Chlorella vulgaris was cultivated by using cheese whey (CW), white wine lees (WL) and glycerol (Gly), coming from local agro-industrial activities, as C sources (2.2 g C L^{-1}) to support algae production under mixotrophic conditions in Lombardy. In continuous mode, Chlorella increased biomass production compared with autotrophic conditions by 1.5-2 times, with the best results obtained for the CW substrate, i.e. 0.52 g L^{-1} d^{-1} of algal biomass vs. 0.24 g L^{-1} d^{-1} of algal biomass for autotrophic conditions. Chlorella showed high protein content (close to 500 g kg^{-1} DM), mixotrophic conditions did not affect amino acid composition in comparison with autotrophy, 43.3 % and 40.8 % respectively, remaining well balanced and rich in essential amino acids. Mixotrophic conditions gave a much higher energy recovery efficiency (EF) than autotrophic conditions, organic carbon energy efficiency (EF_oc) of 32% and total energy efficiency (Eft) of 8%, respectively, suggesting the potential for the culture of algae as a sustainable practice to recover efficiently waste-C and a means of local protein production.

3.1. Introduction

Mixotrophy is a trophic culture method in which microalgae can drive both photoautotrophy and heterotrophy utilizing both inorganic and organic carbon (C) sources (Kang et al., 2004). Inorganic carbon is fixed through photosynthesis, which is influenced by the conditions of illumination. Organic-C is assimilated through aerobic respiration which is affected by the availability of organic carbon (Hu et al., 2012). Some scientists suggested that the specific growth rate of microalgae under mixotrophic cultivation is approximately the sum of those under photoautotrophic and heterotrophic modes (Marquez et al., 1993). However, others believed that the specific growth rate in mixotrophy is not the simple combination of those in photoautotrophy and heterotrophy, and that the two metabolic processes affect each other, contributing to synergistic effects which enhance biomass productivity (Acién et al., 2013). Since organic compounds can be utilized under mixotrophic cultivation, the growth of microalgae does not entirely depend on photosynthesis, therefore light is not the limiting factor for microalgal growth. Under mixotrophic conditions more biomass can be produced for the same light intensity then the overall efficiency of the system increasing (Liang et al., 2009). Mixotrophic cultivation of microalgae can improve the growth rate, shorten the growth cycle, reduce the biomass loss in dark hours due to pure respiration (Park et al., 2012) and enhance lipid and protein productivity (Li et al., 2012; Abreu et al., 2012). Chlorella is one of the few microalgae widely employed for human consumption. It has a high protein content and a balanced amino acid composition (Liu and Chen, 2016) and several authors have demonstrated that mixotrophic conditions enhance lipid or protein content/productivity in Chlorella sp. (Wan et al., 2011; Abreu et al., 2012). Despite mixotrophic cultivation of microalgae providing high biomass production, the cost of

the organic carbon substrate has been estimated to be about 80% of the total cost of the cultivation medium (Bhatnagar et al., 2011). Lombardy Region (North Italy) is in one of the more densely populated (419 inhab. km_2), industrialized and intensively cultivated regions of the EU. In particular, agriculture and the food industry in the Lombardy Region represent the 2.03% of EU28-agricultural/agro industrial-GDP (data of 2013) (Pieri and Pretolani, 2015). These sectors generate large amounts of wastes that need to be disposed of or recycled, to avoid environmental problems. Lombardy Region produces a large amount of wastes from the dairy industry (cheese whey, CW), i.e. 3.3 _ 106 Mg y_1, that represents 36% of the Italian CW production (http://www.clal.it/en/?-section=siero_regioni, visited in October 2016) and 2.75% of the total cheese whey produced in the world. Of this amount only 50% is currently used directly for animal feed or to produce milk-derived products (http://www.lattenews.it/il-siero-dilatte-euna-risorsa-per-diversi-mercati/, visited in October 2016), the remaining part not being traceable or cleaned (Pizzichini et al., 2001). The wine industry also produces large amounts of wastes (wine lees) rich in C that are estimated as 2.52 _ 106 hl (ISTAT, 2015, http://agri.istat.it/sag_is_pdwout/jsp/dawinci.jsp?q=plC260000010 000012000&an=2014&ig=1&ct=607&id=97A|15A|21A|73A, visited in October 2016). These wastes are currently treated by biological processes, for example anaerobic digestion (Riaño et al., 2011) or by aerobic treatment (Petruccioli et al., 2002). To all of this, about 6000 Mg year_1 of glycerol can also be added as the waste stream from the biodiesel industry in Lombardy Region (http://www.assocostieribiodiesel.com/bio/statistiche/elaborated data, visited in October 2016), with only a small part of it being used as a co-substrate for energy production in biogas plants (Robra et al., 2010). All these waste streams are characterized by high C

contents that could usefully be recovered and used to produce new products, avoiding the environmental problems due to their management. The recovery of waste has become a priority in Lombardy Region, Italy and in the EU, where the potential of economic investment in green chemistry topics is also being addressed (e.g. Integrated Research on Industrial Biotechnologies and Bioeconomy – Cariplo Foundation, 2015; Horizon 2020, European Commission). For example, CW has been recently proposed for producing bioplastics via polyhydroxyalkanoates production by using mixed culture (Colombo et al., 2016); wine lees have been used to produce electricity by microbial fuel cells (Sciarria et al., 2015), and glycerol was proposed to produce 4-hydroxymethylfurfural, a molecule useful for pharmaceutical and other material products (Cui et al., 2016). Microalgae production by a mixotrophic approach can be a good candidate for recovering agro-industrial wastes because of the great potential for producing high-added value products useful for many different applications: pharmaceuticals, health, food, feed, nutraceuticals etc. (Abreu et al., 2012). In particular algae production by using Chlorella sp., is of great interest for protein production (Abreu et al., 2012). Lombardy Region has a deficit for protein and about $1x106$ Mg of protein per year are imported from extra-EU regions (e.g. soy meal from Brazil and Argentina) (http:// atlas.media.mit.edu/en/profile/hs92/2304/#Importers, visited in October 2016), so that promoting local protein production could be of interest in reducing external dependence. The aim of this work was to consider the potential to recover regional available waste C-rich streams, i.e. cheese whey, wine lees and glycerol, and to promote local protein-based production by using Chlorella vulgaris cultivated under mixotrophic conditions.

3.2. Materials and Methods

3.2.1 Microalgae strain

The microalgae used in these trials was an indigenous strain of *Chlorella* sp. previously isolated from a digestate storage tank and subsequently characterized (Ledda et al., 2015). Algae inoculum was maintained in 500 ml Erlenmeyer flasks with BG11 Medium (Rippka et al., 1979) under continuous illumination provided by fluorescent cool white lamps with an average irradiance of 25 μE m^{-2} s^{-1}.

3.2.2. Culture medium

The culture medium used during batch and continuous trials was BG-11. In order to prevent bacterial contamination, BG-11 was autoclaved for 90 min at 120 °C before use. For mixotrophic cultivation in both batch and continuous modes, carbon (C) substrates were added to the BG-11 medium in order to reach a final C concentration of 2.2 g L^{-1}. The carbon concentration chosen was selected after preliminary tests using C substrates ranging from 0 to 10 g L^{-1} (Andruleviciute et al., 2014). Three different C substrates were used in this experiment: cheese whey (CW), digestate ultrafiltrate (UF) plus glycerol (Gly) and white wine lees (WWL). Cheese whey was provided by Alimenti Saves (Brescia, Italy) and subsequently stored at -20°C. CW was used after de-proteinization that was performed by using heat treatment at 115°C for 15 min (Dragone et al., 2011) and then by filtration of the flocs formed by using a 0.2 μm Whatman filter. Enzymatic hydrolysis of CW was performed by using β-galactosidase (13.5 units mg^{-1}, Sigma–Aldrich, San Luis, Missouri, USA) at 30°C and pH 4.5, for 24 h in a shake flask at 200 rpm using 65 U of enzyme per g lactose quantified in whey permeate (Espinosa-Gonzalez et al., 2014). Glycerol was obtained from a biogas plant that uses glycerol as

co-substrate for energy production (Azienda Agricola Le Ghiande). Because glycerol lacks N, ultrafiltered digestate rich in nutrient was also used with it, as previously reported (Ledda et al., 2013). White wine lees were sampled in a wine cooperative in northern Italy (Corte Franca, BS). All biomass samples were immediately stored at 4°C and characterized upon arrival.

3.2.3 Microalgae culture growth conditions

Batch culture

Batch trials were carried out both under autotrophic (control) and mixotrophic conditions in four photobioreactors (PBRs) of 2.5 L working volume. The pH was set at 8.4 and it was controlled by using pure CO_2 injection adopting an "on-demand" mode. The temperature was constant and set at 25°C; light was provided by cold fluorescent lamps at an irradiance of 370 $\mu E\ m^{-2}\ s^{-1}$ at PBR surface with 12h:12h photoperiod. The starter inoculum 10% (v/v) with 0.3 g L^{-1} of microalgae was placed in the reactors and culture medium added to start the trial. The PBRs in the batches were named: PBR under autotrophic conditions (BPBR-A), PBR under mixotrophic conditions using as C source cheese whey (BPBR-CW), white wine lees (BPBR-WWL) and PBR glycerol plus ultrafiltrate (BPBR-Gly + UF), respectively. BPBR-Gly + UF was loaded with glycerol as reported before and with UF at 10% v/v in order to provide nutrients, keeping the ammonia concentration below 150 mgl^{-1}, thus avoiding inhibiting conditions (Franchino et al., 2013).

Continuous culture

PBRs were continuously fed with substrates (BG-11 and BG C-enriched) using a Hydraulic Retention Time (HRT) of 5 days. PBRs were maintained at 25°C under constant aeration with an air flux of 10 L min^{-1}, pH of 8.4, this latter adjusted by using pure CO_2 injection, and an incident light on PBR surface of 250 µE m^{-2} s^{-1} and 12h:12h photoperiod. The PBRs under continuous culture were named: PBR under autotrophic conditions (CPBR-A), PBR under mixotrophic conditions using as C sources: cheese whey (CPBR-CW), white wine lees (CPBR-WWL) and glycerol plus ultrafiltrate (CPBR-Gly + UF). Culture conditions were identical to those adopted for batch culture apart from the incident light on the PBR surface which was of 540 µE m^{-2} s^{-1} with 12h:12h light photoperiod. PBRs were continuously fed with the substrates (BG-11 and BG C-substrate enriched) adopting an HRT of 5 days and maintaining the C concentration at 2.2 g C L^{-1} for mixotrophic PBR.

Monitoring Culture

Microalgae concentration was estimated by optical density (OD), measuring the absorbance at 560 nm with a Jeneway 7305 UV-visible spectrophotometer (Bibby Scientific Limited, Staffordshire, ST15 OSA, UK). Dry weight (DW) was determined sampling 10 ml of algae suspension from each PBR. The samples were centrifuged at 4,000 rpm for 10 min and then washed twice with an equivalent volume of distilled water to avoid excess sugars or salts. Culture samples were then filtered through a 1.2-µm filter (GF/C, Whatman Ltd., Maidstone, UK) and dried overnight at 80°C. A good linear correlation fit was obtained (R^2 value of 0.97, $p < 0.05$, n = 24) between the dry weight and OD measurements at 560 nm. The presence of contaminants, mainly

bacteria, was verified by centrifuging the liquid at 3,000 rpm for 15 min to separate microalgae (in the solid fraction, i.e. pellet) from bacteria (in the liquid fraction). The supernatant was then centrifuged at 12,000 rpm for 20 min, and the bacterial pellet was then observed by a light microscope at 100 x with a Zeiss Axio Scope A1 (Carl Zeiss Group, Oberkochen, Germany). Sampling was performed every two days; for continuous cultures, the data were collected from the beginning of the 3rd cycle.

3.2.4 Analytical methods

Ammonia nitrogen (N-NH$_4^+$) and chemical oxygen demand (COD) were determined using fresh material according to *standard methods* for wastewaters characterization (APHA, AWWA, WEF, 2005). All the other analytical evaluations were performed on freeze-dried biomasses. Total Kjeldahl Nitrogen (TKN) was determined according to standard methods (APHA, AWWA, WEF, 2005). Total phosphorus (TP) was determined by inductively coupled plasma mass spectrometry (ICP-MS, Varian, Fort Collins, USA) according to the 3051A and 6020A EPA methods (EPA, 2007). Total lipids content in lyophilized biomass was evaluated by gravimetric assay; in particular, a slightly modified version of the method proposed by Kochert et al., (1978) was used. In brief, after a mechanical disruption of the cells, an aliquot of the freeze-dried sample mixed with 2 ml of chloroform: methanol (2:1 v/v) was centrifuged at 5,000 rpm for 5 minutes. The extracts were transferred to new vials, and the previous step was repeated until the supernatants were cleared, this procedure allowing the maximum lipid extraction. Total lipid content was then gravimetrically measured after solvent evaporation using a rotary evaporator (Büchi R110, Büchi Labortechnik AG., Flawil, Switzerland). The crude proteins of samples were calculated by multiplying the total

Kjeldahl nitrogen by nitrogen-to-protein conversion factors of 5.95 for *Chlorella* sp. (González-López et al., 2010). The amino acid content of algal biomass was determined using the AOAC official method (AOAC - Official method 994.12, 1997). In brief, about 0.2 g of freeze dried sample were hydrolyzed in 6Mol L^{-1} HCl for 22 h at 110° C to free individual amino acids, followed by HPLC analysis (Agilent 1100 Series HPLC). Analyses were performed by automated online pre-column derivatization using an automated liquid sampler and Poroshell 120 column HPH-C18 (*3.0 x 100 mm*, *2.7 µm. P/N 695975-502*). The standard preparation, derivatization process, LC method used was performed according to Agilent Pub. #5991-5571EN (Pub no. 5991-5571EN, July 7 2015, Agilent Technologies). The primary amino acids (OPA-derivatized) were monitored at 338 nm and norvaline was used as ISTD. The secondary amino acids (FMOC-derivatized) were monitored at 262 nm and sarcosine was used as ISTD. The separation was carried out under gradient elution with two mobile phases. Phase A: 10 mMol L^{-1} NaH_2PO_4 + 10 mMol L^{-1} $Na_2B_4O_7$ + 5mM NaN_3, pH 8.2 adjust with HCl 5 Mol L^{-1}, and Phase B: ACN:MeOH:water (45:45:10, v/v/v). The flow rate was 0.62 mL min^{-1}, the column temperature 40°C and injection volume 20 ml. Carbohydrates were estimated by subtracting the percentage of ashes, lipids and crude proteins out of 100% (Wilkie and Mulbry, 2002). The ash content was determined as the residue after ignition at 550°C overnight.

