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Experimental Studies on Microalgae cultivation in urban wastewater: nutrients removal, CO_2 absorption, biomass harvesting and valorisation

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To everyone who

believed in this work.

Contents

Summary

Microalgal biotechnology has received a growing attention in recent years as valuable alternative to conventional processes used to treat wastewater and as suitable method to capture carbon dioxide (CO_2). Moreover, the algal biomass generated during wastewater treatment can be used as sustainable bioresource to produce biofuel, agricultural fertilizers or animal feed; the same biomass, cultivated in more controlled conditions, could be also used for cosmetic and pharmaceutical products. Although this technology is attractive, a certain number of problems need to be solved before a large-scale application. The main purpose of this work has been, actually, to study some critical aspects related to the sustainable microalgal production chain, such as biotic factors (light, nutrients supply), CO_2 utilization, lipids production and biomass harvest.

A wastewater-autochthonous algal culture was used to treat raw urban wastewater in closed photobioreactors under different light intensities and nutrients supply. Nutrients removal were correlated to both biotic (absorption in microalgal biomass, bacteria nitrification) and abiotic processes (ammonia volatilization, phosphate precipitation), caused by increasing values of pH during the cultivation time. High ammonia concentration resulted in nitrite accumulation in the cultivation media, likely due to microalgal cultivation stressed conditions. The best conditions for biomass production and lipids accumulation resulted with low nutrients supply (~ 10 mg NH_4^+/L, ~ 6.5 mg PO_4^{3-}/L) and high light intensity (100 µmol $s^{-1}m^{-2}$). The biomass autoflocculation was investigated at the end of the cultivation period, corresponding to the highest pH value of the cultivation media. The highest biomass recovery of 72% was obtained for the lowest light intensity (20 µmol $s^{-1}m^{-2}$) and nutrients supply conditions.

The same wastewater-autochthonous algal culture was further cultivated in an open system (i.e. 200 L pilot-scale raceway pond), using urban wastewater as growth medium, to analyse its CO_2 capture potential by applying different gas input flowrates (i.e. 0.2, 0.4 and 1.0 L/min). Biomass growth, inorganic carbon and nutrients absorption were also studied during the cultivation start-

up and its semi-continuous feeding conditions. Low gas flowrates favoured the fixation of bio-available CO_2, while high gas flowrates favoured the CO_2 absorption in the open system, also corresponding to the highest microalgal productivity (28.3 g $d^{-1}m^{-2}$ at the gas flowrate of 1.0 L/min).

The combined cultivation of microalgae and yeast was conducted in batch conditions and in the open system, with the final purpose of increase the total lipids concentration of the produced biomass. Urban wastewater was used as cultivation medium. Yeast growth was monitored only during the first days of the cultivation because of the low availability of readily assimilable organic substrates in the medium. Microalgae growth showed a 3 days long initial lag phase and a subsequent linear growth, when nutrients were completely depleted showing removal rates of 2.9 $mgN \cdot L^{-1} \cdot d^{-1}$and 0.96 $mgP \cdot L^{-1} \cdot d^{-1}$ respectively . The cultivation induced a natural bactericidal and antifungal action at the end of the cultivation period (14 days). The highest lipids content was measured at the end of the cultivation (i.e. 15% lipids/dry weight) and resulted mainly composed of arachidic acid.

Bioflocculation was studied as harvesting technique since it is low cost and not toxic for the biomass. Microalgae bioflocculation was studied through their interaction with filamentous cyanobacteria. The filamentous cyanobacteria were obtained by the cultivation of the wastewater-autochthonous algal culture in specific operating conditions of light, temperature, growth media and cultivation mode. The filamentous cyanobacteria showed a natural flocculation-tendency also at pH 7, so their cultivation with microalgae was studied in order to enhance the biomass harvesting through bioflocculation. Microlagae and cyanobacteria were cultivated in synthetic media and in pre-filtered urban wastewater. Natural flocculation occurred for cyanobacteria and enhanced the microalgae harvesting which resulted trapped in cyanobacteria mats; anyway, the suspended microalgae limited the clarification of the growth media. The natural cyanobacteria flocculation-tendency was further investigated by applying two different mixing systems (air bubbles and shaking moment) and different initial biomass concentrations. Results showed a better flocculation performance in case of air bubbles mixing and with high initial biomass concentration. Moreover, the best condition for the natural biomass settling occurred at the end of the biomass exponential growth phase.

Abbreviations

PBR photobioreactor

vvm gas volumetric flowrate per unit volumetric culture medium

NER net energy ratio

DIC dissolved inorganic carbon

COD chemical oxygen demand

LHC light harvesting antenna complexes

TSS total suspended solids

EPS extracellular polymeric substances

WW wastewater

WWTP wastewater treatment plant

BBM Bold's Basal Medium

OD Optical Density

DO Dissolved Oxygen

T Temperature

DCW Dry cells weight

v/v volume/volume

FAME Fatty acid methyl ester

Chl a Chlorophyll a

GHG Greenhouse gas

HRT hydraulic retention time

Ibc initial biomass concentration

Chapter 1

Background and aim of the study

Currently, the world is facing many challenges: environmental pollution, global warming, increasing energy demand and malnutrition are some of them. Microalgal technology is an emerging field, which has the potential to mitigate these problems.

Nowadays, wastewater treatment is receiving an important attention since uncontrolled discharges of either untreated or not adequately treated wastewater have produced eutrophication in aquatic ecosystems and pollution of groundwater resources [1–4]. In developed countries, biological and physical technologies are mostly used for wastewater treatment, but recent stricter environmental regulations are forcing existing facilities to move towards advanced technologies [5,6]. In addition, conventional wastewater treatments require high costs and are responsible for greenhouse-gas emission, therefore more sustainable alternatives, eco-friendly and cost-effective, are recommended [7–10]. Microalgae-based processes offer several advantages over conventional wastewater treatment methods, for instance they can recycle nutrients by assimilation within microalgae, capture CO_2 thanks to their autotrophic metabolism and produce valuable biomass [11]. Inorganic nitrogen and phosphorus are particularly difficult to remove from wastewater. Due to the ability of microalgae to use both these nutrients

for their growth, microalgae are particularly useful to reduce the concentration of inorganic nitrogen and phosphorus in wastewater [12–15].

Global warming is induced due to the increasing emissions of the greenhouse gases (GHGs) as a result of anthropogenic activities, causing severe changes to the global climate. In addition to methane as well as nitrous oxide and other fluorinated gases, carbon dioxide (CO_2) is one of the major constituents of GHG emissions. The European Union (EU-28), in 2015, emitted 3.47 billion tonnes of CO_2, which is 1.3% higher than in 2014 (3.42 billion tonnes of CO_2) [16]. Microalgae transform gaseous CO_2 into their cellular components such as carbohydrates, lipids, and proteins in a process called photosynthesis. In this way, microalgae help in mitigating the effect of global warming by capturing the CO_2 from the earth's atmosphere [17–20]. Compared to terrestrial plants, microalgae exhibit faster growth rate, and their photosynthetic efficiency can potentially exceed 10%, which is 10–50 times greater than terrestrial plants [21–23]. Most of the solutions for atmospheric CO_2 reductions are primarily focused on CO_2 utilisation following capture; but only biological conversion is capable of a direct CO_2 mitigation [24]. Utilisation of CO_2, as a feedstock for other production processes, offers opportunities to offset part of the significant capital investment associated to capture CO_2.