The specific growth rate µ (day $^{-1}$) was calculated from the Equation (1):

$$\mu \ = \ 1/t \ln \left(Xf/Xo \right) \qquad [1]$$

in which *Xo* and *Xf* are the concentrations of cells (g L^{-1}) at the beginning and at the end of the batch run, respectively, and *t* (days) is the duration of the run.

Daily biomass productivity (Dp as mg L^{-1} d^{-1}) during the culture period was calculated by the Equation (2):

$$Dp = (Xf - Xo)/t \qquad [2]$$

Nitrogen removal (Nre %) was calculated according to Equation (3):

$$Nre = (No - Nf)/No\% \qquad [3]$$

in which No is the nitrogen concentration at the beginning and Nf is the nitrogen concentration at the end of the experiment.

3.2.6 Energy Balance

Light intensity as photosynthetically active radiation (PAR) was detected by a light meter by using an LI-190 quantum sensor (LI-COR, Lincoln, Nebraska, USA). Energy provided as organic carbon (Ec) and energy contained in the biomass produced (combustion enthalpy) were calculated on the base of the biochemical composition according to Lehninger (1985). Light energy (E_l) (kJ) supplied to the reactor was calculated as:

$$E_l = PAR*Sr*t \qquad [4]$$

where PAR is the PAR supplied to the reactor, Sr is the reactor surface exposed to the light and t is the time (h) of exposition to the light.

Y_{SE} biomass yield on the supplied energy to culture (g kJ^{-1}) was calculated as:

$$Y_{SE} = Y/(E_c + El) \qquad [5]$$

where Y is biomass yield, Ec is the energy provided as organic carbon and E_l is the light energy supplied to the reactor

P_{SE} that represents the protein yield on the supplied energy to culture (g kJ^{-1}) was calculated as:

$$P_{SE}= (Y*Crude\ Protein\ content)/(E_c+El)\quad [6]$$

where Y is biomass yield, Ec is the energy provided as organic carbon and E_l is the light energy supplied to the reactor

EF_t is the total Energy Efficiency due to light and C supplied and was calculated as:

$$EF_t =(Y*biomass\ enthalpy)/(E_c+El)\quad [7]$$

where Y is biomass yield, Ec is the energy provided as organic carbon and E_l is the light energy supplied to the reactor

EF_{oc} is the Organic Carbon Energy Efficiency and was calculated by the following formula:

$$EF_{oc} = ((Y_{mixo}-Y_{autotroph}) *biomass\ enthalpy)/(E_c)\quad [8]$$

where Y_{mixo} is biomass yield of mixotrophic cultures, $Y_{autotroph}$ is biomass yield of autotrophic cultures and Ec is the energy provided as organic carbon. In the energy balance calculation energy consumption due to mixing and gas exchange was not considered because in a fullscale plant it is very limited, i.e. 0.5–7 kJ L^{-1} day (Acién Fernández et al., 2013), which is less than 5% of the input light energy considered in this work.

3.3. Results and discussion

3.3.1 By-products characterization.

Principal chemical features of the organic streams used for the mixotrophic trials are reported in Table 3.1. CW, Gly and WWL were characterized by the high presence of easy degradable C, as suggested by BOD_5/COD values that ranged from 0.65 to 0.89. The UF substrate contains low degradable C (BOD_5/COD ratio of 0.35) (Table 3.1) but the UF was used only as a nitrogen source and total C dosed accounted for less than 2%

of total C of the Gly + UF mix. Nitrogen in CW and WWL was in organic form and only a small percentage of it was present as ammonia; however, UF contains nitrogen in the ammonia form. The P content was highest in CW and UF, while in Gly it was under the detection limit.

Table 3.1. Chemical characteristics of carbon substrates used for mixotrophic trials.

	pH	DM	TOC	TKN	N-NH$_3$	P tot	COD	BOD	BOD/COD
		g kg^{-1}	g L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	g O$_2$ L^{-1}	g O$_2$ L^{-1}	
CW[a]	5.26	158±6	55.1±1.6	805±48	103±9	400±20	147±7	97±4	0.66±0.04
UF	8.40	3.11±0.19	0.63±0.04	1377±41	1155±35	300±18	1.52±0.03	0.54±0.03	0.36±0.02
Gly	6.81	902±72	737±10	udl[b]	udl	udl	1680±154	1307±360	0.78±0.22
WWL	2.83	16.3±0.8	67.7±2.0	219±4	32.2±1.3	150±8	181±11	162±13	0.9±0.1

[a]CW: Cheese Whey, UF: Ultrafiltrate, Gly: Glycerol, WWL: White Wine Lees

[b]udl: under detection limit

3.3.2. Biomass production under autotrophic and mixotrophic conditions

Batch trials

Batch trials were performed in order to detect kinetic growth parameters and to assess the suitability of the carbon streams to support mixotrophy. Unfortunately, culture contamination by bacteria occurred within 4-8 d so that mixotrophic batch trials were stopped before the stationary phase was reached. Nevertheless, trials data revealed that the maximum growing rates (μ_{max}) obtained under mixotrophic conditions for trials BPBR-CW and BPBR-WWL were higher than that of the control. On the other hand, trial BPBR-GLY + UF did not show significant differences when compared to BPBR-

A, while the BPBR-GLY showed a μ_{max} lower than control. The literature has reported how mixotrophic conditions increase bacterial and fungal culture contamination (Ceron-Garcia, 2000). Chlorination and the use of antibiotics, such as penicillin, streptomycin and chloramphenicol were reported to prevent contamination (Ceron-Garcia, 2000). The addition of these chemicals to reducing contamination of cultures was not chosen in this work because another strategy was selected, i.e. continuous algae culturing (see next section). By doing so, organic carbon dosed for mixotrophic algae growth was dosed, daily reducing bacteria growth because of high competition for C by algae that were kept at high growth rate conditions (Liu and Chen, 2016).

Continuous trials

Results obtained in continuous trials run for five weeks, indicated that both biomass concentration and productivity significantly increased for mixotrophic trials with respect to the autotrophic controls (Table 3.2): the CPBR-CW productivity was about twice than that of the autotrophic control, while CPBR-Gly+UF and CPBR-WWL productivities were about 1.5-times higher than that obtained with the control. These data were consistent with the literature (Table 3.3) that reported a biomass productivity for *Chlorella* sp. under mixotrophic cultivation with cheese whey, increasing by 1.6 to 2.9 times over that of photoautotrophic cultivation (Abreu et al., 2012). The CPBR-Gly + UF trial, although it gave good algae productivity was characterized by the high bacterial contamination, as discussed, also, for the batch trials. Therefore, this trial was interrupted on the 18th day losing all biomass produced (contamination did not allow us to separate algae biomass to be used for chemical characterization).

Table 3.2. Growing parameters detected during algae growth: continuous trials

	C_b:	D	Y	Inflow TOC	Outflow TOC	Biomass TOC
	g L^{-1}		g L^{-1} d^{-1}	mg L^{-1}	mg L^{-1}	g kg^{-1} DM
CPBR-A	1.21±0.02a[a]	0.2	0.24±0.03a	0.0	42±1b	521±10a
CPBR-CW	2.59±0.04c	0.2	0.52±0.02c	2231±30a	253±12d	587±47b
CPBR-Gly+UF	1.67±0.03b	0.2	0.33±0.04b	2285±94a	180±7c	477±5a
CPBR-WWL	1.75±0.05b	0.2	0.35±0.02b	2360±109a	26±3a	498±20a

[a] Means followed in the same column by the same lower-case letter are not statistically different (p <0.05) according to Tukey test.

Table 3.3. Comparison of mixotrophic performances of Chlorella cultivated under different condition according to literature and in this work.

Microalgae strain	Carbon source	Carbon amount	Culture conditions	Biomass	Mixotrophic performance respect to control	Reference
		g L^{-1}		g L^{-1}	g L$^{-1}_{mixotrophic}$ /g L$^{-1}_{autotrophic}$	
Chlorella vulgaris	Cheese whey	2.2 (hydrolized)	Continous	2.59	2.1	
Chlorella vulgaris	Glycerol+UF	2.2	Continous	1.67	1.4	This work
Chlorella vulgaris	White wine lees	2.2	Continous	1.75	1.4	
Chlorella vulgaris	Glucose	4	Batch flasks	1.4	3.5	Heredia-Arroyo et al., 2011
Chlorella vulgaris	Cheese whey	0	Batch	1.22		Abreu et al., 2012
		10 no-hydrolized	Batch	1.98	1.6	
		10 hydrolized	Batch	3.58	2.9	
	Glucose + galactose	10	Batch	2.24		
Chlorella vulgaris	Glycerol + glucose	5+2	Batch	2.6	1.8	Kong et al., 2013
	Glycerol	1		0.62	7.6	
freshwater Chlorella sp	Glucose	2	Fed batch	1.35	3.2	Cheirsilp et al., 2012
marine Chlorella sp.		2	Fed batch	1.41	2.3	

Chlorella sp	Glycerol	2	Batch	1.65	1.0	Andrulevici ute et al., 2014
Chlorella sorokiana	Glucose	6	Batch	4.57	4.6	Li et al., 2014
Chlorella sorokiana	Glucose	10	Batch	0.61	1.5	Wan et al., 2011
Chlorella sp	Acetate	10	Batch	0.99	3.96	Liang et al., 2009
	Glucose	10	Batch	1.7	6.8	
	Glycerol	10	Batch	0.72	2.88	

All biomass productivities obtained in mixotrophic conditions were higher than that of the control (CPBR-A). Even though the total C substrate dosed in the culture medium was the same for all treatments studied (C = 2.2 g L^{-1}), algae production differed (Table 3.2). This fact could be due to the different C-availability characterizing substrates, i.e. BOD_5/COD ratio (Table 3.1). Nevertheless, CPBR-CW, which was characterized by the lowest BOD_5/COD, i.e. lowest C availability, showed the highest biomass productivity, followed by CPBR-Gly + UF and CPBR-WWL treatments. Therefore, it can be concluded that algae productivity did not depend only on C availability but it also depended on other causes, i.e. nutrient availability. N mass balance proposed in Table 3.4 indicated that nitrogen uptake (NU) increased with mixotrophy because of higher biomass productivity than control (Table 3.2). On the other hand, there was no correlation between NU and biomass growth under mixotrophic conditions, indicating that N did not influence algae growth under these conditions and that it was not a limiting factor. More interesting was the fact that P content in the inflow medium (Table 3.4) was significantly higher for CPBR-CW than for the other CPBRs, and that this circumstance coincided with the higher biomass productivity (Table 3.2). The very good correlations found for Inflow TP vs. biomass productivities (r = 0.98, P< 0.05; n = 4) (Table 3.2 and 3.4), indicated that effectively a higher P content in the culture medium

allowed higher biomass production. Again, data in Table 3.4 suggest that a higher TP content in the substrate determined a larger P consumption, such as confirmed by the very good correlation found for the total P consumed (Influx TP – Outflux TP) (Table 3.4) vs. Outflow TP (r = 0.98, P < 0.05; n =4). Linear correlations found indicated that P was not luxury consumed as its consumption was accompanied by a linear increase of the biomass production (total P consumed vs. algae productivity: r = 0.97, P < 0.05; n = 4). These results found confirmation in the literature: when nutrients are provided in non-limiting amounts and light is the growth-limiting factor, most algal species display a constant phosphorus content of about 10 g kg^{-1} DM (Kaplan et al., 1986). Powell et al., (2008) reported phosphorus in the biomass up to 31.6 g kg^{-1} DM in the case of high P availability, while a minimum P content of 4.1 g kg^{-1} DM was reported when using P-depleted medium. Litchman et al., (2003) reported that P limitation can even halve the kinetic parameter of algae growth. Now taking into consideration P content in biomass obtained in this work (Table 3.3) and reference data previously reported, biomasses obtained for treatments CPBR-Gly+UF and CPBR WWL, showed a typical P content determining P-limiting condition (P < 10 g kg^{-1} DM). This fact confirmed that low P content in the substrate was responsible for the limited algae growth. Thus TP content in input medium was the driving factor affecting algae growth under mixotrophic conditions while carbon availability (BOD$_5$/COD) did not cause any differences in algae kinetic growth parameters and final biomass concentration. Therefore, the results of this work indicate that mixotrophic conditions allowed more biomass production with respect to autotrophic conditions, using different C-substrates with Chlorella. Biochemical characterization reported in Figure 3.1, indicated that mixotrophy did not affect the biochemical composition of the biomass produced in comparison with

autotrophic conditions. These results differ from those reported by Liang et al., (2009) who found a decrease of lipid content (relative content) in mixotrophic cultures compared with the autotrophic ones, although the total lipids productivity in mixotrophy was approximately six times higher (27 mg L^{-1} day^{-1}) than that obtained by autotrophy (4 mg L^{-1} day^{-1}). Again, Kong et al., (2013) reported an increase (six times) in the lipid productivity and in lipid content (relative content) for mixotrophy in comparison with autotrophy when glycerol and glucose were added to the culture medium.

Table 3.4. N and P content in growing media and algal biomasses.

		CPBR-A	CPBR-CW	CPBR-Gly+UF	CPBR-WWL
Inflow TKN	mg L^{-1}	247±13b[a]	281±18c	143±9a	256±18b
Outflow TKN	mg L^{-1}	181±6d	63±5b	13±2a	98±3c
Biomass TKN	g kg^{-1}	71±1a	76±5a	68±4a	81±1a
Inflow TP	mg L^{-1}	7.21±0.01a	23.2±0.2d	10.1±0.1b	13.1±0.3c
Outflow TP	mg L^{-1}	1.53±0.10c	4.12±0.05d	0.81±0.05b	0.42±0.03a
Biomass TP	g kg^{-1}	12.2±0.6c	14.3±0.8d	6.33±0.17a	7.91±0.21b

[a]Means followed in the same line by the same lower-case letter are not statistically different ($p < 0.05$) according to Tukey test.

In this work lipid content, expressed on a biomass dry matter basis, did not show any significant changes for the three treatments with respect to the controls (Figure 3.1). Daily lipid productivity was 2 times higher than that of autotrophy for CPBR-CW and only slightly higher (1.2 fold) for both CPBR-GlyUF and CPBR-WWL treatments. At the same time, protein content, on a dry matter basis, did not show significant changes for the three treatments studied compared with the autotrophic culture (Figure 3.1). On

the other hand, total protein productivities were significantly increased by 2.3, 1.7 and 1.3 times compared with those obtained by autotrophy for CPBR-CW, CPBR-WWL, and CPBR-GlyUF, respectively. These results have been reported, also, for other microalgae: Hu and Gao (2003) found higher protein productivity in mixotrophic cultures than control for *Nannochloropsis*. El-Sheekh et al., (2012) working with *Chlorella vulgaris* and *Scenedesmus obliquus* reported a higher protein content under mixotrophic condition than under autotrophic and heterotrophic culture, concluding that the protein content depended on carbon provided. More recently Kadkhodaei et al., (2015) provided data for *D. salina* showing four times more protein production than control, by adding glucose to the medium.

Figure 3.1. Biochemical characterization of microalgae biomass
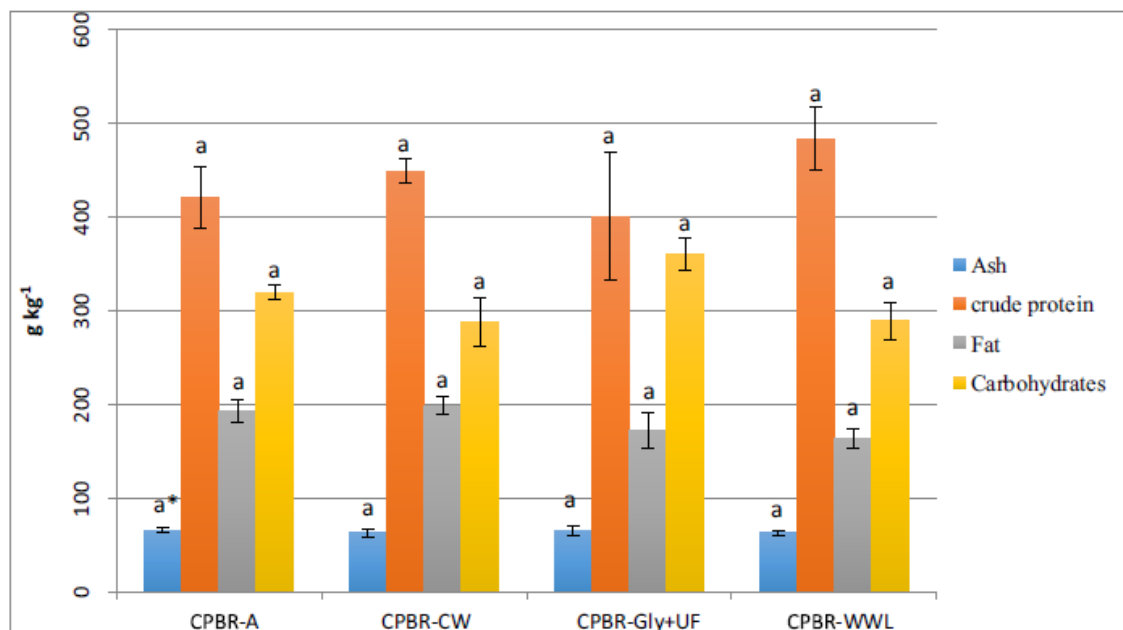
*Instagrams referred to a single biochemical component with the same lower-case letter are not statistically different (p<0.05) according to Tukey test

3.3.3 Energetic balance

Mixotrophic culture allowed higher biomass production in comparison with autotrophic condition because of the C supplied. The efficiency by which this added energy was transformed into microalgae biomass can be evaluated by performing a complete energy mass balance. To do that the total energy supplied to the system (radiant plus chemical energy) was compared to the energy content in the microalgae biomass (Table 3.5) taking into consideration total biomass produced. Results obtained showed a significant increase in the specific biomass yield (g) per energy unit (kJ) (Y_{SE}) for CPBR-CW, i.e. 0.007 g kJ^{-1}, with respect to the control, i.e. 0.004 g kJ^{-1}. Yang et al., (2000) reported a Y_{SE} of 0.007 g kJ^{-1} for mixotrophy; moreover they found that mixotrophy gave the most efficient utilization of energy for biomass production compared to autotrophy and heterotrophy. Considering the total efficiency of the energy transformation (EF_t) into biomass, the CPBR-CW trial was outstanding with 14% value. If we consider only the energy supplied by the carbon source and the biomass produced because of that added energy, the energy transformation (EF_{OC}) increased to 32%. CPBR-Gly+UF and CPBR-WWL treatments showed total energy efficiencies (EF_t) close to the control (8%) and the EF_{OC} equal to 8% (Table 3.5).

Table 3.5. Energy balance: continuous trial

		CPBR-A	CPBR-CW	CPBR-Gly+ UF	CPBR-WWL
Biomass enthalpy[b]	kJ g DM^{-1}	19.8±0.2a	19.8±0.1a	19.4±0.4a	19.1±0.6a
E_l[c]	kJ day^{-1}	144±2a	144±2a	144±2a	144±2a
E_c[c]	kJ day^{-1}	0a	43.4±1.1b	51.5±2.0c	43.2±1.4b
E_b[c]	kJ day^{-1}	11.9±0.5a	25.7±1.1c	16.1±0.6b	16.8±0.8b
EF_t[c]	%	8%	14%	8%	9%
EF_{oc}	%	-	32%	8%	11%
Y_{SE}[c]	g kJ^{-1}	0.004±0.001a	0.007±0.001b	0.004±0.001a	0.005±0.001a
P_{SE}[c]	g kJ^{-1}	0.002±0.000a	0.003±0.000b	0.002±0.000a	0.002±0.000a

[a]Means followed in the same line by the same lower-case letter are not statistically different ($p < 0.05$) according to Tukey test.
[b]Combustion enthalpy of biomass and carbon medium was calculated on the base of biochemical composition according to Lehninger (1985).
[c]E_l: light energy (as PAR) supplied to the reactor each day (kJ d^{-1}) calculated as PAR supplied to the reactor * reactor surface exposed to light; E_c: Total chemical energy supplied to the reactor by carbon supply (kJ d^{-1}); E_b: Energy content in the algae biomass produced in one day (kJ d^{-1}); EF_t: Total Energy Efficiency (light and C supplied); EF_{oc}: Organic Carbon Energy Efficiency; Y_{SE} biomass yield on the supplied energy to culture (g kJ^{-1}); P_{SE} Protein yield on the supplied energy to culture (g kJ^{-1}).

In these cases the use of carbon was less efficient because of both less P supplied (see Chapter 3.2.2) and the presence of high bacterial contamination. Results obtained in this work were in line with those previously reported in the literature. Yang (2000) and Ren et al., (2014) reported efficiency conversion of light energy (EF_t) into biomass under autotrophic conditions in the range of 1-8 %. However, energy conversions of 14.6 % (Ren et al., 2014) and 18% (Yang, 2000) were reported for mixotrophic conditions. Reference data for (EF_{oc}) ranged between 18% (Yang et al., 2000) to 45% (Ren et al., 2014) and are consistent with the data found by this work, i.e. EF_{oc} for CPBR-CW of

32% (Table 3.5). These results are impressive if compared with data reported in the literature for unicellular heterotrophic microorganism such as *Candida utilis*, for which a 58% of energy efficiency has been reported (Trinh et al., 2009).

3.3.4 High biological-value protein production by Chlorella *sp. by using C waste streams: a speculative approach.*

The availability of local waste streams rich in organic C and the findings of this work outlined the possibility to build up a sustainable local protein production chain by using microalgae under mixotrophic conditions. Protein production using *Chlorella* assumes importance because of both its high content of protein and the biological value of the proteins, that are rich in high-quality essential amino acids (Buono et al., 2014). The amino acids (AA) compositions of protein obtained by cultivating *Chlorella* under autotrophic and mixotrophic conditions in this study are reported in Table 3.6. Data indicate that mixotrophic conditions did not affect amino acid composition in comparison with autotrophy, so that it remained well balanced and rich in essential amino acids that accounted for more than one third of the total amino acid contents. It was also interesting to observe the elevated biological value of *Chlorella*-AA, in comparison with both vegetal (soybean) and animal protein (beef meat) (Table 3.6).

Table 3.6. Amino acid composition

	PBRC-A	PBRC-CW	PBRC-Gly+UF	PBRC-WWL	Soybean[b]	Beef[b]	Commercial chlorella[c]	Spirulina[d]
Amino acid				% DM				
Lysine[a]	1.96 ± 0.05	2.40 ± 0.01	3.65 ± 0.04	2.22 ± 0.05	2.60	1.57	3.43	3.05
Histidine[a]	0.75 ± 0.01	0.87 ± 0.03	0.51 ± 0.03	0.98 ± 0.11	1.05	0.6	1.08	1.01
Phenylalanine[a]	2.41 ± 0.16	2.26 ± 0.14	1.21 ± 0.14	2.51 ± 0.03	2.06	0.78	2.36	2.7
Leucine[a]	3.77 ± 0.02	3.47 ± 0.00	3.87 ± 0.03	3.89 ± 0.10	3.23	1.44	4.32	5.41
Isoleucine[a]	1.55 ± 0.02	1.78 ± 0.05	1.42 ± 0.08	1.96 ± 0.05	1.89	0.85	1.99	3.49
Methionine[a]	0.55 ± 0.03	0.53 ± 0.07	0.58 ± 0.04	0.56 ± 0.01	0.53	0.48	1.28	1.36
Valine[a]	2.45 ± 0.14	2.60 ± 0.08	2.54 ± 0.06	3.11 ± 0.10	2.01	0.89	2.91	3.32
Threonine[a]	2.32 ± 0.27	2.34 ± 0.12	1.46 ± 0.07	2.46 ± 0.01	1.62	0.81	2.53	29.9
Arginine	4.64 ± 0.04	4.57 ± 0.29	3.89 ± 0.05	3.70 ± 0.31	3.02	1.12	3.08	4.55
Alanine	3.58 ± 0.04	3.69 ± 0.01	3.49 ± 0.05	3.93 ± 0.02	1.77	1.03	4.32	4.40
Glycine	2.20 ± 0.04	2.28 ± 0.14	2.77 ± 0.15	2.74 ± 0.10	1.74	0.86	2.96	3.03
Proline	2.54 ± 0.04	2.60 ± 0.12	3.23 ± 0.05	2.01 ± 0.13	2.28	0.67	2.37	2.27
Gultamic acid	4.41 ± 0.12	5.67 ± 0.07	4.10 ± 0.11	5.14 ± 0.03	7.77	2.71	6.18	8.90
Serine	1.98 ± 0.20	1.83 ± 0.04	2.08 ± 0.13	2.15 ± 0.07	2.13	0.71	2.06	2.78
Tyrosine	1.28 ± 0.02	1.41 ± 0.10	0.96 ± 0.03	1.47 ± 0.01	1.31	0.64	1.98	2.79
Aspartic acid	3.53 ± 0.16	4.14 ± 0.01	2.77 ± 0.11	4.37 ± 0.14	4.86	1.59	4.72	6.12
Cystine	0.84 ± 0.02	0.91 ± 0.04	0.12 ± 0.06	0.92 ± 0.02	0.55	0.23	0.73	0.57

[a] essential amino acid.
[b] FAO (1970).
[c] chlorella pills available on the market.
[d] Handbook of microalgal culture: Applied Phycology and Biotechnology (2013).

Now, taking into consideration the total amount of wastes available in the Lombardy Region and studied in this work, the algae yield obtained (g L^{-1}) (Table 3.2) and the total protein content in algae (on a dry matter basis: $g_{protein}$ kg^{-1} algae) (Figure 3.1), it could be calculated that about 103×10^3 Mg of high quality protein could be produced. These data mean that for each kg of waste-C it would be possible to produce 0.52 kg of protein. Obviously this calculation it is only speculative, but it gives an idea of the potential for producing high quality local protein from algae by recovering local C-rich waste streams.

3.4 Conclusion

Mixotrophic algae cultivation by using agro-industrial C-rich wastes represents an interesting approach to boost algae production with particular interest for protein production. Chlorella *sp.* has been reported in this work to improve algae/protein production under mixotrophic conditions compared to the autotrophic condition. A speculative calculation indicated that the total recovery of the waste-C stream studied could produce 103×10^3 Mg of a high quality protein, this amount being estimated as 10% of the total protein imported by the Lombardy Region, suggesting the possibility of promoting a local production chain.

4. Carbon and nutrient recovery in the cultivation of Chlorella vulgaris: A life cycle assessment approach to comparing environmental performance.

Microalgae cultivation is arousing interest for its ability to provide biomass for food, feed and energy. Microalgae are more efficient in converting solar energy into biomass than terrestrial plants, and microalgae cultivated in a mixotrophic mode showed a higher biomass productivity. This work aimed to evaluate the environmental impacts of the cultivation of microalgae in autotrophy and mixotrophy and to define under what conditions mixotrophic cultivation gives the best environmental performance. To make this comparison, primary data of Chlorella vulgaris cultivation in autotrophy and mixotrophy were used. The scenarios considered were autotrophy (Scenario 1); mixotrophic cultivation on cheese whey, (Scenario 2); and mixotrophic cultivation using dairy wastewater (cheese wastewater) (Scenario 3). In addition, since commercial nitrogen fertilizers are one of the major contributors to the environmental impact of Chlorella production, two other scenarios were modelled: autotrophy on recovered nitrogen from digestate (Scenario 4) and mixotrophic culture on recovered nitrogen and carbon (Scenario 5). The mixotrophic growth of microalgae was shown to be an environmentally effective process (i.e. it showed a decrease of the impact categories), when the organic carbon provided had no other allocation and could be considered free of a cost burden. The cultivation of microalgae on recovered nitrogen and recovered

carbon was found to decrease the CO2 emission by almost 60% and similar decreases were obtained for the other impact categories in comparison with autotrophy. A value of CO2 emission equal to 1.05 kg CO2 eq. kg-1 of microalgae was achieved for Scenario 5, and a decrease of more than 50% was assessed for the impact categories: Marine eutrophication, Human toxicity, Freshwater ecotoxicity, Marine ecotoxicity and Fossil fuel depletion