Carbon fixed by microalgae is incorporated into biomass, which in turn could be utilized for a variety of applications including bioenergy, chemicals, and food production [25–27]. Indeed, under stressful environmental conditions, microalgae are capable to accumulate large amounts of lipids [28], which are suitable for biodiesel production through transesterification [29,30]. It has been shown that the use of biofuel could reduce carbon emissions and may help to increase energy security [31,32]. Compared to plant-based biofuel crops, microalgae can grow in a wider variety of wastewater sources (industrial, agricultural or urban) and recycle nutrients already present in waste streams to produce new biomass [15,33,34]. Growing algae in wastewater offers numerous economic and environmental advantages, providing one of the most sustainable ways to produce biodiesel derived from microalgae. Wastewater usage eliminates competition for fresh water, saves cost for nutrients supply since nutrients are in abundance in wastewater, provides the treatment of the wastewater by assimilating organic and

inorganic pollutants into their microalgae cells, and eliminates the CO_2 emissions associated with wastewater treatment [35–38].

Harvesting microalgal biomass from growth medium is a significant challenge in microalgal biomass production technologies. This is mainly due to the small size (5 - 50 µm) [39], the negative charge (about -7.5 to -40 mV) on the algae surface that results in dispersed stable algal suspensions especially during the growth phase [40], low biomass concentrations (0.5 - 5 g/L) and mass densities similar to water [41]. In algal biofuel research, the development of a cost effective harvesting method is one of the most challenging aspect [42], indeed it is currently a key factor that limits the commercial use of microalgae. It has been reported that 20–30% of the total production cost is used in biomass harvesting [43]. Current harvesting strategies includes mechanical, electrical, biological and chemical based methods [44]. However, the limitation of this type of approach had contributed to very high costs, mostly due to energy requirement for equipment at massive scale operation [45]. Thus, to minimize the energy consumption of harvesting microalgae, an innovative technological approach is required. A promising approach is represented by bioflocculation methods, which consist in biomass flocculation induced by extracellular polymer compounds such as polysaccharides and proteins derived from microalgae and other microorganisms [46]. Bioflocculation is a non-toxic and low-cost harvesting method; anyway, challenges in the scale-up of this technique have to be faced [47,48].

The concepts expressed above could be summarized by the scheme reported in Figure 1 that includes microalgae cultivation in a circular economy process: microalgae cultivation is promoted by light and CO_2 availability; the harvested biomass can be valorised as bioproducts or source of biofuels, which are used for anthropic activities through combustion; emitted CO_2 in the atmosphere could be recirculated in the microalgal cultivation.

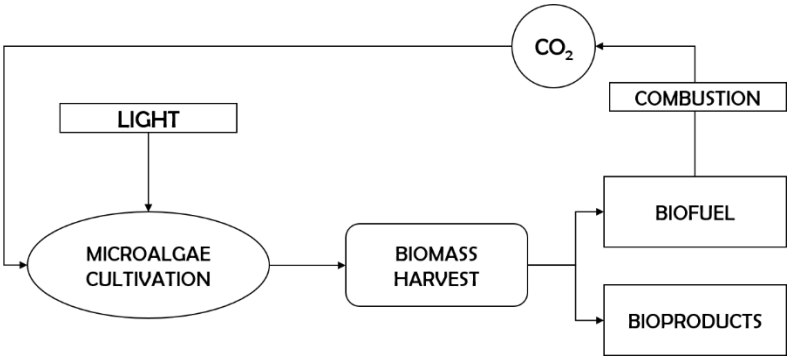

Figure 1. – Circular economy for microalgae cultivation.

The present phD project is aimed at studying each different phase of the reported scheme in order to optimize it with an innovative approach. The microalgae inoculum that has been used in this study is autochthonous of municipal wastewater since its cultivation has been principally conducted in wastewater medium. Specific objectives of the project are:

- Cultivation of the inoculum in open and closed systems;
- Analysis of abiotic parameters, such as pH, light and nutrients availability on biomass growth and lipids production;
- Analysis of possible alternatives to enhance the content of lipids in biomass;
- Study of the potentiality of CO_2 capturing in open microalgal system;
- Study of eco-sustainable and low-cost harvesting methods, enhancing bioflocculation technique.

In order to fulfil the above objectives, the following work-tasks were conducted.

1. Microalgae cultivation in closed system using municipal wastewater as growth substrate in order to study the effect of light intensity and nutrients supply on biomass production, lipids accumulation and settleability characteristics (Chapter 3).

2. Microalgae cultivation in open system using municipal wastewater as growth substrate in order to study the biomass growth phases and the CO_2 capture for different input fluxes (Chapter 4).
3. Combined microalgae and yeast cultivation in open system and in urban wastewater for urban wastewater treatment and potential biodiesel production (Chapter 5).
4. Bioflocculation studies for eco-sustainable and low cost biomass harvesting: bioflocculation for filamentous cyanobacteria and microalgae in synthetic medium and urban wastewater (Paper I, Chapter 6); influence of different initial biomass concentrations and mixing method on filamentous cyanobacteria bioflocculation tendency (Paper II, Chapter 6);

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Chapter 2

Origin and diversity of microalgae

Microalgae are photosynthetic microorganisms, the basic plants present in abundance in the nature. Algae are divided into five main groups, namely Chlorophyceae, Rhodophyceae, Phaeophyceae, Cyanophyceae and Bacillariophyceae. Characteristics of each algae group are summarised in Table 1 [1].

Table 1. – The characteristics of algae groups

Algae Group	Common name	Characteristics
Chlorophyceae	Green algae	(i) Estimated 6000-8000 species (ii) 90% live in freshwater rather than marine (iii) Ranging from tiny unicellular and colonial organisms to large macroscopic weeds (iv) In monophyletic group as the terrestrial plants
Rhodophyceae	Red algae	(i) Estimated 4000-5000 species (ii) 90% live in marine (iii) Ranging from unicellular to macroscopic algae often found on rocky shore

		(iv)	In monophyletic group as the terrestrial plants
Phaeophyceae	Brown algae	(i)	Estimated 1500-2000 species
		(ii)	Almost all live in marine
		(iii)	Ranging from giant kelps to smaller intertidal seaweeds
		(iv)	Grow in rocky intertidal zone
Cyanophyceae	Blue-green algae	(i)	Prokaryotic cell
		(ii)	Present in almost all feasible habitats
		(iii)	CO2 and Nitrogen fixers since billion years ago
Bacillariophyceae	Diatoms	(i)	12000 known species
		(ii)	Single celled with macroscopic in size
		(iii)	Grow in seas, lakes and moist soils and out of glass (silicon dioxide or silicon)

Microphytes or microalgae can be prokaryotic (Cyanophyceae) or eukaryotic (Chlorophyta) organisms, photosynthesize light and are among the oldest living microorganisms. They can grow rapidly in a wide number of environments such as in freshwater, wastewater, and marine environment as well as in extreme milieus [2]. The sizes of microalgae are ranging from micrometers to millimeter and its size depends on the species [3]. About 200,000-800,000 algae species exist, of which, only around 50,000 has been described [4].

Metabolism of Microalgae

Microalgae can grow under different conditions depending on the source of energy and the carbon used, as it is summarized in Table 2. **Photoautotrophic** microalgae growth occurs under visible light, having inorganic carbon in the culture medium as the only carbon source. Photoautotrophic cultivation can be used in both open and closed systems, producing polysaccharides, proteins, lipids and hydrocarbon compounds through photosynthesis. In photoautotrophic conditions microalgae achieve a high photosynthetic efficiency as well as growth rate, comparable with those of terrestrial plants [5].