4.1. Introduction

Microalgae are photosynthetic microorganisms which convert sunlight, water, carbon dioxide, inorganic N and P into algal biomass and thus into valuable organic compounds such as lipids (in particular Poly Unsaturated Fatty Acids), proteins, pigments, biopolymers, animal feed products, nutraceuticals and pharmaceuticals (Pulz and Gross, 2004). Microalgae proved to be more efficient in converting solar energy into biomass than terrestrial plants, showing a photosynthetic efficiency higher than 8% of PAR (Photosynthetic Active Radiation) (Huntley et al., 2007; Yang et al., 2000; Zijffers et al., 2010; Ren et al., 2014). Microalgae generally grow photoautotrophically, using light as the only energy source, which they convert into chemical energy (Wang et al., 2016), but they can also use heterotrophic metabolism, in which microalgae use organic carbon as an energy source (Morais et al., 2009), and mixotrophic metabolism. Mixotrophy allows microalgae to be grown by means of both the photoautotrophic and heterotrophic pathways by using both inorganic and organic carbon sources (Kang et al., 2004). The assimilation of CO_2 is influenced by light (photosynthesis), while the assimilation of organic compounds takes place through aerobic respiration (Hu et al., 2012). In natural ecosystems, this behavior seems to be a rule rather than an exception and it may be

considered to be the backup alternative for obtaining energy when photosynthesis is impossible because illumination is insufficient or because other limiting factors occur in oligotrophic environments. Some scientists have suggested that some kind of synergetic mechanisms are involved: i.e. that in mixotrophic cultivation, photosynthesis and aerobic respiration act in synergy, enhancing biomass productivity (Yu et al., 2009; Acién et al., 2013). Microalgae cultivated in a mixotrophic mode showed an improvement in their growth rate, a reduction in the length of the growth cycle and biomass losses in the dark hours, and an augmentation in biomass productivity due to the supplementation of photoautotrophy with carbon substrates (Park et al., 2012; Wan et al., 2011; Xiong et al., 2010; Ogbonna et al., 2000). For instance the biomass productivities of *Nannochloropsis oculata*, *Dunaliella salina*, *Chlorella sorokiniana*, *Spirulina platensis* and *Scenedesmus obliquus* cultivated in mixotrophy with supplementation of glucose were 1.4 up to 8.7 times higher than for the same microalgae phototrophically cultivated (Chen et al., 1997; Mandal et al., 2009; Wan et al., 2011). The carbon source for mixotrophy should consist of soluble molecules which are easy to take up and process, such as sugars, glycerols and alcohols (Znachor & Nedoma, 2010; Perez-Garcia et al., 2011). Creating value from waste and by-products represents an interesting solution for making use of low-cost C-sources for the mixotrophic approach. Cheese whey is a liquid resulting from the precipitation of milk casein in the cheese-making process: 80 million Mg of cheese whey are produced every year in the EU and 30 million Mg in North America (Nikodinovic-Runic et al., 2013). Cheese whey has high carbon content (COD = 50–150 g L^{-1}) (Salati et al., 2017) and lactose is the main component (Prazeres et al., 2012). Due to these features, cheese whey has been already considered as a substrate for algal growth (Abreu et al., 2012; Girard, 2014; Espinosa- Gonzalez, 2014; Salati et al., 2017). As well as carbon-rich by-products, the agriculture and livestock sectors produce large amounts of effluents, particularly

animal manure wastewaters, which are extensively available all over the world and can lead to severe pollution issues if not properly managed (Zhou, 2014). Every year in the EU-27 more than 1,500 million Mg of livestock wastes are produced (Choi et al., 2014). Effluents from poultry, piggery and dairy farms and the digestates produced by the anaerobic digestion process contain huge quantities of nitrogen, phosphorus and organic carbon. Some authors have obtained data to support the possibility of recovering and re-using nutrients from digestates through the cultivation of microalgae (Franchino et al., 2013; Ledda et al., 2015a; Ledda et al., 2015b; Salati et al., 2017). In this context, microalgae-based processes may represent a chance to recover nutrients (C, N and P) which would otherwise be wasted (de Godos et al., 2009; Mulbry et al., 2008), promoting both the circular economy and more sustainable production systems. To evaluate the environmental benefits of this circular model, Life Cycle Assessment (LCA) methodology is an effective tool which is increasingly being used. LCA is a methodology that aims to analyze (and compare) products, processes, or services from an environmental perspective (ISO 14040 and ISO 1044 standards, 2006 (ILCD 2010)), providing a useful and valuable tool for production systems evaluation As LCA allows the comparing of metrics based on a standardised procedure, and as LCA has already been applied to evaluate microalgae processes, it has been used in this paper to compare the different options for carbon and nutrient recovery for microalgae growth and the corresponding environmental impacts and opportunities. LCA uses a specific functional unit according to the product or service under investigation: this unit provides the reference to which all flows in the assessment are referred. The system boundaries define where the process starts and ends and which flows are being accounted for. Published LCA studies on microalgae production have mainly focused on biodiesel production and downstream operations. Yuan et al., (2015) reported GHG emissions of 71 g CO_2-equivalent per MJ of biodiesel (best case) with cultivation and oil extraction dominating energy use and emissions, in a scenario which included cultivation, harvesting, fat extraction with hexane and transesterification. Bauer et al., (2016) investigated the variation in GHG emissions for low and high N_2O emissions scenarios, reporting GHG emissions of 41.36

and 51.7 g CO2eq MJ^{-1}, respectively. Some references report LCA taking into account microalgal culture using nutrients from wastewater and centrate (Udom et al., 2013; Rothermel et al., 2013). Other works report on LCA studies which considered microalgae grown on recovered CO_2 from flue gas and combustion (Collet et al., 2011; Shimako et al., 2016). Zaimes et al., (2013) carried out a wide comprehensive life cycle assessment (LCA) for multiple microalgal biomass production pathways, consisting of a combination of cultivation and harvesting options in different locations. Ventura et al., (2013) assessed the energy input and the CO_2 emission for four scenarios of microalgae bioenergy production: biodiesel production, biodiesel with an anaerobic digestion system, biogas production and supercritical gasification of algae. The above-mentioned examples give an idea of both the variability of the algae production systems and the complexity and differentiation of the systems in which the algae facilities can be assessed in LCA studies. Within this framework, a valuable methodological effort was contributed by Bradley et al., (2016) to outline a harmonised approach in order to counterbalance the existing high flexibility of defining the boundary conditions and functional units in microalgae LCA studies. The study was intended to provide a framework within which comparisons could be feasible. Approaching mixotrophic cultivation of microalgae, Adesanya et al., (2014) considered in their sensitivity analysis of biodiesel production, the mixotrophic cultivation of *Chlorella vulgaris* grown on glucose compared with the reference scenario of autotrophic culturing. They demonstrated significant savings in GWP (Global Warming Potential) and FER (Fossil-Energy Requirements), which they attributed to the highly dense cultures causing less energy demand in algae processing. The same work underlined the need for research on low-cost carbon substrates coming from industrial and agricultural wastes in order to carry out economically viable mixotrophic cultivation. So as to support this approach with new data, in the effort of compare microalgae cultivation scenarios, this work describes an LCA study that highlights the environmental benefits to be obtained while producing algae under mixotrophic conditions by recovering both C (cheese whey) and N (digestate derived-N).

4.2. Methods and tools

4.2.1 Goals and scope definition

The objective of this work was to evaluate the environmental impacts of microalgae culture in autotrophy and mixotrophy, including scenarios with and without nutrient (nitrogen and phosphorus) recovery from waste products, and to define the conditions under which the mixotrophic culture offers the best environmental performance. Five different scenarios were considered in the evaluation, considering autotrophy, mixotrophy and nutrient recovery (Table 4.1). This aim determines the system boundaries of the research: i.e., there is no ambition to perform a detailed and exhaustive LCA of algae processing, but to highlight specific differences in the two different methods of culturing, using the primary data on cultivation in autotrophy and mixotrophy. The research questions at the basis of the work are: i. is mixotrophic culturing effective to reduce the environmental impacts of algae production? ii. Within which boundaries? iii. Which points need to be enhanced?

Table 4.1. Resume of the scenarios considered in the LCA

| Scenario	Description and assumption
1. Autotrophic growth	Microalgae were grown using autotrophic metabolism by using chemical nitrogen supply (nitrogen fertilizer at global market price).
2. Mixotrophic growth on carbon by-products	Mixotrophic growth of microalgae on cheese whey as carbon source. The microalgae use the carbon of the cheese whey to grow. The COD value of the cheese whey is 147.000 mg L^{-1}, as reported by Salati et al., 2017. Chemical nitrogen is used (nitrogen fertilizer at global market price). Economic allocation of environmental burdens of milk was used. The yield of cheese on milk, for hard cheese is around 7%, cheese whey gross price was assumed equal to 18 € Mg^{-1} (average market value), gross price of milk 300 € Mg^{-1}, finally gross price of produced cheese 4,080 € Mg^{-1}. The cheese whey was considered transported from cheese factory to the algae plant, a distance of around 10 km. This distance was calculated according to the density of cheese

	factories in northern Italy, and is the distance allowing at least 6-8 factories to provide the cheese whey to the algae facility.
3. Mixotrophic growth on cheese wastewaters	Mixotrophic growth of microalgae on cheese factory wastewater (washing waters) as carbon source. The COD value of the wastewater is in the range of 4000-5000 mg L^{-1}. No economic value was attributed to cheese wastewaters and the microalgae culture was assumed to be performed close to the cheese factory site, i.e. no transportation was considered.Nitrogen was supplied by using chemical nitrogen supply (nitrogen fertilizer at global market).
4. Autotrophic growth on recovered nitrogen	Microalgae were grown with autotrophic metabolism by using recovered nitrogen, i.e. a proper amount of digestate was added to the culture medium. No economic value was attributed to this material. Transportation was considered within 10 km, the distance is likely in high density livestock areas with many anaerobic digestion plants such as Lombardy region (400 plants in 2013, Manenti et al., 2016). Ammonia emission considered (5.5 g referred to FU), according to Woertz et al., 2009.
5. Mixotrophic growth on cheese factory wastewaters and recovered nitrogen	Mixotrophic growth of microalgae on cheese wastewater by using recovered nitrogen (digestate). No economic value was attributed to wastewaters and the microalgae growth was performed close to the cheese factory site, i.e. no transportation needed. No economic value was attributed to cheese wastewater. Transport was considered within 10 km. Ammonia emission considered (6 g referred to FU), according to Woertz et al., 2009.

4.2.2 System boundaries

The system considered in the LCA includes the inputs and outputs of material and energy for the production step of algae growing (i.e. the management of reactors) (Fig. 4.1). Production steps considered in this work were the cultivation of microalgae in reactors and the transport of input materials to the algae facility, i.e. standard market transport for fertilizers and distance within 10 km for the transport of both the carbon source used for mixotrophy and the recovered nutrients (digestate). The recovered nutrients are assumed to come from digestate (Table 4.1), a material derived from the anaerobic digestion of slurry, containing nitrogen and phosphorus in form and amounts which are suitable for microalgae growth (Ledda et al., 2015a). The distance for the

cheese whey and digestate transport to the algae factory, i.e. 10 km, is a likely average distance for the considered location (see Table 4.1, Scenarios 2 and 4 for further details). Figure 4.1 describes the process included in the analysis. This distance was calculated according to the density of cheese factories in northern Italy, and it represents the distance allowing at least 6-8 factories to provide the cheese whey to the potential algae facility. The capital goods needed for production were excluded, since previous LCA studies have shown that the impact due to algae infrastructure is negligible compared to the other system processes (Clarens et al., 2010).
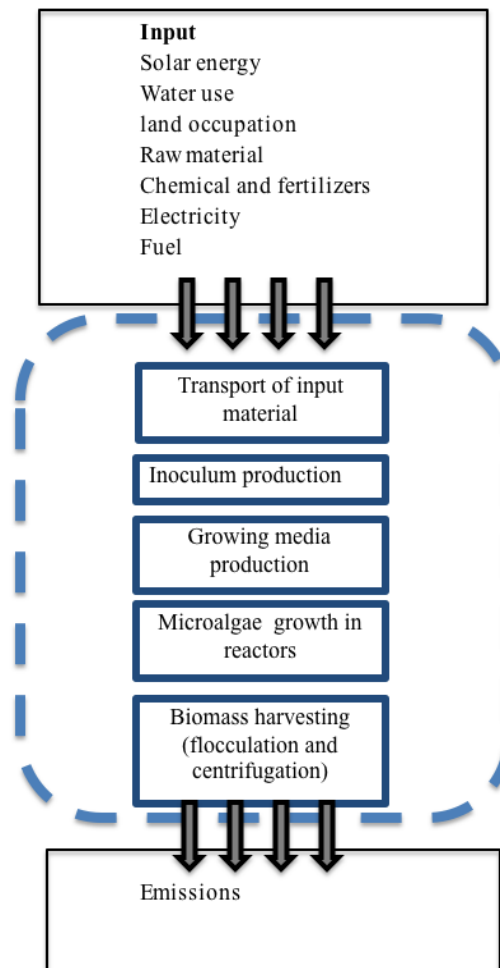
Figure 4.1. Systems boundaries

4.2.3 Functional unit

The functional unit (FU) provides the reference to which all data in the assessment were normalized. The mass-based functional unit is the most commonly used method in LCA studies of agricultural systems (Bacenetti et al., 2015; Yan et al., 2015); in this study the algae production was intended for food - and not for energy - therefore one kg of produced *Chlorella* biomass (dry weight) after algae cultivation and harvesting, was chosen as the functional unit. Smetana et al., (2017) provided data on GWP referred to unit of mass and unit of protein, but a lot of studies on microalgae cultivation up to now have focused on producing biodiesel; these studies proposed the energy unit (MJ) (microalgae enthalpy) to express GHG value. In this work some comparisons were provided in section 2, i.e. Results and Discussion, using $CO_{2eq.}$ referred to MJ, according to the biomass enthalpy of 19.8 MJ kg^{-1} measured on the biomass (Salati et al., 2017).

4.2.4 Inventory analysis

All the information related to the nutrients used, the carbon added for mixotrophic growth, the productivity obtained, the water consumption, and all the factors associated with the production of microalgae biomass, were obtained from previously performed direct measurements (Salati et al., 2017). Table 4.2 lists the main data used for the calculations and their sources. Algal composition and growth rates were derived from laboratory trials comparing autotrophic and mixotrophic growth of *Chlorella vulgaris* by using cheese whey as the carbon source (Salati et al., 2017). Production yields based on carbon, light energy supplied, biomass composition, water and nutrient demand, and data on quality of discharged water, came from continuous trials performed for five

weeks (Salati et al., 2017). Microalgae photosynthetic yields applied to full-scale solar insolation values averaged over a 10-year period which were obtained from ENEA (ENEA, 2017), and the corresponding surface required was calculated. Cultivation of the freshwater algae strain *Chlorella vulgaris* was modelled in a virtual algae production facility of 10 Ha, in which 60% of the surface was for solar harvesting (open ponds) and the rest for infrastructure-related demands. Nitrogen was supplied to the culture medium according to the nitrogen content in the biomass (Salati et al., 2017); N uptake efficiency was considered equal to 90%. Nitrogen emissions associated with algae cultivation were computed according to Woertz et al., (2009) for ammonia, when digestate was provided, while nitrous oxide emissions were computed according to Fagerstone et al., (2011) and Mezzarie et al., (2013). In Table 4.1, the different scenarios (Scenario 1 to 5) considered and related assumptions are summarized. Finally, cheese whey is a milk-derived product, having a specific market allocation and value, thus it cannot be considered cost burden-free, so the environmental burdens associated with the production of the carbon source used for mixotrophic algae growth (cheese whey) were allocated according to economic value, i.e. in proportion to the economic value of the products (ISO 14041) between cheese and cheese whey. The underlying concept is that the total amount of cheese whey coming from cheese production is high but has low economic value, since cheese is the main product in terms of value. The allocation of burdens according to mass relations is not significant. The yield of cheese from milk, for hard cheese, is around 7%; cheese whey gross price is 18 € Mg^{-1}, milk 300 € Mg^{-1}, finally the gross price of produced cheese is 4,080 € Mg^{-1} (average values in 2016), thus the resulting allocation factors of environmental burdens of 6% and 94% were calculated for cheese whey and cheese respectively. Previous studies approaching

cheese and milk LCA used economic allocation of environmental burdens (Berlin, 2002). In Scenario 3 the C-source economic value was considered equal to zero as it referred to cheese factory wastewater. Background and foreground data come from Ecoinvent v. 3.3 (Wernet et al., 2016).