Heterotrophic growth takes place when organic carbon is used as a food and energy source. Hence, light is no longer required for cell growth [6]. Microalgae cultured under these conditions grow in the "dark", adapting rapidly to new culture media [7]. Under heterotrophic cultivation, lipids productivity is much greater (about 20 times) than in the photoautotrophic cultivation [5]. This increased lipids productivity depends on the microalgae species, the culture medium composition, and other growth parameters. Since light is not required for cell growth, heterotrophic cultivation is considered less expensive for cell growth. In heterotrophic cultivation, both reactor design and scale-up are relatively less challenging. It is also anticipated that heterotrophic culture may be suitable for the processing of large volumes of wastewater effluents [8].

Microalgae can also grow with cells metabolizing organic compounds as a carbon source in presence of light. This microalgae culture is designated as **Photoheterotrophic** [9]. In a photoheterotrophic metabolism, microalgae use light and an organic carbon for growth [10]. While photoheterotrophic microalgae cultivation has been reported to produce hydrogen [11], there is, however, no report using photoheterotrophic cultivation for lipid or biodiesel production.

Finally, when both organic and inorganic carbon is used as a food and energy source without and with visible light, these growths are named as **Mixotrophic** growth and **Mixophototropic** growth respectively. In mixotrophic cultivation, microalgae can grow autotrophically or heterotrophically depending on the light availability and the carbon source concentration [13]. A mixotrophic metabolism can be facultative and/or obligatory. For some microalgae species, the mixotrophic specific growth rate is close to the sum of the photoautotrophic and the heterotrophic specific growth rates [14]. Das et al. [12] studied the two growth phases of *Nannochloropsis sp*. under photoautotrophic conditions followed by mixotrophic conditions. The mixotrophic cultivation resulted in a biodiesel productivity higher than that achieved when cells are cultured under photoautotrophic conditions. Yeh and Chang [9] investigated both growth and lipid productivity of *Chlorella vulgaris* with various culture media. Results showed that under mixotrophic conditions, both lipids content and productivity are enhanced if compared to other cultivation approaches.

Table 2. – Summary of microalgal cultivation conditions.

Cultivation condition	Energy source	Carbon source
Phototrophic	Light	Inorganic Carbon
Heterotrophic	Organic carbon	Organic carbon
Photoheterotrophic	Light	Organic carbon
Mixotrophic	Light and Organic carbon	Inorganic and Organic carbon

Microalgae cultivation systems

Open systems, closed systems and the attached-growth systems are the mainly used microalgae and cyanobacteria cultivation systems. The choices of systems are dependent on the products to be achieved and the strains to be cultivated [15–18].

Open systems

Open pond is the most common used system for large-scale microalgae cultivation due to its low cost and ease in operation and maintenance. It is commonly used for industrial application, to produce significant amount of products for commercial purposes at relatively low cost [1]. The open pond is commonly designed with a superficial area of 0.2–0.5 ha and a width of 0.25m for commercial microalgal production [19]. Open pond systems present high surface area per volume ratio, thus performing a high CO_2 mitigation. Additionally, if the nutrients source used is wastewater, coupled with CO_2 supplied from flue gas, the open pond system is usually applied, providing effective wastewater treatment. However, this system presents the main following two disadvantages: (i) when CO_2 is supplied in bubbles the shallow depth and the short times of contact impede a high gas/liquid mixing efficiency and as a consequence the CO_2 absorption is low; (ii) photosynthesis in algae cell is carried out with low spatial efficiency because of sharp decrease of light penetration. Therefore, process failures are frequent in open pond systems: broth evaporation and species invasion are responsible for the loss of algae in culture medium [19]. These problems are the key

restraint factors in putting open ponds into microalgae cultivation for energy on a large scale.

Raceway pond

It looks like race track, usually shallow with 15–25 cm in depth. It is equipped with paddle wheel agitation, to ensure good circulation and nutrients homogenisation. In addition, the flow of culture is controlled and governed with baffles placed in the flow channel [20,21]. The liquid velocity of the ponds is set to reach more than 30 cm per second [22]. Raceway ponds are currently the most commonly used large scale cultivation systems for commercial scale. It is used for culturing *Chlorella sp*., *S. platensis*, *Haematococcus sp*. and *Dunaliella salina*. If compared to closed photobioreactor systems, raceway cultivation produces less biomass of *Chlorella sp.* and *Spirulina sp*. due to carbon limitation, since only 5% of carbon is directly transferred from the atmospheric air [23]. In order to achieve outstanding carbon sequestration, the microalgal farms can be placed surrounding the industrial plant and therefore utilise efficiently CO_2 from the flue gas [24].

Closed systems

Closed photobioreactors (PBRs) have gained much interest by researchers due to better control of cultivation parameters and capability to satisfy carbon requirement. PBR cultivation has achieved high photosynthetic efficiency and biomass productions compared to open pond system [25]. These advantages are even more important if the desired microalgae are used for pharmaceutical purposes or highly selective products applications [26]. PBRs are designed in configurations accomplishing the following two targets: (i) maximise photosynthetic efficiency and CO_2 mass transfer rate; (ii) minimise cultivation dark zone and power consumption [27].

Airlift photobioreactor

In airlift PBR, the liquid volume in the vessel is separated into two connected zones by baffle. The liquid is moved in the circulatory flow caused by the CO_2 supply at the bottom of the reactor [22]. It gave the most CO_2 fixation efficiency due to its relatively better mass transfer and circulation [28]. This provides the high cycling of medium with low surface area exposed to light radiation, thus resulting in minimum photoinhibition. With pressurised gas–

liquid system used to generate fine bubbles into the reactor, CO_2 concentration can be regulated easily rather than using baffles as in open pond system. Additionally, in optimised PBR, microbubbles perform the highest surface area to volume ratio for enhancing the CO_2 mass transfer rate and the slow rising in the medium, leads to a better dissolution of gas into liquid [28]. The microbubbles, actually, can rise gradually and get dissolved in the medium, whereas macrobubbles rise rapidly and burst on the surface of medium to the atmosphere. Airlift PBR is effectively in gas hold-up. On the other hand it is challenging to scale-up as it presents a high cell shear effect, complex liquid flow pattern and high operational cost. Furthermore, it makes temperature difficult to control. In order to mitigate the effects of these limitations, split column airlift PBR was introduced recently, characterized by a temperature control system and light transport located internally at the centre of the airlift PBR [26]. This central flat plate provides illuminated surface in the medium, serves as central baffle to prevent dark zone and functions as heat exchanger to ensure better temperature control. Fernandes et al. (2014) reported that the biomass productivity of microalgae cultivated in spit column airlift PBR was 15– 36% higher than in conventional bubble column.

Tubular photobioreactor

Tubular PBR system is the most noticeable system for large scale outdoor cultivation [28]. Tubular PBRs are made up of transparent materials and placed in outdoors under sunlight radiation. The microalgae are cultivated in the vessel, permitting the addition of air, CO_2, and nutrients into the medium and O_2 removal by reactor. The medium is circulated through tubes and back to reservoir in high turbulent flow, by using mechanical pump. The flow rate in the tube is ranging from 30 to 50 cm s^{-1}, to ensure CO_2 distribution, light/dark cycle and prevent cell deposition [22]. A portion of microalgae is usually harvested after it circulated through solar collection tubes. The tubes are generally 5–20 cm in diameter to enabling sunlight penetration, thus reaching high microalgal productivities [19,22]. Though it is often considered as the most suitable for microalgae cultivation, the reactor size and length are limited to parameter control, O_2 removal and CO_2 depletion [28]. To date, the maximum capacity that it can achieve is about 20 L. Further increase in concentration culture has resulted in the increase of tube length and diameter.

Thus, it is difficult to scale up and the only solution is to multiply the reactor units.