Table 4.2. Data used for calculation: literature source are indicated to outline primary data, references and assumption.

Parameter	Unit	Autotrophic culture	Mixotrophic culture	Data source
Incident energy considered for the scenario	MJ m^{-2} year^{-1}	5040	5040	Incident average energy in mediterranean location
Rate of light energy conversion into biomass (on energy base) based on average PAR	%	8	14	Primary data, Salati et al., 2017
Rate of organic carbon conversion into algae biomass (on energy base)	%		32	Primary data, Salati et al., 2017
PAR average value of incident light	%	48%	48%	Escobedo et al., 2011
Algae concentration in medium	g L^{-1}	1.2	2.6	Primary data, Salati et al., 2017
Algae µ	d^{-1}	0.2	0.2	Primary data, Salati et al., 2017
N input	g kg^{-1} algae	78.1	83.6	Primary data, Salati et al., 2017
P input	g kg^{-1} algae	13.4	15.7	Primary data, Salati et al., 2017
Organic C input	g kg^{-1} algae	0	850	Primary data, Salati et al., 2017
CO_2	kg kg^{-1} algae	2.2	2.2	Putt et al., 2011. Waste CO2 considered as cost burden free.

|				Assumption
Distance for carbon transport	km	10	10	Assumption
Distance for digestate transport	km	10	10	Assumption
Flocculants iron sulphate	kg kg algae^{-1}	0.00728	0.00207	
Water demand	m^3 kg algae^{-1}	0.83	0.39	Primary data, Salati et al., 2017
Soil occupation	m^2 kg algae^{-1}	0.23	0.13	Primary data, Salati et al., 2017
Land use change		Assumption that the land use has changed from scrub land to industrial area		
Energy demand for mixing and CO_2 injection	MJ kg^{-1} algae	15.6	7.2	Medium value according to the reference Acien et al., 2013.
Nitrogen released in water	g kg^{-1} algae	7.1	7.6	Primary data, Salati et al., 2017
Phosphorus released in water	g kg^{-1} algae	1.25	1.59	Primary data, Salati et al., 2017
Organic carbon released in water (TOC)	g kg^{-1} algae	35	98	Primary data, Salati et al., 2017
N_2O released in atmosphere	Kg kg^{-1} algae	0.00156	0	Fagerstone et al., 2011, Mezzari et al., 2013
NH_3 released in atmosphere	g kg^{-1} algae	0	0	Different value used for scenario 4 and 5 according to Woertz et al., (2009)

4.2.5 Impact assessment

In the Life Cycle Impact Assessment (LCIA) phase, emissions and resource data identified during the LCI (Life Cycle Inventory), are sorted into classes according to the effects they have on the environment and these are translated into indicators that reflect environmental pressures and resource scarcity. A cause-effect pathway links the relationship between the emission of a chemical and its potential effects. Analysis was performed using the ReCipe 2008 method (Goedkoop et al., 2009). The method addresses eighteen impact categories at midpoint level: Climate change, ozone depletion, terrestrial acidification, freshwater eutrophication, marine eutrophication, human toxicity, photochemical oxidant formation, particulate matter formation, terrestrial ecotoxicity, freshwater ecotoxicity, marine ecotoxicity, ionizing radiation, agricultural land occupation, urban land occupation, natural land transformation, water depletion, mineral resource depletion, fossil fuel depletion. All the mid point categories in the ReCipe method were used, in order to assure a complete and robust comparisons of scenarios. The software SimaPro was used for the computational implementation of the inventories (Goedkoop et al., 2010).

4.3. Results and discussion

Environmental impacts for both autotrophic and mixotrophic growth conditions (Scenario 1 and 2) are compared in Figure 4.2. The total GHG emissions for each kg of algae biomass at the industrial unit gate were of 3.06 kg of CO_{2eq} and of 1.92 kg $CO_{2eq.}$ for algae grown under autotrophic and mixotrophic conditions, respectively.
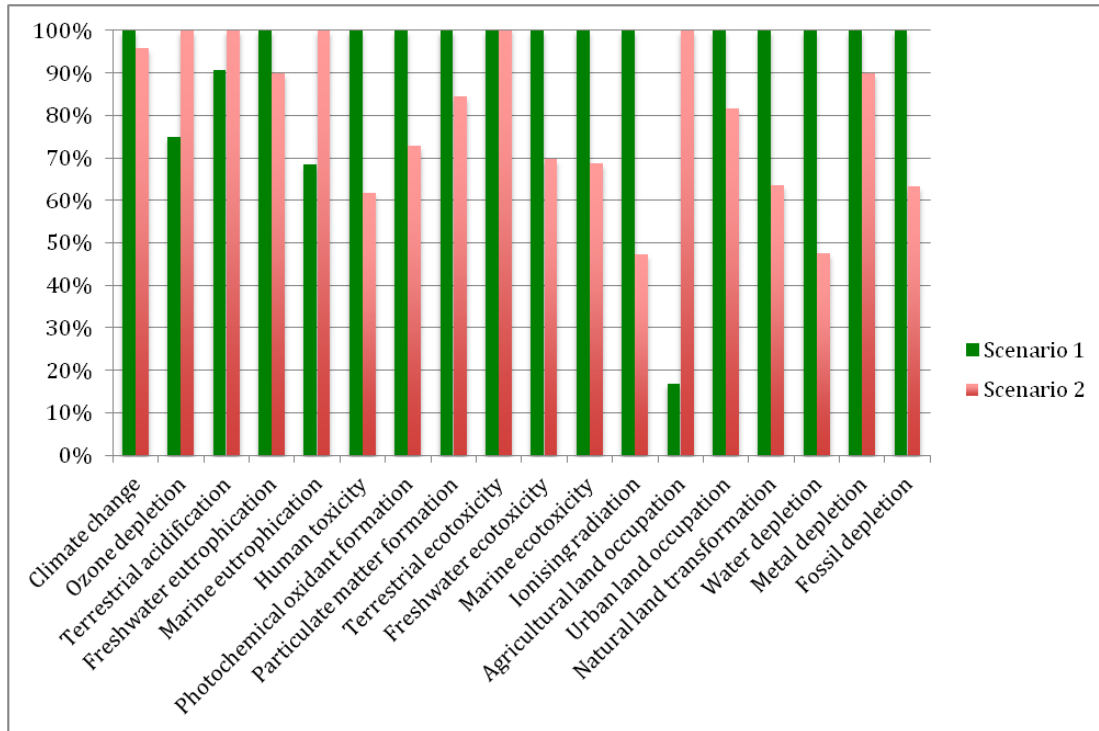
Figure 4.2. Comparison between impact categories of scenario 1 2 (ReCipe 2008). The 100% value represents the highest value reached by a scenario for that impact category. The lower values of the same category are standardized to that higher value to facilitate comparisons.

Mixotrophic culture relies on the efficient conversion of both light energy and chemical energy provided by organic carbon, leading to higher biomass production and overall energy conversion efficiency. The performance of a microalgal culture can be evaluated through the conversion efficiency (EF_t), i.e. the amount of energy supplied to the culture transformed into algae biomass (eq. 1)

$$EF_t = Energy\ in\ biomass\ /\ (energy\ from\ light\ +\ energy\ from\ organic\ carbon) \qquad (1)$$

Salati et al., (2017) reported the total efficiency of the energy transformation (EF_t) into biomass in mixotrophic trials to be 14%, to be compared to 8% efficiency reported for the autotrophic condition. Ren et al., (2014) and Yang (2000), reported EF_t of 14.6% and 18% for mixotrophic conditions, and these data are not far from those reported in

this work. To what extent the increased production determined a better environmental performance depended on the different factors such as the cost burden of the carbon source and the effect that carbon addition had on both environmental emissions and avoided products or impacts of the process. Environmental burdens for milk production were included in the environmental account for algae production, according to the economic value of cheese whey compared with that of milk (see Table 4.1, Scenario 2). The main environmental burdens of cheese whey production are: nutrient runoff, CH_4 emission because of ruminating cattle, emission of ammonia from manure and emission due to the materials used at the farm (such as fertilizers or chemicals). As summarized in Fig. 4.2, there was a decrease of environmental impacts (13 out of 18 indicators decreased) in mixotrophic cultivation. $CO_{2eq.}$ emission decreased by only 4%: on one hand the higher algae productivity decreased the specific $CO_{2eq.}$ emission, but on the other hand the environmental burdens associated with whey production increased: the result is a slight decrease in the final $CO_{2eq.}$ emission. Freshwater eutrophication refers to the increase of nutrients leading to excessive primary productivity and biodiversity losses. In the mixotrophic scenario the impact category decreases due to the higher productivity but at the same time other contributions due to whey production, such as manure managing and field cultivation are accounted for in the results. The net result is a 10% decrease. Human toxicity describes the potential harm of a unit of chemical released into the environment, expressed as 1,4 dichlorobenzene equivalents. This impact category in the scenarios considered is affected mainly by electricity input and nitrogen input, thus the mixotrophic scenario results in a 38% decrease due to the lower input of energy (electricity) for each kg of algae biomass produced. Again, a decrease is recorded in photochemical oxidant formation: this refers to secondary air pollution

formed by the reaction of sunlight with emissions from fossil fuel combustion, expressed as Non-methane Volatile Organic Compounds Equivalent (NMVOCV). In the scenarios considered, the category is affected mainly by electricity input (combustion of fossil fuel), nitrogen input and the use of agricultural machinery burning diesel; the mixotrophic scenario has more diesel burning for agricultural machinery but less input in electricity and N, resulting in a final 27% decrease. Particulate matter formation refers to the emission of NOx, NH_3, SO_2, or primary $PM_{2.5}$ to the atmosphere, followed by atmospheric transformation in the air, expressed as PM10 equivalent. In Scenarios 1 and 2 the main items contributing to this category are: electricity, nitrogen fertilizer and in Scenario 2, traction for the production of feed. The mixotrophic scenario results in a 16% decrease. Ecotoxicity describe the toxicity potential of toxic substances by modelling fate, exposure and effects in soil, freshwater and marine compartments: Terrestrial ecotoxicity remained almost unchanged in the two scenarios, while Freshwater and Marine ecotoxicity were mainly affected by electricity input and nitrogen fertilizer input, decreased by around 30% in mixotrophy. Water depletion takes into account the amount of water consumed from surface water bodies or groundwater. This impact category in the two scenarios is affected mainly by the use and discharge of water of the open ponds used for algae growth. The mixotrophic scenario results in a 52% decrease. Finally, the depletion of non-renewable resources (metal and fossil fuel): as for many other categories the main items contributing are electricity, nitrogen and phosphate fertilizers. The mixotrophic scenario results in a 10% decrease for metal depletion and 37% for fuel. The decreases in the impacts were due to the higher biomass productivity of mixotrophy compared with autotrophy, and thus to the decrease of the contribution of the main production inputs of microalgae (electricity and fertilizers) per

biomass unit weight. Nevertheless, the use of cheese whey increased the other environmental impacts. This was because cheese whey production represents a farm activity, so the mixotrophic approach, which included it as the C source, determined an increase of Agricultural Land Occupation (+496%) in comparison with the autotrophy figures. Terrestrial acidification describes changes in soil chemical properties following the deposition of nutrients (nitrogen and sulfur) in acidifying forms. It assesses the environmental impact of nitrogen oxides (NOx), ammonia (NH_3), and sulfur dioxide (SO_2): in the mixotrophic scenarios an increase of 10% is outlined, due to the agronomic inputs (fertilizers and manure spreading) needed for feed in the production of cheese whey. The same can be said for the Marine Eutrophication category, referring to the amount of N that will end up in coastal waters, causing an increase in primary productivity. A net increase of 46% is outlined in mixotrophy due to N use in feed culturing and its release in freshwater. Finally, also the Ozone Depletion value increases of 33% due to diesel consumption for soil tillage. To better understand within which boundaries mixotrophic culturing was highly effective, cheese factory wastewaters (Scenario 3), i.e. wastewaters derived from factory cleaning containing diluted cheese whey (COD 2-10 g L^{-1}, Carvalho et al., 2013) instead of cheese whey, were considered. Cheese wastewaters could be proposed for growing algae, avoiding the need for the wastewater depuration process and/or its transportation for discharge. In this case, as wastewater has no economic value, the environmental burdens of the organic carbon provided to the microalgae were not taken into account in the microalgae production and were considered to be entirely accounted for in the cheese production process (as waste treatment). As expected, this scenario (Scenario 3) showed a significant decrease of all the environmental impacts considered (Table 4.3): CO_2 emission decreased by