Flat plate photobioreactor

Flat plate PBR has large surface area per volume ratio allowing large irradiated zone, high cost effective, large cultivation volume and excellent biomass productivities. Flat plate PBR has achieved short light path and steep light gradients, and can be further enhanced by addition of baffles for aeration towards light gradient [28]. The aeration rate using bubbling technique can be expressed in gas volumetric flow rate per unit volumetric culture medium (vvm). The optimum aeration rate of 0.023–1.000 vvm was proposed for 5% (v/v) or 10% (v/v) CO_2 aeration and 0.05 vvm is appropriate for flat-plate PBR [19]. Flat plate PBR is scalable, reaching 1,000–2,000 L capacity. Limitations include difficulties in temperature control, low growth rate near the wall and hydrodynamic stress [22].

Bag photobioreactor

Bag PBR is a semi-continuous PBR, cultivating microalgae in transparent polyethylene bags. The bags are hung and placed in the cage with multiple partitions, located under the sunlight. The air is sparged from the bottom of the bags, together with sealing the bags in conical shape at the bottom, to prevent settling of cells [22]. This is commonly used in lab scale before proceeding to outdoor pilot plant.

Biofilm cultivation

Biofilm cultivation method achieves rapid development based on the fact that microorganisms tend to grow attached to the containers. The biofilm cultivation is different if compared to suspended systems. The dense algal cells are immobilized and attached onto artificial supporting material(s), and the liquid medium is supplied to the biofilm to keep the algal cells in wet conditions. This method is originally used to wastewater treatment [29]. Many studies proved that the biofilm system is a promising method for its long-term stability, contamination freedom and low overall energy consumption [30–33]. Recently, by combining this immobilized biofilm method with light dilution PBR structures, Liu et al. [18] proposed a novel ‘attached cultivation’ technology for cultivation of Nanochloropsis, Cylindrotheca. Recently this method has been successfully applied to

cultivation of *Haematococcus* [30] and *Botryococcus* [34]. In order to fully utilize the solar light, they reported an array bioreactor structure to realize dilution of strong solar light and then the highest biomass productivity of 50–80 gm^{-2} d^{-1} obtained outdoors for *Scenedesmus obliquus*, corresponding to the photosynthetic efficiency of 5.2–8.3%.

In general, open pond reactors and photobioreactors have merits and shortcomings respectively. Algae characteristics, geological environment and target products should be considered when the method for cultivation is chosen. Raceway ponds present the lowest capital costs [18,35]. However, they need large surface areas, thus competing with food crops. Photobioreactors are easier to control and reach a higher amount of biomass if compared with raceway ponds. On the other hand, photobioreactors present the highest capital and operational costs. Jorquera et al. [36] presented a comparative analysis among open raceway ponds, tubular and flat-plate photobioreactors for production of biomass by life-cycle method. The results showed that both flat-plate photobioreactors and raceway ponds showed net energy ratio (NER) > 1, thus workable for mass cultivation. Ideally, NER> 7 is considered economically feasible for algae biofuels [37]. Therefore, highly efficient cultivation systems and extensive utilization are still under research [38].

Microalgae growth parameters

Generally, the parameters that influence microalgae growth are as follow: (i) the concentration of macronutrients; (ii) the CO_2 concentration; (iii) the flow rate; (iv) the culture media temperature; (v) the light intensity and (vi) the photosynthetic efficiency. Figure 1 reports a schematic overview of factors and conditions affecting microalgae growth [39].

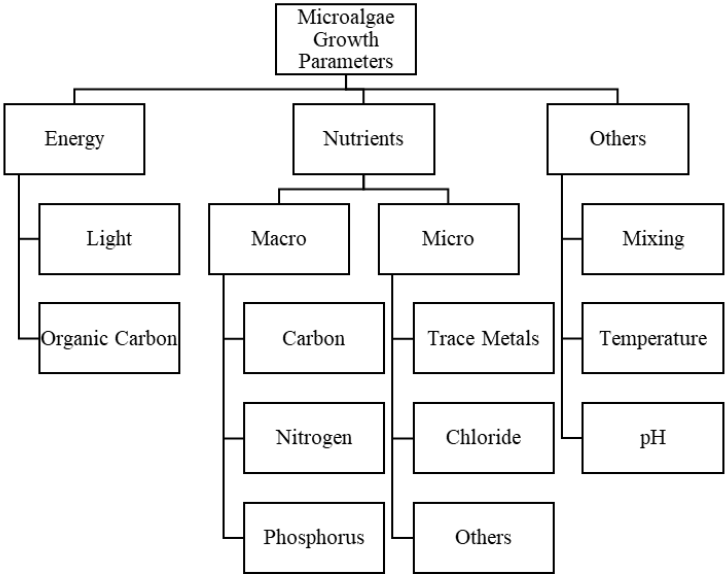

Figure 1.-. Schematic diagram of microalgae growth parameters including energy sources, nutrients and other factors.

Nutrients for microalgae growth

Carbon, nitrogen and phosphorus are the three essential nutrients for biomass growth. Apart from carbon, which can be obtained from atmospheric air or CO_2 sparging, microalgae assimilate sufficient nitrogen and phosphorus from medium for their metabolic activities. Nitrogen present in form of ammonium is the primary nitrogen source for microalgae assimilation [22,40]. Moreover, phosphorus is the element required for photosynthesis, metabolisms, formation of DNA as well as ATP and cell membrane. Phosphorus is available in the medium in the form phosphate and normally supplied in excess as it is not readily bioavailable. Other inorganic salts and trace elements like metals and vitamins are usually added into the medium for effective photosynthetic activity [1]. Micronutrients required in traces include silica, calcium, magnesium, potassium, iron, manganese, sulphur, zinc, copper, and cobalt, although the supply of these essential micronutrients rarely limits algal growth when wastewater is used [41]. If nutrients are not available in the water source, the addition of commercial fertilizers can

significantly increase production costs, making prohibitive the price of algae derived fuel [42]. For this reason, wastewater is an attractive resource for algae production.

Carbon

Carbon is an essential element for microalgal production, comprising approximately 50% of its organic biomass, and growth can become limiting when the demand for carbon exceeds supply [43]. Inorganic carbon can be used as a carbon source under phototrophic and mixotrophic conditions. During photosynthesis light energy is initially converted into chemical energy, which is then used to assimilate CO_2 for the formation of carbohydrate molecules. The stoichiometric formula for photosynthesis is:

Light energy + 6 H_2O + 6 CO_2 = $C_6H_{12}O_6$ + 6O_2

In water medium, dissolved inorganic carbon (DIC) could be found as three species, such as CO_3^{2-}, HCO_3^-, CO_2; their concentration is regulated by pH values and temperature (Figure 2) [44]. Microalgae preferentially uptake CO_2 through passive diffusion, over the other dissolved inorganic carbon species which require active transport and the assistance of metabolically expensive carbon concentrating mechanisms [45].

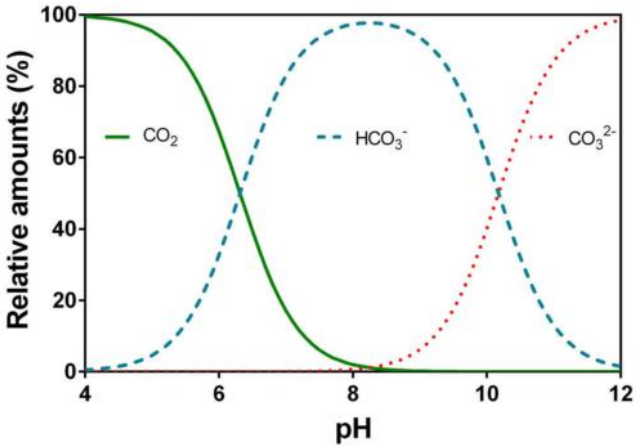

Figure 2. – Relative speciation (%) of carbon dioxide (CO_2), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) in water as a function of pH.