37% in comparison with autotrophy, resulting in 1.93 kg kg^{-1} algae biomass. Again, the higher yield in biomass production than under autotrophic conditions reduced the Land Use (natural and agricultural) by 38% and 24% respectively, and the Water Depletion by 52%. These results highlight that the best environmental performance in producing algae biomass consisted in recovering dilute C-rich waste streams (cheese wastewater) which have no economic value and so they have no alternative uses. The contribution of the production inputs to the total environmental impacts was outlined for Scenarios 1 and 3 in Figs. 4.3 and 4.4, respectively. Electricity and nitrogen supplies were the main production factors and the main contributors to the Eco-indicator values, both in autotrophic and mixotrophic conditions. The management of ponds affects the impact categories of Freshwater Eutrophication and Marine Eutrophication, i.e. nutrients content in the discharged water and the emission of ammonia and N_2O. It is interesting to note that according to Mezzari et al., (2013), the presence of organic carbon in the algae growth medium prevents the production of N_2O, recovering heterotrophic denitrification activity and suppressing N_2O emission. N_2O production has been reported to have a wide range of variation in autotrophic culture according to Dissolved Oxygen (DO) level, i.e. from 0.002% to 0.39% of total nitrogen present in the medium (Fagerstone et al., 2011). The nitrogen used for algae growth contributes significantly to CO_2 emission because of the large amount of energy necessary to produce fertilizers and their transportation (IPCC, (2006); therefore, N input negatively affects all the environmental impact categories (IPCC, 2006). According to previous work (Franchino et al., 2013; Ledda et al., 2015a; Ledda et al., 2015b; Salati et al., 2017) there was an opportunity to substitute for synthetic nitrogen (commercial fertilizers), the nitrogen which can be obtained from animal slurry or digestate, while paying attention to not

exceeding the right amount of ammonia for algal growth (Munoz et al., 2005; Sepúlveda et al., 2015). To highlight the effect of nitrogen recovery needed to produce microalgae biomass, the growing process was modelled assuming that N was provided by digestate (Scenario 4 and 5) according to the previous assumption made regarding N consumed by biomass and N efficiency. The recovery of N from digestate (Scenario 4) and of both N (from digestate) and C (from cheese wastewater) (Scenario 5), led to a strong decrease of all the impacts categories considered in comparison with autotrophy based on using synthetic fertilizers (Fig. 4.5): $CO_{2eq.}$ emissions decreased from 3.07 to 2.26 kg $CO_{2eq.}$ kg^{-1} algae biomass using recovered N, and to 1.05 kg $CO_{2eq.}$ kg^{-1} algae biomass using both recovered N and recovered C for mixotrophy. Different values for $CO_{2eq.}$ emissions for algae grown in autotrophic conditions during the culturing phase are reported in Table 4.4: they are reported as referred to the energy unit of 1 MJ (the energy content of the biomass). The amounts of energy consumption reported were quite variable: in this work, the electricity consumption was modelled conservatively high and this affected the results in term of $CO_{2eq.}$ emission. However, we think that it can be a reliable reference to start ameliorating the economic and environmental efficiency of microalgae production. Other works showed lower $CO_{2eq.}$ emission thanks to the assumption of low electricity consumption achieved by optimized agitation systems, gas exchange and also thanks to nutrients recovery. Results obtained from Scenario 1 of this work ranged within the high values reported in the literature, because of high-energy consumption attributed to the use of commercial fertilizers. The low GHG value for Scenario 5 showed better performance with respect to the reference LCAs, even those which considered low energy consumption and the use of recovered nitrogen (Yuan et al., 2015; Mendeiros et al., 2015). Finally, the data are consistent

with Adesanya et al., (2017): in the sensitivity analysis they found a value of 4.2 kg $CO_{2eq.}$ for each kg of biomass in the hybrid autotrophic cultivation system and 1.7 kg $CO_{2eq.}$ for each kg of biomass when cultivating microalgae in mixotrophic conditions.
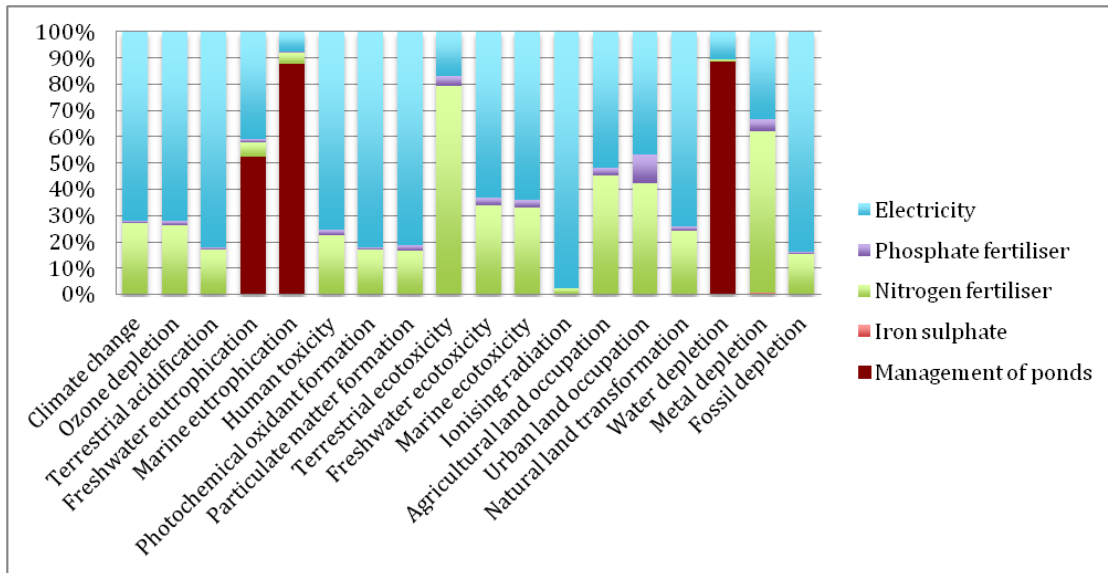
Figure 4.3. Process contribution to impacts categories in autotrophic culture scenario 1.
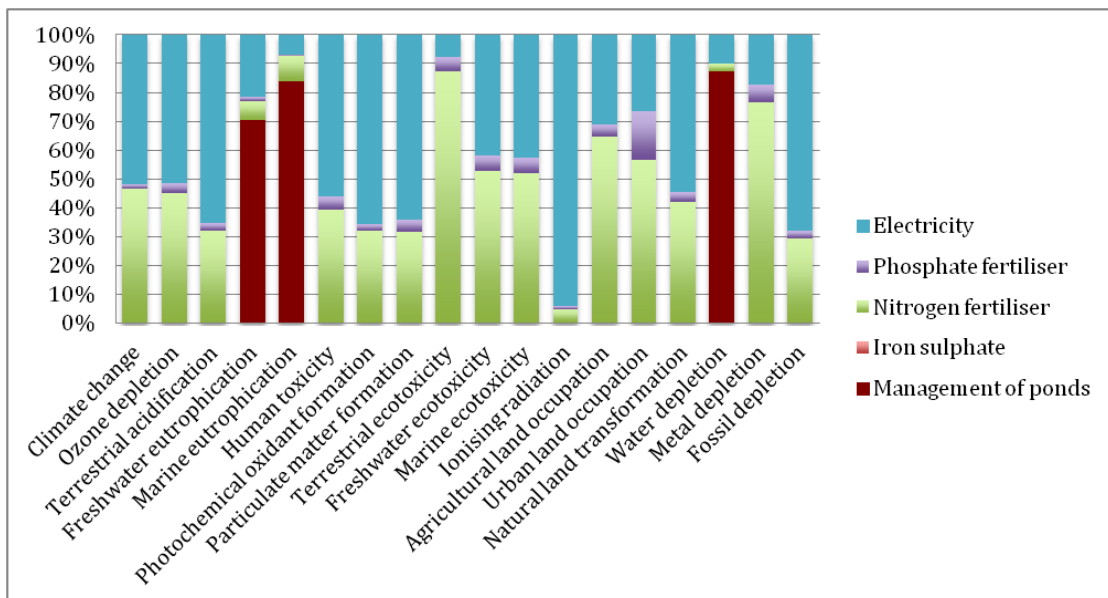
Figure 4.4. Process contribution to impacts categories in mixotrophic culture scenario 3.
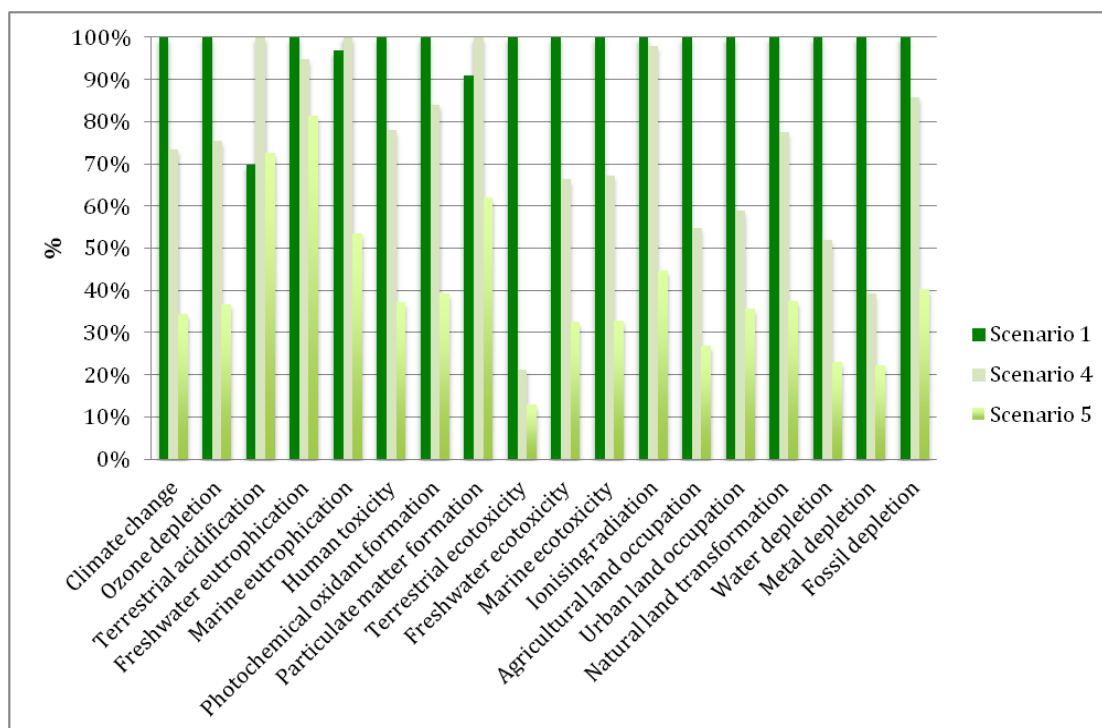
Figure 4.5. Comparison of all the scenarios (2-3-4-5) respect to the control (scenario 1).

Table 4.3. Characterization at the midpoint level Recipe (H) for Scenario 1 2 3 4 and 5

Impact category	Unit	Scenario 1	Scenario 2	Scenario 3	Scenario 4	Scenario 5
Climate change	kg CO_2eq	3.07	1.93	1.93	2.26	1.05
Ozone depletion	kg CFC-11 eq	0.0000002	0.0000001	0.000000	0.000000	0.000000
Terrestrial acidification	kg SO_2eq	0.02	0.01	0.01	0.03	0.02
Freshwater eutrophication	kg P eq	0.0023	0.0020	0.0020	0.0022	0.0019
Marine eutrophication	kg N eq	0.007	0.003	0.003	0.007	0.004
Human toxicity	kg 1,4-DB eq	0.93	0.57	0.57	0.73	0.35
Photochemical oxidant formation	kg NMVOC	0.01	0.01	0.01	0.01	0.00
Particulate matter formation	kg PM10 eq	0.007	0.004	0.00	0.01	0.00

Terrestrial ecotoxicity	kg 1,4-DB eq	0.0003	0.0003	0.00034	0.00007	0.00004
Freshwater ecotoxicity	kg 1,4-DB eq	0.03	0.02	0.02	0.02	0.01
Marine ecotoxicity	kg 1,4-DB eq	0.03	0.02	0.02	0.02	0.01
Ionising radiation	kBq U235 eq	1.27	0.59	0.59	1.24	0.57
Agricultural land occupation	m^2a	0.09	0.07	0.07	0.05	0.02
Urban land occupation	m^2a	0.02	0.02	0.0162	0.0119	0.0072
Natural land transformation	m^2	0.0004	0.0003	0.0003	0.0003	0.0002
Water depletion	m^3	0.94	0.45	0.45	0.49	0.22
Metal depletion	kg Fe eq	0.08	0.07	0.07	0.03	0.02
Fossil fuel depletion	kg oil eq	0.73	0.41	0.41	0.63	0.30

Table 4.4. GHG emission for algae culturing: this work and literature data.

GHG (g CO_2 eq MJ^{-1})	Electricity (MJ kg^{-1} biomass)	N Supply	CO_2 supply	N_2O emission	References
70	3.41	Recovered	Recovered	Not considered	Yuan 2015
50-140[a]	4.0	Commercial fertilizers	Recovered	Not considered	Zaimes (2013)
41.3		-	Recovered	Not considered	Connelly (2015)
51.7		-	Recovered	Considered	Bauer (2016).
32.9	1.79	Recycle	Recovered	Not considered	Liu (2013).
	0.83	Recovered	Recovered	Not considered	Collet (2011).
124	3.8	Recovered	Recovered	Not considered	Mendeiros et al., (2015)
63	3.8	Recovered	Recovered	Not considered	Mendeiros et al., (2015)

155	15.6	Commercial fertilizers	Recovered	Considered	This work Scenario 1
149	7.2	Commercial fertilizers	Recovered	Considered	This work Scenario 2
97	7.2	Commercial fertilizers	Recovered	Considered	This work Scenario 3
114	15.6	Recovered	Recovered	Considered	This work Scenario 4
53	7.2	Recovered	Recovered	Considered	This work Scenario 5

[a]Different location considered, value modified from NET GWP (CO_2 in biomass subtracted from the total GWP) into GWP of production.

4.4. Conclusion

The Life Cycle Analysis showed that in mixotrophic growth of microalgae particularly when the recovered carbon had no alternative use, (so that it could be considered cost burden-free, as in Scenarios 3 and 5) environmental impacts categories were largely reduced. Electricity and nitrogen supplies represented the production inputs causing the main environmental impacts of mixotrophic microalgae cultivation. The cultivation of microalgae on recovered nitrogen and carbon decreased CO_2 emissions by almost 60% and lowered the other Eco-indicators in comparison with autotrophy.

Acknowledgments

5. Organic wastes as alternative to CO_2 for producing mixotrophic microalgae enhancing lipid production.

Veronesi D., D'Imporzano G., Menin B., Salati S., Adani F.

In this work, white wine lees (WWL), cheese whey (CW) and glycerol (GLY) were used as carbon (C) sources to mixotrophically support the production of the microalga Nannochloropsis salina, replacing CO_2 supply. By doing so algae was allowed to grow on C sources dosed at 2 g L^{-1}, 3 g L^{-1} and 4 g L^{-1} of C, in the presence and absence of CO_2 supply. WWL and CW were not able to support algae growth due to a fungal contamination that was genomically identified, while GLY gave interesting results in particular with 3 g L^{-1} of C. GLY-C was able replacing CO_2-C completely when this latter was omitted, showing an algal biomass production similar to those obtained in autotrophy. If CO_2-C was provided jointly with GLY-C, biomass production and lipids content increased more than 30% and 23% respectively, compared to autotrophy.