In addition, the organic carbon found in nutrients can be used as a carbon source for microalgae growth as well as for the energy requirement. The specific role of the organic carbon depends strongly on the microalgae metabolism. This ability of the microalgae to use organic carbon is relevant when microalgae are cultivated in wastewaters under mixotrophic conditions [46]. Zhu et al. [47] investigated the growth of *Chlorella zofingiensis* in integrated fresh water and piggery wastewater (animal wastewaters) using tubular photobioreactors. Results showed that the growth rate of the microalgal biomass augmented by increasing the initial COD (chemical oxygen demand) concentration. Perez-Garcia et al. [8] reviewed the metabolism of glucose, glycerol, acetate, and other carbon sources for heterotrophic microalgae cultivation. These authors showed the significant flexibility of microalgae towards various carbon source media. Although the biomass productivity is higher under mixotrophic conditions, CO_2 capture occurs according to a lower rate. However, under such operating conditions, both organic carbon and CO_2 are utilized as part of the cell composition. This competition for carbon sources may reduce, as a consequence, the CO_2 amount consumed by microalgae.

Nitrogen

Nitrogen compounds, especially ammonium (NH_4^+) and nitrate (NO_3^-), are important substrates for microalgae growth. These compounds contribute to more than 10% of the microalgal biomass. Additionally, urea and nitrite are other forms of nitrogen compounds but the latter is considered toxic at high concentrations [48].

Organic nitrogen is found in a variety of biological substances, such as peptides, proteins, enzymes, chlorophylls, energy transfer molecules (ADP, ATP), and genetic materials (RNA, DNA) . Organic nitrogen is derived from inorganic. In Figure 3 [49], a simplified scheme of the assimilation of inorganic nitrogen sources including nitrate (NO_3^-), nitrite (NO_2^-), nitric acid (HNO_3), ammonium (NH_4^+), ammonia (NH_3), and nitrogen gas (N_2) is reported. Microalgae play a key role in converting inorganic nitrogen to its organic form through a process called assimilation. In addition, cyanobacteria are capable to convert atmospheric nitrogen into ammonia by means of fixation. Assimilation, which is performed by all eukaryotic algae, requires inorganic nitrogen, thus being solely in the forms of nitrate, nitrite, and ammonium.

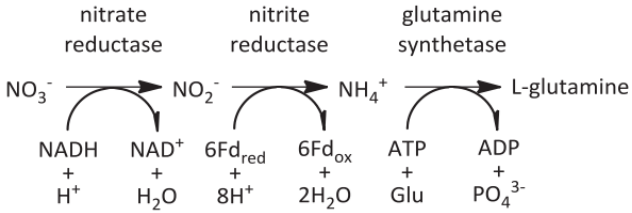

Figure 3. – Simplified schematic of the assimilation of inorganic nitrogen.

As shown in Figure 3, translocation of the inorganic nitrogen occurs across the plasma membrane, followed by the reduction of oxidized nitrogen and the incorporation of ammonium into amino acids. Nitrate and nitrite undergo reduction with the assistance of nitrate reductase and nitrite reductase, respectively. Nitrate reductase uses the reduced form of nicotinamide adenine dinucleotide (NADH) to transfer two electrons, resulting in the conversion of nitrate into nitrite. Nitrite is reduced to ammonium by nitrite reductase and ferredoxin (Fd), transferring a total of six electrons in the reaction. Thus, all forms of inorganic nitrogen are ultimately reduced to ammonium prior to being incorporated into amino acids within the intracellular fluid. Finally, using glutamate (Glu) and adenosine triphosphate (ATP), glutamine synthetize facilitates the incorporation of ammonium into the amino acid glutamine. Ammonium is thought to be the preferred form of nitrogen because a redox reaction is not involved in its assimilation; thus, it requires less energy. Studies have shown that, in general, algae tend to prefer ammonium over nitrate, and nitrate consumption does not occur until the ammonium is almost completely consumed [50]. Therefore, wastewaters with high ammonium concentrations can be effectively used to rapidly grow microalgae.

Although ammonia is an excellent source of N for algal growth [51], free ammonia is toxic to most strains of microalgae due to the uncoupling effect of ammonia on photosynthetic processes in isolated chloroplasts [52]. The speciation of ammonia and ammonium is strongly dependent on pH (Figure 4), therefore algal strains may not be significantly inhibited by free ammonia at low pH while considerable inhibition may occur at pH values of 9.0 or higher [53]. The ammonium tolerance of different algae species varies from 25 µmol NH_4^+–N L^{-1} to 1000 µmol NH_4^+–N L^{-1} [54]. A potential solution to

this problem in wastewater treatment facilities is to decrease the ammonia concentration in the algal growth reactor by diluting the high ammonia wastewaters with other wastewater sources, such as nitrified secondary effluent [39].

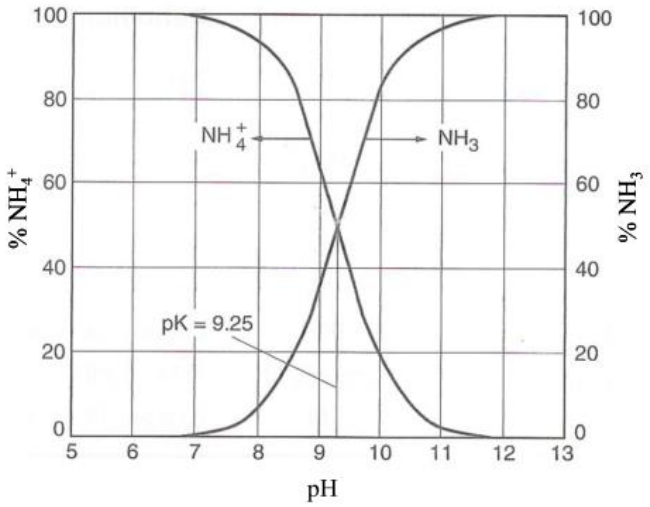

Figure 4. – Ammonium and ammonia distribution as a function of pH

Ammonium is not only removed by cell metabolism, but also by ammonia stripping, where significant amounts of ammonia can be volatilized at increased pH and temperature. Garcia et al.[55] showed that ammonia stripping was the most important mechanism in high growth rate algal ponds operating at various hydraulic retention times. It was also reported that when high rate algal ponds were exposed to warm climate, ammonia release accelerated even when the pH was below 9 [49].

Phosphorous

Phosphorus is also a key factor in the energy metabolism of algae and is found in nucleic acids, lipids, proteins, and the intermediates of carbohydrate metabolism [49]. Inorganic phosphates play a significant role in algae cell growth and metabolism. During algae metabolism, phosphorus, preferably in

the forms of $H_2PO_4^-$ and HPO_4^{2-}, is incorporated into organic compounds through phosphorylation, much of which involves the generation of ATP from adenosine diphosphate (ADP), accompanied by a form of energy input [56]. Energy input can come from the oxidation of respiratory substrates, the electron transport system of the mitochondria, or in the case of photosynthesis, from light. Phosphates are transferred by energized transport across the plasma membrane of the algal cell. Microalgae not only utilize commonly inorganic forms of phosphorus, but some varieties of them are capable to use the phosphorus found in organic esters for growth [57]. Although orthophosphate is generally recognized as the limiting nutrient in freshwater systems, many cases of eutrophication are triggered by superfluous phosphorus, which can result from runoff of wastewater [58]. Similar to the removal of nitrogen, it should be noted that phosphorus removal in wastewater is not only governed by the uptake into the cell, but also by external conditions such as pH and dissolved oxygen [49]. Phosphorus cannot exist in a gaseous state, thus phosphate will precipitate from the medium as a result of elevated pH (Figure 5) and high dissolved oxygen concentration [59].