5.1. Introduction

Nowadays, the importance that microalgae could play in several sectors such as agriculture, animal feeding, green chemistry, nutraceutics, pharmaceutics and bioenergy production, it is increasingly recognized (Pulz and Gross 2004). To growth and reproduce, these microorganisms require an energy source (light), a carbon source (CO2 for autotrophy microalgae) and a growth medium (water plus nutrients) (Blair et al., 2014). This is the common modality of algae cultivation, i.e. photoautotrophy, in which

algal cells use light energy to extract protons and electrons from water, reducing CO_2 to organic molecules. Photoautotrophic cultivation is limited by high production costs. In particular, nutrients and CO_2 supplies are the major production costs in a full-scale microalgae production plant (Acién et al., 2012). To reduce the high production costs, several authors suggested the use of wastewaters as culture medium (Park et al., 2010; Ledda et al., 2015). Some other studies suggested, as feasible alternative to the photoautotrophic cultivation, the exploitation of the ability of some algae strains to grow under mixotrophic conditions using raw materials rich in carbon (C) and nutrients (Xiong et al., 2010; Wan et al., 2011). Mixotrophic cultivation is the mode by which microalgae can drive both photoautotrophy and heterotrophy and can utilize both inorganic and organic carbon sources (Kang et al., 2004). Inorganic carbon is fixed through photosynthesis which is influenced by illumination conditions, while organic compounds are assimilated through aerobic respiration, which is affected by the availability of organic carbon (Hu et al., 2008). Some scientists suggested that the specific growth rate of microalgae under mixotrophic cultivation is approximately the sum of those under photoautotrophic and heterotrophic modes (Marquez et al., 1993) whereas others believed that the specific growth rate in mixotrophy is not the simple combination of the two modes (Vonshak et al., 2000; Yu et al., 2009). The same authors indicated that the two metabolic processes (i.e., photosynthesis for photoautotrophy and aerobic respiration for heterotrophy) affect each other under mixotrophic cultivation, determining a synergistic effect that enhances biomass productivity. The flexibility of some algal strains to switch their C nutritional mode based on substrate availability and light conditions is one of their inherent evolutionary advantages (Venkata Mohan et al., 2014). Therefore, mixotrophy represents a good solution to obtain both high biomass

and high value compounds production, recovering C-rich wastes (Ogbonna et al., 2000; Yang et al., 2000). In addition, the ability of some algae to recover nutrients (N and P) growing on wastewaters (Salati et al., 2017), could improve both economic and environmental sustainability of the process. To accomplish viable and cost-effective results, the cheapest carbon enrichment to microalgal medium should be adopted.

The present study focused on the possibility to use alternative carbon sources for the mixotrophic growth of Nannochloropsis salina, instead of the usual CO2 supply to support microalgae production, reusing and valorising different organic carbon wastes, monitoring the effects on growth and biomass composition of microalgal cultures, grown with and without CO2 feeding.

5.2. Materials and methods

5.2.1 Organism and medium

Nannochloropsis salina - strain SAG 40.85 - was acquired from Sammlung von Algenkulturen, Pflanzenphysiologisches Institut (Universität Göttingen, Germany). The cultures were grown in f/2 medium (Guillard and Ryther 1962; Guillard 1975), which was enriched with 1.875 g $NaNO_3$ l^{-1} and 0.135 g $NaHPO_4$ l^{-1}. Inocula were prepared batch-wise and were grown under aseptic conditions in Erlenmeyer flasks of 150 mL under constant aeration and mixing by using filtered air (filter of 0.2 μm) with a continuous illumination of 50 μE m^{-2} s^{-1} provided by fluorescent white tubes, at a controlled temperature of $22 \pm 1°C$.

5.2.2 Organic carbon sources

Three different C-waste streams were selected: white wine lees (WWL), glycerol (GLY) and cheese whey (CW). WWL were derived from a wine cooperative in Corte Franca (BS), Italy. GLY was obtained from a local biogas plant that uses crude glycerol as co-substrate for biogas production. CW was sampled from an agro-food industry in northern Italy and successively stored at -20°C. Before being used, CW was subjected to a deproteinization performed by heat treatment at 115 °C for 15 min (Dragone et al., 2011) and successive filtration using 0.2 μm filters. Thereafter, CW was hydrolyzed by using β-galactosidase (Sigma-Aldrich, San Luis, Missouri, USA) from *Aspergillus oryzae*. Enzymatic hydrolysis was performed at 30 °C and at pH of 4.5 for 24 h (Abreu et al., 2012) in a shake flask at 200 rpm using 65 U of enzyme per grams of lactose quantified in whey permeate, such as reported by Espinosa-Gonzalez et al., (2014). Before their use, all selected carbon sources were chemical characterized in terms of pH, dry weight (DW), chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN) and ammonium nitrogen (N-NH$_4^+$), according to the methods reported for wastewater sludges (IRSA CNR 1994).

5.2.3 Experimental procedures

Nannochloropsis salina SAG 40.85 was grown in batch mode in bubble columns photobioreactors (PBRs) of 0.045 m internal diameter, 0.5 m height with a working volume of 0.5 L. After 15 days of batch culture, C-substrates were added when all the cultures were in exponential phase. Cultures were incubated at 20°C, air-aerated with a flow rate of 5 L min^{-1} and illuminated with a 12:12 light photoperiod with an irradiance of 260 μmol m^{-2} s^{-1}. Cultivation system was equipped with an automatic control of the

pH, which remained constant at 8 ± 0.3, by using pure CO_2 injection dosed "on demand" according to pH value. Screening tests were carried out in order to check if *N. salina* was able to mixotrophically grown using all the selected streams, testing them one-by-one at different carbon concentration, as in the following reported: 5.1 g L^{-1}, 7.7 g L^{-1} and 10.2 g L^{-1} of GLY (2 g L^{-1}, 3 g L^{-1} and 4 g L^{-1} of carbon); 29.4 mL L^{-1}, 44.1 mL L^{-1} and 58.58 mL L^{-1} of WWL (2 g L^{-1}, 3 g L^{-1} and 4 g L^{-1} of carbon); 36.5 mL L^{-1}, 54.8 mL L^{-1} and 73 mL L^{-1} of CW (2 g L^{-1}, 3 g L^{-1} and 4 g L^{-1} of carbon). Experiments were carried out in triplicate with a control trial that was performed by cultivating algae under autotrophic condition. Results from the screening tests were used in order to study the effect of CO_2 supply and deprivation on growth and biomass composition of *N. salina*.

5.2.4 Analytical methods

Microalgae growth was determined measuring the DW by sampling 5 mL of algae culture from each PBRs every two days. Algae suspension was then centrifuged at 4,000 rpm for 15 minutes. The supernatant obtained was then used for nutrients uptake evaluation by using a spectrophotometric analytical kit (NANNOCOLOR®, Macherey-Nagel, Germany). The algae pellet was washed twice with an equivalent volume of distilled water (in order to avoid any overestimation caused by salt) and then filtered by using pre-weighed Whatman GFC filter of 1.2 μm, previously dried at 65°C overnight. The lipid content of freeze-dry biomass was gravimetrically determined after subsequent solvent extraction (chloroform: methanol 2:1 v/v) and centrifugation of the solution this latter obtained by using a rotary evaporator (Büchi R110, Büchi Labortechnik AG., Flawil, Switzerland). Crude protein content was measured by

determination of total nitrogen by Kjeldahl method. Total nitrogen value was converted into proteins multiplying by specie-specific nitrogen-to-proteins conversion factors of 4.87 for *Nannochloropsis salina* (Templeton et al., 2015). Carbohydrate content was determined by the slightly modified phenol-sulphuric acid method of DuBois et al., (1956). Briefly, an aliquot of freeze-dry sample was treated with 5 ml of concentrated sulphuric acid (98 % w/w) and 1 ml of phenol (6%, w/v). Samples were cooled to room temperature and the absorbance of the final blend was measured at 490 nm by a Jeneway 7305 UV-visible spectrophotometer; then carbohydrates were quantified by comparative estimation with a calibration curve obtained using glucose.

5.2.5 Contaminant identification

Additionally to microscopic examination, a molecular analysis was performed to identify the fungal contaminant mycelium. Genomic DNA was extracted and purified from 250 mg of freeze-dry fungal mycelium using the NucleoSpin® Soil (Macherey-Nagel, Düren, Deutschland) extraction kit, following manufacturer's instructions. DNA quantity and quality were assessed by means of NanoDrop 1000 Spectrophotometer. Fungal-specific PCR primers developed for analysis of the ITS region were used for the DNA amplification: the forward primer ITS1f CTTGGTCATTTAGAGGAAGTAA and the reverse primer ITS2 GCTGCGTTCTTCATCGATGC. PCR amplification was performed in a total volume of 25 µL containing 1.5 µL of total genomic DNA normalized at 5ng/ml, 0.25 µL of forward and reverse primer at 10µM 2X GoTaq® Hot Start Colorless Master Mix (Promega, Fitchburg, United States) containing GoTaq® Hot Start DNA Polymerase supplied in 2X Colorless GoTaq® Reaction Buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 4mM MgCl2.

Amplification was run in a GeneAmp PCR system 2700 (Applied Biosystems, Massachusetts, USA) as follows: 1 cycle of 3 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 58 °C and a 30 s extension step at 72 °C plus final extension of 5 minutes at 72°C. The PCR products were analyzed by electrophoresis on a 1.5% (w/v) agarose gel stained with 2 µl of Midori Green DNA Stain solution (Nippon Genetics Europe GmbH, Düren; Germany). Expected band size of 230 bp was sequenced by Sanger sequencing on both strands and manually checked.

5.2.6 Statistical analysis

All statistical analyses were performed using analysis of variance (ANOVA) with the Tukey test used to compare means (SPSS statistical software, SPSS Chicago IL).

5.3. Results and Discussion

5.3.1 Effect of carbon wastes on growth and molecular identification of the contaminant

Preliminary results shown that *N. salina* was unable to growth mixotrophically on WWL and CW: algal cells did not consume the organic C provided and so that culture was affected by a fungal contamination. From previous work (Salati et al., 2017) a *Chlorella sp.* was able to use CW and WWL with good growth performance producing local proteins, while the strain used in this work didn't show the ability to growth on the same C-wastes, which means that the capacity to growth on particular C-source it's species-specific. In order to identify the contaminant a molecular analysis was performed. The consensus ITS sequence obtained as described in paragraph 5.2.5, was aligned and compared with published reference strains using the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information

(http://blast.ncbi.nlm.nih.gov). The alignment between the query and reference sequences showed the best match (97% of identity) with a *Fusarium sp.* SSU rRNA sequence. *Fusarium sp.* is recognized as a typical contaminant of marine culture (Palmero et al., 2009). Regarding GLY substrate, it was assimilated by algal cells at all the concentrations tested, allowed for higher biomass production than that under autotrophic condition. Table 5.1 reports some works on the mixotrophic growth of *Nannochloropsis* strain, and all have worked only with simple carbon compounds such as glucose, glycerol, sodium acetate and ethanol (Sforza et al., 2012; Xu et al., 2004; Xu et al., 2004; Cheirsilp and Torpee, 2012; Das et al., 2011; Gim et al., 2016; Hu et al., 2003). No data are available about the possibility to use more complex substrates to support the mixotrophic growth of this strain. At the same time, none of the works selected have take into consideration the possibility to substitute CO_2 with an organic carbon source. It was therefore decided to investigate the effect of GLY on the growth of the selected alga as an alternative C source to CO_2.

5.3.2 Mixotrophic growth on glycerol without CO_2 supply

Based on the results obtained in previous trials, it was decided to start the PBRs culture in autotrophy with CO_2 supply provided during the exponential growth phase until the 15th day, i.e. when organic carbon was added at different concentration (section 5.2.3) and CO_2-feeding was stopped in mixotrophic PBRs. Figure 5.1a showed that biomass productions obtained in mixotrophic PBRs trials was similar to that obtained under autotrophic condition or slightly lower. The best concentration of GLY was 3 g L^{-1} of carbon with which *N. salina* has reached a maximum biomass-DW similar to that obtained in autotrophic conditions. i.e. biomass DW of 4.20 ± 0.17 g L^{-1} and of 4.30 ±

0.08 g L^{-1} for autotrophy and mixotrophy, respectively (Table 5.1). Productivity values were also very similar for different PBRs, i.e. 0.16 ± 0.01 g DW L^{-1} d^{-1} for autotrophy as well as mixotrophy with 2 g L^{-1} and 3 g L^{-1} of carbon, while the trial with 4 g L^{-1} of C had a productivity of 0.14 ± 0.01 g DW L^{-1} d^{-1}. These values are in according with Gim et al., 2016 that found a daily biomass productivity of 0.17 g L^{-1} d^{-1} in mixotrophic conditions. Both biomass DWs produced and daily biomass productivity measured for the trial performed under mixotrophy condition and fed with 4 g L^{-1} of carbon, were slightly lower than correspondent values measured for autotrophy trials. However, as highlighted in Figure 5.1b, organic carbon was completely absorbed by *Nannochloropsis salina* at all concentrations provided. These results suggested that mixotrophic metabolism was activated and that glycerol was used as carbon source for algae growth instead of CO_2.

5.3.3 Mixotrophic growth on glycerol with CO_2 supply

In this second experiment, glycerol was added to the media of the mixotrophic PBRs on day 15 while continuing CO_2 supply. All mixotrophic trials showed higher biomass production than that for autotrophic control (Figure 5.1c). The best performance was obtained for the trial performed by adding 3 g L^{-1} of GLY-C with a biomass DW of 4.40 ± 0.21 g L^{-1} and 6.59 ± 0.32 g L^{-1} for autotrophy and mixotrophy, respectively (Table 5.1). Daily biomass productivities were higher for all the mixotrophic PBRs than those obtained under autotrophy condition (Table 5.1). Both algal biomass DWs and productivities were higher than those of trials without CO_2 supply. This is likely due to the dual metabolisms, i.e. photosynthesis plus aerobic respiration of carbon substrates, which may have had a synergistic effect enhancing biomass production (Park et al.,

2012; Vonshak et al., 2013). Again, glycerol was completely used for all the C concentrations considered after 12 days (Figure 5.1d). By comparing carbon uptake (Figures 5.1b and 5.1d), it was clear that when CO_2 was supplied, the carbon absorption (glycerol-C) was faster than when CO_2 was not supplied. Interesting was the fact that more than half of the total carbon dosed was consumed after only 7 days in trials with GLY and CO_2. This fact can be explained by considering the higher growth rate of algal cells when both photoautotrophic and heterotrophic metabolisms are efficiently performed simultaneously (Wan et al., 2011). Comparing data obtained in this work with other similar studies (Table 5.1) it's easily to note that we have obtained higher performance in terms of biomass production (DW). This fact could be ascribed to the higher light intensity used in this work i.e. 260 µmol m^{-2} s^{-1}, that is almost two folds higher by the maximum value reported, used by Das et al., 2011. In addition none of the authors reported in Table 5.1, use a system for CO_2 supply based on the microalgal demand according to pH value, which is the better system to support the photosynthetic activity of microalgal cells minimizing any leak of CO_2.

Table 5.1. Culture conditions and growth results of *Nannochloropsis* strain obtained in this work, in comparison with literature data.