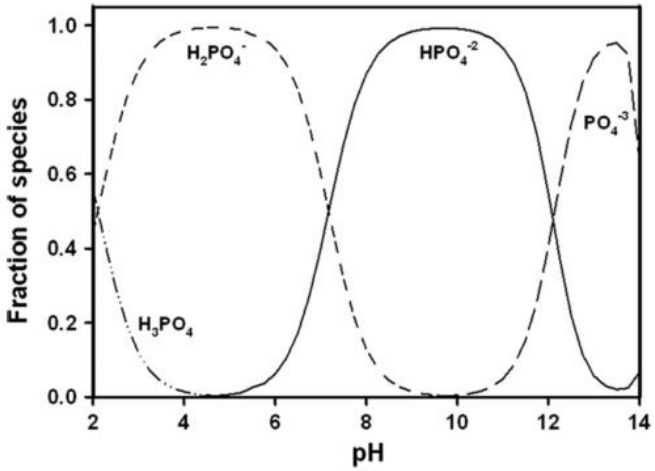

Figure 5. – P speciation calculated using total P concentration of 100 µM.

Microalgae cells tend to store the excess amounts of phosphorus as polyphosphate granules. Thus, these species can be used by microalgae during phosphate starvation conditions for cell growth [39]. As a result, the reduction of phosphates may affect the photosynthesis process and the lipid production [60].

N:P ratio

N:P ratios can be used to predict the nutrients limitation status, although amounts, especially of P, can be available completely to the microalgae metabolism [61]. For freshwater microalgae, N and P potentially co-limit the production over a wide range of N:P ratios: from 10 to 30, while ratios above 30 suggest P limitation and below 10 suggest N limitation [61]. Total concentrations of NH_4^+–N, within the range of 20 and 250 gm-3, did not affect the specific growth rate and maximal cell densities of *Chlorella vulgaris* [62], however, when nitrogen is limiting, based on N:P ratios, cell division of *C. vulgaris* becomes inhibited, resulting in a 3-fold decrease in biomass growth compared to cultures with stoichiometrically balanced N:P ratios [63]. Improved nutrients removal and significantly greater biomass growth yields were achieved in a marine fish farm wastewater photobioreactor when initial N and P concentrations were stoichiometrically balanced [64]. Nitrogen or phosphorus limitation can negatively impact on the primary productivity of microalgae. Parameters used to describe primary production, including photosynthetic efficiency under low light (a), the maximum rate of photosynthesis (Pmax), as well as a cell's ability to dissipate excess photon energy to prevent photo damage, have been shown to be sensitive to nutrients limitation, with photosynthesis efficiency decreasing and energy dissipation increasing when N or P limitation was enhanced [65]. Typical N:P ratios in wastewater suggest that phosphorus is rarely limiting algal growth but nitrogen may become limiting under certain conditions [43]. However, nitrogen is likely the only nutrient responsible for limiting growth in wastewater pond when carbon and light are not limiting [48]. Both the N:P ratio and their total concentrations vary according to the wastewater characteristics. N and P load into the open pond can affect the nutrients removal efficiency and the overall water quality of the effluent discharge [43].

Light

The availability and amount of light are important factors of the photosynthesis process on the microalgae growth. In this context, other parameters affect the efficiency of light utilization such as the density of the culture and the cell pigmentation [66]. In open and outdoor cultivation systems, sunlight is directly applied to the culture media. Thus, in open and outdoor systems, growth limitations are due to low and unequally distributed light radiation. On the other hand, in indoor lab scale closed systems, photobioreactors can result in a much higher microalgae growth rate using fluorescent lamps. This artificial irradiation source provides higher density of radiation with an overall modest productivity of biomass [5]. Thus, the microalgae scale-up production, still requires the development and the implementation of sustainable radiation source of light. Rubio et al. [66] developed a model to describe the effects of light radiation on the photosynthesis process of microalgae growth. It was observed that high lipid production can be achieved when light utilization is enhanced. Thus, density of radiation and radiation utilization efficiency are important factors in algae culture, in addition to others, such as proper selection of microalgae strains and other growth parameters [67].

Light limitation is regarded as one of the main control parameters for microalgal performance in open ponds [43]. While nutrients can be stored and recycled by the cell, photons can be only absorbed once and have to be instantaneously transformed into chemically bound energy, or dissipated out of the cell again. In order to maximise productivity it is important to understand how the operational conditions of open ponds affect both the availability of light as well as the efficiency of light absorption and utilisation by the microalgae. The light reaching the surface of the pond varies on diurnal and seasonal scales. At any given point in time, the amount of light available to the microalgae for photosynthesis is governed by both the degree of attenuation within the pond and internal self-shading within the cell. Light passing through the water column declines exponentially with depth as the microalgae absorb or scatter the light. The high biomass concentration in open ponds affects the amount of light that can reach the bottom of the pond, often causing that up to one third of the water column receive insufficient light to support net photosynthesis. High concentrations of non-microalgal particulate matter in the wastewater can further increase light attenuation in

open ponds [43]. This high attenuation means that cells near the surface are exposed to supersaturating light, thus requiring them to dissipate excess photos to prevent photodamage, whereas cells near the bottom of the water column receive from little to no light. In both conditions, photosynthesis is suboptimum, negatively impacting the biomass yield [1].

The efficiency of light absorption by the microalgal cells is a function of their size as well as their intracellular pigment concentration. Light harvesting pigments used by microalgae to capture light are organised in light harvesting antenna complexes (LHC) that are associated with the photosystem reaction centres [68]. Light energy is absorbed by the pigments and is transferred to a reaction centre where photochemistry takes place. When light absorption exceeds the biochemical capacity of the reaction centre the LHC dissipates the excess energy as heat or fluorescence [69]. In order to facilitate this energy transfer, the photosynthetic membrane is tightly packed with pigment-binding proteins which can result in dense packaging of pigments within the LHC [70]. When light becomes limiting, microalgal cells increase their concentration of light harvesting pigments, in particular chlorophyll, in order to capture the available light. However, this can lead to internal self-shading, named as ‘‘package effect’’, where light absorption efficiency per unit chlorophyll decreases with increasing chlorophyll content [68].

The required light/dark photoperiods are typically ranging from 12/12 to 16/8 h. These light/dark periods are important as the photo-induced damage caused by over-illumination of excessive photon flux can be repaired during the dark period. *S. obliquus* has shown high photosynthetic rate with increasing light/dark frequencies [71]. The light/dark of 10 Hz frequency cycles for *S. platensis* and *S. dimorphus* cultivation enhanced the microalgae productivity that increased by 43% and 38%, respectively [22]. Additionally, microalgae do not acclimate to a definite light/dark period. This is dependent on the nature of microalgae species, its acclimated state, frequency of changing light/dark period, and the duration of exposure [26,71]

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Mixing and aeration

Optimal mixing is required to enhance CO_2 distribution and simultaneously strip O_2 that, otherwise, could inhibit the photosynthesis [1]. Various

cultivation associated with mixing strategies have been applied, as follow: (i) mechanical stirring systems like paddle wheel and baffles; (ii) gas injection like bubble diffuser; (iii) membrane-sparged device. With optimised aeration and stirring system, *Chlorella sp*. achieved CO_2 bioconversion efficiency of 58%, 27%, 20% and 16% under the CO_2 concentration of 2%, 5%, 10% and 15% (v/v), respectively [19]. The mixing systems, although contribute to transfer CO_2 from gas to liquid, present some disadvantages, such as: (i) loss of CO_2 to the atmosphere; (ii) bio-fouling of membrane and diffusers; (iii) shear damage to cells; (iv) large energy input; and (v) poor mass transfer to relatively low interfacial surface area. The appropriate flow and mixing have to be set to achieve elevate CO_2 fixation performance [1].