Microalgae strain	Trophic way	Carbon source	Carbon amount (g L^{-1})	Cultivation system	CO$_2$ supply	Light intensity (µmol m^{-2} s^{-1})	Productivity (g L^{-1} d^{-1})	DW (g L^{-1})	Lipid (% DW)	References
Nannochloropsis salina	*Mixotrophy*	Glycerol	10	Batch	Air flow	100 (Continuous)	-	0.43	-	Sforza et al., 2012
	Autotrophy	-	-	Batch	5% (v/v)			2.53		
	Mixotrophy	Glycerol	10	Semibatch				2.1		
	Autotrophy	-	-	Semibatch	5% (v/v) light - 0% night	100 (12:12 h day-night)		1.3		
	Mixotrophy	Glycerol	10	Semibatch				2.05	50	
Nannochloropsis sp.	*Autotrophy*	-	-	Batch	Air flow	80 (Continuous)	-	0.8	27	Xu et al., 2004
	Mixotrophy	Glucose	5	Fed batch				1.1	31	
Nannochloropsis salina	*Autotrophy*	-	-	Batch	Not reported	73 (Continuous)	-	0.36	5.6 (EPA % DW)	Xu et al., 2004
		Glucose	5.4					0.51	4.5 (EPA % DW)	
	Mixotrophy	Ethanol	1.4					0.45	-	
		Sodium acetate	2.5					0.32	-	
Nannochloropsis sp.	*Autotrophy*	-	-	Batch	Air flow	42 (16:8 h day-night)	-	0.35	25	Cheirsilp and Torpee, 2012
	Mixotrophy	Glucose	2					1.2	25.5	
			10					3.8	19.3	
Nannochloropsis sp.	*Autotrophy*	-	-	Batch	Air flow	140 (12:12 h day-night)	0.03	0.59	14.74 (FAME % DW)	Das et al.,., 2011
	Mixotrophy	Glucose	2				0.09	0.77	15.00 (FAME % DW)	
	Mixotrophy	Sucrose	2				0.1	0.82	14.76 (FAME % DW)	
	Mixotrophy	Glycerol	2				0.11	0.8	19.06 (FAME % DW)	

Nannochloropsis oculata	*Autotrophy*	-	-	Batch	No	80 (12:12 h day-night)	-	0.54	26.5	Gim et al., 2016
	Mixotrophy	Glucose	3.6				0.17	1.69	37.3	
Nannochloropsis sp.	*Autotrophy*	-	-	Batch	air flow	50 (Continuous)	-	0.46	7	Hu et al, 2003
		-	-		0.28%			0.63	9	
	Mixotrophy	Sodium acetate	0.16		air flow			0.5	7	
	Mixotrophy				0.28%			0.6	8	
Nannochloropsis salina	*Autotrophy*	-	-	Batch	Yes	260 (12:12 h day-night)	0.16	$4.20 \pm 0.17b$[a]	35.6	This work
			2				0.16	$3.92 \pm 0.19a$	34.6	
	Mixotrophy	Glycerol	3		No		0.16	$4.30 \pm 0.08b$	45.7	
			4				0.14	$3.44 \pm 0.32a$	44.8	
	Autotrophy	-	-		Pure CO$_2$ on-demand according to pH value		0.18	$4.40 \pm 0.21a$	35.5	
			2				0.22	$5.32 \pm 0.19b$	34.4	
	Mixotrophy	Glycerol	3				0.24	$6.59 \pm 0.32d$	46.6	
			4				0.23	$5.78 \pm 0.12c$	42.5	

[a]Values in the same column followed by the same letter are not statistically different at $p < 0.05$ according to Tukey test.
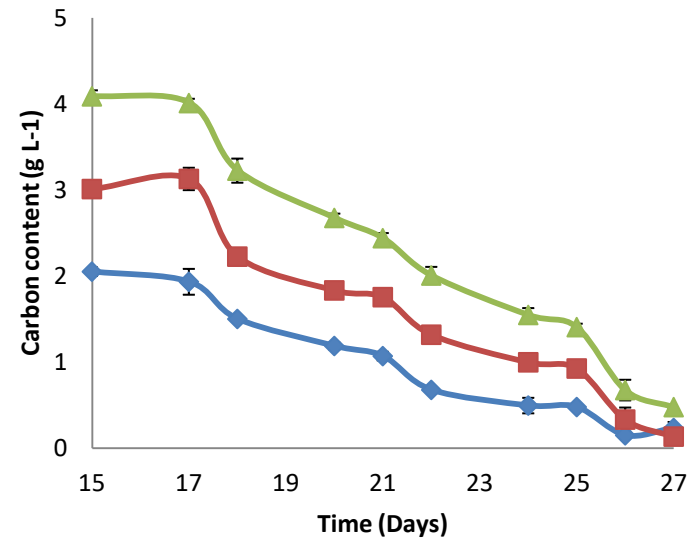
Figure 5.1. *Nannochloropsis salina* growth and organic carbon uptake: (**a**) growth curve with the addition of glycerol and the stop of CO_2 supply; (**b**) organic carbon uptake of *Nannochloropsis salina* without CO_2 supply; (**c**) growth curve with the addition of glycerol with CO_2 supply; (**d**) organic carbon uptake of *Nannochloropsis salina* with CO_2 supply.
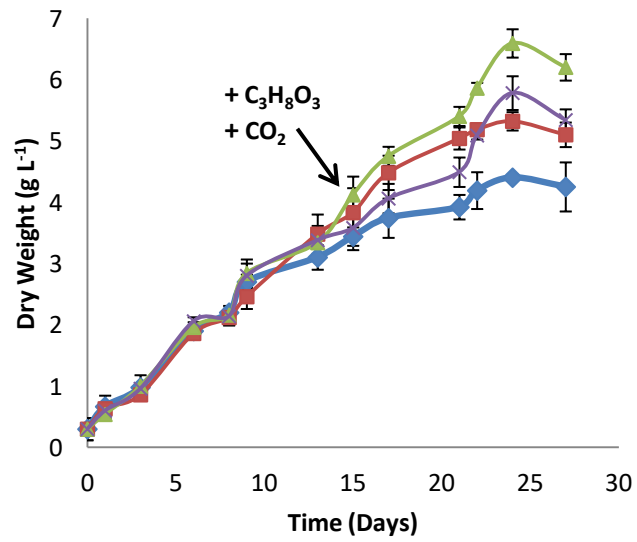
13
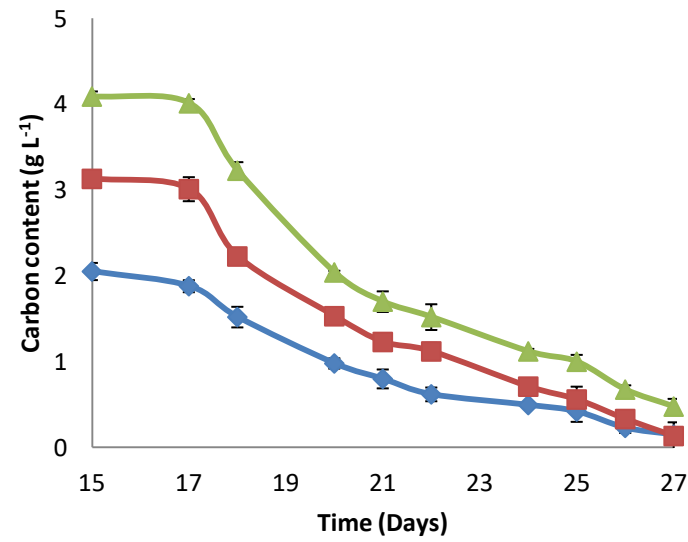
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128
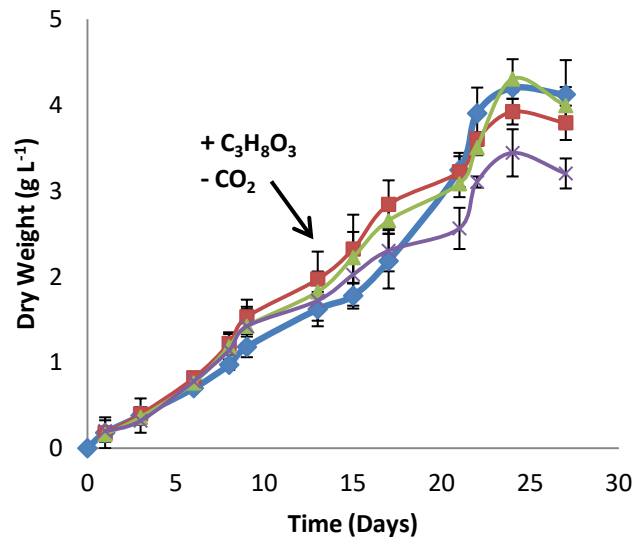
5.3.4 Biomass composition

The biochemical compositions of the lyophilized biomass obtained for each experiment is reported in Table 5.2. In both tests performed with and without CO_2 supply, trials grown under mixotrophic condition showed some changes in the final biomass composition with particular references to lipid content. It is well knows that the addition of organic carbon source stimulates the lipids (Cerón García et al., 2005; Heredia-Arroyo et al., 2011) and pigments (Ip et al., 2004) accumulation in several microalgal strains. In this study higher lipid amounts were recorded for mixotrophic trials where 3 and 4 g L^{-1} of GLY-C was added. Regardless of CO_2 feeding, when 2 g L^{-1} of C was provided, the total lipid content was very similar to that of autotrophic control (35.6 ± 0.2 % DW and 34.6 ± 0.2 % DW for autotrophic and mixotrophic condition, respectively). The dose of 2 g L^{-1} C was probably not sufficient to trigger to mixotrophic metabolism changing, also, biochemical composition. As reported in Table 5.2, the highest lipid content was obtained in PBRs with 3 g L^{-1} and 4 g L^{-1} of C (biomass of 45.7 ± 1.8 % DW and of 46.6 ± 1.6 % DW for experiment with and without CO_2, respectively). Therefore, the higher content of GLY-C was able to affect algal biomass and lipid accumulation. Liang et al., 2009 observed an increase in lipid content of *Chlorella vulgaris* cultured with an increasing concentration of glycerol, i.e. the lipid content increased from 22% DW with 1% (w/v) glycerol addition, to 32% DW with 2% (w/v) glycerol supplementation. The same authors reported that the additions of 5% (w/v) and 10% (w/v) of glycerol exerted inhibition. Andruleviciute et al., 2014 stated that compared to autotrophic conditions, the use of glycerol for microalgal cultivation increased the lipid content for several algal species, but also found that the continuous addition of glycerol cause a decrease in lipid content. Results presented in this work and

data from literature seem to indicate that substrate inhibition is strain-dependent Liang et al., 2009.

Table 5.2. Biomass composition of *Nannochloropsis salina* grew on glycerol with and without CO_2 supply.

CO_2 supply	Carbon $(g\ L^{-1})$	Lipid content (% DW)	Protein content (% DW)	Carbohydrates content (% DW)
+	0	$35.6 \pm 0.2a$[a]	$13.3 \pm 0.7a$	$17.7 \pm 0.8b$
-	2	$34.6 \pm 0.3a$	$13.1 \pm 0.1a$	$16.1 \pm 0.1b$
-	3	$45.7 \pm 1.8b$	$14.1 \pm 0.2a$	$5.2 \pm 0.7a$
-	4	$44.8 \pm 3.4b$	$12.3 \pm 0.6a$	$6.3 \pm 1a$
+	0	$35.5 \pm 2.1a$	$12.5 \pm 0.9a$	$18.9 \pm 2b$
+	2	$34.4 \pm 1.7a$	$13.7 \pm 0.5a$	$18.5 \pm 1.4b$
+	3	$46.6 \pm 1.8b$	$16.3 \pm 0.6b$	$6.5 \pm 0.5a$
+	4	$42.5 \pm 1.1b$	$13.8 \pm 0.5a$	$7.5 \pm 0.7a$

[a]Values in the same column followed by the same letter are not statistically different at $p < 0.05$ according to Tukey test.

In particular in this study the inhibition threshold for *N. salina* was found to be 4 g L^{-1} of C (Figures 5.1a) as biomass produced was the lowest under mixotrophic condition compared to 3 g L^{-1} of GLY-C that was the best concentration for the mixotrophic growth of the tested strain, both in presence and absence of CO_2. Regarding the crude protein content, no differences were evident for mixotrophic condition at all GLY-C concentrations tested and autotrophic condition, except for the PBRs with 3 g L^{-1} of GLY-C plus CO_2 in which the final proteins content, i.e. 16.3 ± 0.6 % DW, was slightly

higher than the others (Table 5.2). Carbohydrate contents were higher in algal culture cultivated autotrophically than mixotrophically (Table 5.2). Li et al., 2012 showed that accumulation of lipids in algal cell occurred through the conversion of either starch or carbohydrates to lipids, but conversion depended by microalgal strains, i.e. different strains have different mechanism to switch from carbohydrate pathway to lipids production Minhas et al., 2016. Furthermore, in this study, all trials performed without CO_2 addition reached final carbohydrate content slightly lower than that obtained with mixotrophic condition with CO_2 addition. This fact may be explained taking into consideration that under autotrophic condition the cell's efficiency to store light energy as carbohydrates is often limited by the availability of inorganic carbon (Blifernez, 2012).

5.4. Conclusions

Nannochloropsis salina displayed the inability to growth mixotrophically on WWL and CW, while it grew very well on GLY improving biomass and lipid production. Results shown that when CO_2 was not provided, GLY-C was able to replace CO_2 when it was dosed at 3 g L^{-1} C. The use of C-rich wastes may be a viable way to produce microalgal biomass at low cost, increasing the productivity of final biomass and target compouds e.g. lipids, but not all substrates are suitable and the adaptability to some C-substrates is species-specific. However, the possible contamination by other microorganism e.g. fungi, that can affect the microalgal growth and the final quality of the biomass, is a real risk related to mixotrophic way.

CHAPTER 5 – Conclusions

Microalgae production under mixotrophic conditions could be a good way to reduce the total production cost of microalgae biomass, increasing the productivity and the quality of the final biomass, supporting the economic feasibility of the whole system. The use of some organic streams coming from the agrofood sector is an opportunity to support microalgae growth, depuring and valorizing the C-rich wastes. On the other hand, not all the streams are suitable as substrate to support the mixotrophic growth of microalgae and not all the strains are able to perform the mixotrophic metabolism. From my results, a minimal carbon concentration was required to trigger mixotrophic metabolism. Time plays a key role since an adaptation period is required to allow mixotrophic metabolism to start. Particular attention should be paid to the correct dosage of carbon concentration and the timing of addition. The main problem is related to the high risk of contamination from other microorganisms, even at laboratory scale, and so lead this production process at industrial scale, where is very difficult to maintain favorable conditions for the growth of pure cultures, probably remain far remove from reality, less than work on closed photobiorectors where the contamination risk is lower than the common open ponds system, normally used to produce microalgae biomass at large scale.

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