In order to overcome the high light attenuation in open ponds, mixing is essential as it ensures all cells are at least briefly exposed to saturating light at frequent time scales, allowing for high productivities to be supported in the light limited pond [43]. Ideally, cells in the euphotic zone should be optimally exposed to light for the very short duration required for the light-reaction, then moved into the dark zone while being replaced by cells from the dark zone receptive to incoming photons [72]. Laminar flows are common along the long channels of full-scale open ponds, and both photosynthetic efficiency and microalgal productivity can be reduced [73]. Several studies have successfully demonstrated that, increasing vertical mixing, microalgal photosynthesis and productivity increased, thanks to optimized light/dark cycles, named as ‘‘flashing light effect’’ [74]. A layer of water with reduced velocity, termed the boundary layer, surrounds each microalgal and the thickness of this layer affects the rates of nutrients diffusion and gas exchange between cell and external environment [75]. Increased mixing can promote nutrients uptake under limiting conditions, leading to enhanced growth [76]. Grobbelaar (1994) [77] showed that increased turbulence enhances the exchange rates of nutrients and metabolites between microalgal cells and the surrounding environment in photobioreactors. When coupled with increased medium frequency light/dark cycles, the increased exchange of nutrients resulted in higher productivity and photosynthetic efficiency [77]. Mixing also prevents sedimentation of cells on the bottom of the ponds. Mixing frequency as well as mixing velocity, are likely involved to play a critical role for maintaining desirable large colonies in open ponds. Understanding how the frequency of mixing events affects the performance of microalgae,

including photosynthesis efficiency, productivity rate, nutrient removal efficiency, as well as physiological and morphological adaptations, is important for enhancing wastewater treatment and biomass yields [43].

Culture pH

The pH of the culture media is an important factor affecting algae growth. Usually, acidic media (pH 5–7) is favorable for the growth of freshwater eukaryotic algae while alkaline media (pH 7–9) is beneficial for the growth of cyanobacteria (blue-green algae) [39]. Microalgae species grow well in optimal pH ranges. *Synechococcus sp*. and *Spirulina platensis* grow at optimal pH 6.8 and pH 9, respectively, meanwhile *Chlorella sp*. can tolerate pH below 4 [19,78].

In open systems, pH varies over the day, increasing with photosynthetic reduction of carbon and decreasing overnight with respiration [43]. Afternoon pH values above 10 are not uncommon in open ponds, particularly during summer [79]. High pH in ponds can negatively affect microalgal photosynthetic rates in several ways. As mentioned above, pH shifts the DIC species equilibrium with a reduction of available CO_2 with increasing pH. In addition, elevated pH interferes with the cell's ability to maintain the activity of the RuBisCO enzyme catalysing photosynthetic carboxylation, thus limiting photosynthesis [80]. High pH also results in the dissociation of ammonium ion to free ammonia, which inhibits microalgae growth over certain threshold [53]. Elevated pH can also negatively impact photosynthesis and growth of microalgae through the alteration of membrane transport processes, metabolic function and uptake of trace metals [81]. Flocculation of some microalgal species can occur under elevated pH, which may negatively impact on light absorption, photosynthesis and nutrient uptake, even if it can make easy the biomass harvesting [82]. Elevated pH can also negatively impact on wastewater treatment through the inhibition of aerobic bacteria, whose growth is increasingly inhibited at pH > 8.3 [53].

Furthermore, if 10–20% (v/v) of CO_2 from flue gas is supplied, pH of medium can be reduced reaching a value of 5.5 [19,83]. To certain extent, this can be counterbalanced by CO_2 uptake from microalgae which will undoubtedly cause pH rising. Furthermore, some microalgae species are unable to withstand the acidic condition set by the carbonic acid formed from CO_2 dissolution in medium [21]. Sodium hydroxide and calcium carbonate are

usually used to adjust pH reaching its optimal range, thus aiming to provide excellent CO_2 bioconversion and biomass production [84].

Culture temperature

Temperature has noticeable effects on the microalgae growth and biomass production, because it affects the metabolic process and the biological reaction rate [24]. Seasonal and daily fluctuations of climate conditions make it difficult to control the temperature within a specific range for outdoor microalgae cultivation. This is especially relevant, in cold days, when the relatively low temperature affects the microalgae growth. This problem can be faced by developing microalgae culture in conjunction with a relatively warm CO_2 source. On the other hand, in summer days when the temperature is very high, evaporative cooling can be used to favour the best conditions for microalgae culturing. This can be achieved by spraying water into the cultivation tank [24]. Indoor cultures have actually the advantage to better control the temperature. Therefore, by placing the photobioreactor in a special room with a set temperature, the microalgae medium can be kept at a set temperature [39].

Most microalgal species have an optimum temperature range between 15 and 25 °C, and at temperatures above or below this range biomass yields are negatively affected. At sub-optimum temperatures, microalgal photosynthesis saturates at lower light intensities, whereas at supra-optimum temperatures respiration and photorespiration rates rapidly increase [85]. Variations in both temperature and solar radiation have been found to affect nutrients removal rates of microalgae [86]. Temperature also affects the solubility of gases in the pond water, including O_2 and CO_2, as well as pond pH [43].

Microalgae for wastewater treatment

The use of algae to treat wastewater has been in vogue for over 40 years: one of the first descriptions of this application has been reported by Oswald [87]. The use of microalgae for the treatment of municipal wastewater has been a subject of research and development for several decades. An extensive work has been conducted to explore the feasibility of using microalgae for

wastewater treatment, especially for the removal of nitrogen and phosphorus from effluents [88,89], which would otherwise result in eutrophication if discharged into lakes and rivers [6]. Concentrations of several heavy metals have also been found to be reduced by the cultivation of microalgae, which is a subject discussed extensively by Munoz et al. [90]. Biological treatment enhances the removal of nutrients, heavy metals and pathogens and furnish O_2 to heterotrophic aerobic bacteria to mineralize organic pollutants, using in turn the CO_2 released from bacterial respiration (Figure 6, [90]). Photosynthetic aeration is therefore especially interesting to reduce operational costs and limit the risks for pollutant volatilization under mechanical aeration. Recent studies have furthermore shown that microalgae can support the aerobic degradation of various hazardous contaminants [90,91]. The mechanisms involved in microalgae nutrients removal from industrial wastewaters are similar to those of domestic wastewaters treatment [49].

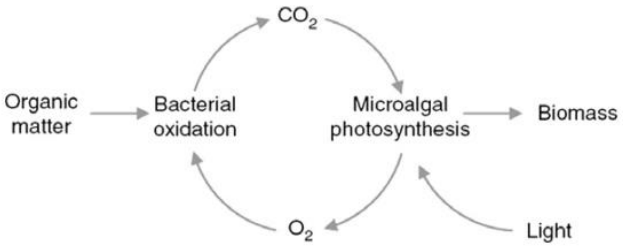

Figure 6. – Principle of photosynthetic oxygenation in BOD removal process.

Microalgae harvesting

The technology used for the recovery of microalgae is considered to have the most influential effect on the economy of microalgae production [92,93]. The selection of harvesting technology is dependent on many factors including cells type, their density and size, alongside downstream processing requirements and the value of the end products [93]. Many harvesting techniques have been developed over the past four decades; however they can generally be broken down into technologies that are used in a one or two stage process [94]. During the primary or bulk harvesting, the biomass is concentrated to 2–7% total suspended solids (TSS); this can be achieved

using flocculation, flotation and/or sedimentation. This is followed by a secondary dewatering or thickening step, which produces an algal cake with 15–25% TSS, this is achieved with filtration or centrifugation, and is often more energy intensive than primary harvesting [5,93]. For almost applications, microalgal harvesting generally comprises the two-step concentration method: thickening and dewatering (Figure 7, [94]). These stages are crucial to obtain thick algal slurry from the initial suspension and to enable further downstream processes [60,95]. Microalgal harvesting currently involves mechanical, chemical, biological and, to a lesser extent, electrical based methods. It is very common to combine two or more of these methods to obtain a greater separation rate at lower costs. In fact, the combination of flocculation–sedimentation with centrifugation can significantly reduce process costs [96]. Biological approaches are emerging techniques that can lead to further reduction of operational costs. Mechanical methods are the most reliable and therefore the most commonly used to harvest microalgal biomass [97,98]. However, these methods are often preceded by a chemical or biological coagulation/flocculation thickening stage to improve effectiveness and to reduce operation and maintenance costs [94].

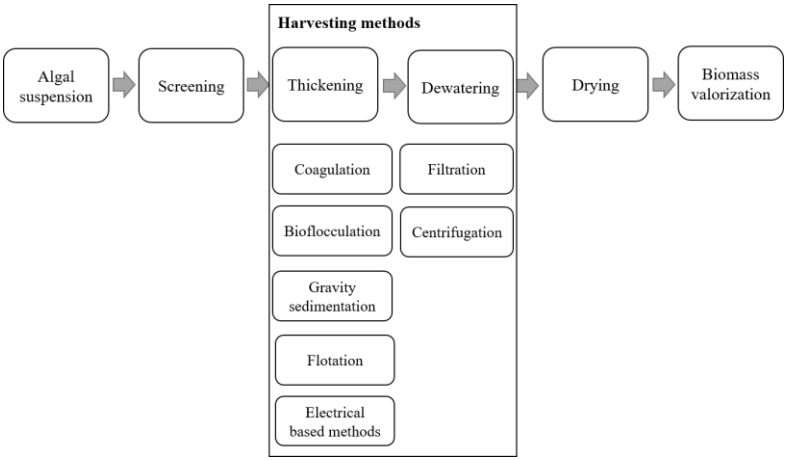

Figure 7. – Diagram of microalgal harvesting and drying techniques.

In table 3 [94] are reported the main advantages and disadvantages of the most commonly used methods for microalgal harvesting that are described in the following sections.

Table 3. – Advantages and disadvantages of different harvesting methods applied to microalgal biomass.

Harvesting methods	**Advantages**	**Disadvantages**
Chemical coagulation/flocculation	• Simple and fast method • No energy requirements	• Chemical flocculants may be expensive and toxic to microalgal biomass • Recycling of culture medium is limited
Auto and bioflocculation	• Inexpensive method • Allows culture medium recycling • Non-toxic to microalgal biomass	• Changes in cellular composition • Possibility of microbiological contamination
Gravity sedimentation	• Simple and inexpensive method	• Time-consulting • Possibility of biomass deterioration • Low concentration of the algal cake
Flotation	• Feasible for large scale applications • Low cost method • Low space requirements • Short operation times	• Unfeasible for marine microalgae harvesting
Electrical based processes	• Applicable to a wide variety of microalgal species • Do not require the addition of chemical flocculants	• Poorly disseminated • High energetic and equipment costs
Filtration	• High recovery efficiencies • Allows the separation of shear sensitive species	• Possibility of fouling increases operational costs • Membrane should be regulatory cleaned

		• Membrane replacement and pumping represent the major associated cost
Centrifugation	• Fast method • High recovery efficiencies • Suitable for almost all microalgal species	• Expensive method • High energy requirements • Suitable only for the recovery of high value products • Possibility of cells damage due to high shear forces

Chemical coagulation/flocculation

Due to the negative electric charges on cell surface and the small particle size of most algae, natural sedimentation rates can be very slow. Coagulation–flocculation is the process of aggregating single cells to larger flocs, thus overcoming the hurdle of repulsion with equicharged particles [99]. Coagulation–flocculation has been extensively researched and is commonly used since it is standard practice in water treatment and mining operations. The coagulation–flocculation process can be induced by adding coagulating metal salts (e.g. alum or ferric chloride) that ionize in the liquid and neutralize the surface charge of the algae. At a high pH, metal hydroxides are formed, which tend to precipitate on the flocs and cause physical linkages between algae, thus increasing the density of the biomass [99]. Specialized polymers work in a similar way, stabilizing the algal cells' electronegative charge thanks to the polymer adsorbing onto the surface of cell walls, which links and binds cells together; this process is known as bridging [100]. In addition, these polymers tend to neutralize and also may reverse the sign of electric charge on the surface of the algae, creating a compatible surface for electrostatic interaction between differently charged cells [101]. Chemical flocculation is carried out by adding chemicals of two different natures: inorganic or organic. The majority of inorganic chemical flocculants are based on multivalent cations such as aluminium sulphate, ferric chloride and ferric sulfate. Organic flocculants are derived from polyacrylamide or polyethylene imine. These polymers can be cationic, anionic, or non-ionic. Polymer dosage significantly affects the flocculation efficiency: less than the

optimum amount will result in weak bridging, thus resulting in flocs that will easily be broken up, whereas if the dosage is too high bridging potential can be reduced due to electrostatic/static hindering [102]. The toxicity of the chemicals used in flocculation is often problematic as biomass can be contaminated, limiting its applicability e.g. for food or feed purposes downstream.

Auto and bioflocculation

Despite being different phenomena, it is common to refer to auto and bioflocculation as being the same concept [94]. Autoflocculation (flocculation merely by pH increase) is an attractive alternative, as it is low cost, low energy, non-toxic to microalgae and does not require the use of flocculants, enabling simple medium reuse [103]. Autoflocculation is induced at high pH, typically above pH 9 [104], caused by the consumption of dissolved carbon dioxide. In this condition, the cell wall can interact with divalent cations [97]. An increase of pH causes super-saturation of calcium and phosphate ions, resulting in a positively charged calcium phosphate precipitate which will result in a neutralization of the negatively charged algae cells [97]. It has been proven that a pH value higher than 10 creates rapid aggregates in certain microalgae species. Indeed, Knuckey et al. [105] noticed that above pH 10 the flocs formed had a more "robust" structure and settled faster than those of a lower pH. Settling efficiencies of 97±2% were also achieved at pH 10 for *Scenedesmus* [105]. Although it occurs often on a lab scale, autoflocculation still needs to be demonstrated at a significant scale [106] and a more comprehensive understanding of the mechanisms involved and how to control them is required.

Bioflocculation relates to microalgal flocculation caused by secreted biopolymers, especially by EPS (extra polymeric substances) [107]. Flocculants produced by bacteria can be an important economical step towards sustainable microalgal based biofuel production. Bioflocculation eliminates the need for chemical flocculants, which represent an expensive, non-feasible and toxic alternative. However, co-culture of microalgae with bacteria, fungi or flocculating microalgae results in microbiological contamination, interfering with food or feed applications of microalgal biomass [101]. In the case of biofuel production, the added microorganisms may even contribute to the increase in lipid yields [108]. The success of

microbial flocculation depends on the production of EPS by the bacteria in high concentrations and the ability of microalgae to attach to them to form flocs [109]. Microbial flocculants have been widely used for wastewater treatment, as the wastewater can provide the necessary carbon source for flocculating microorganisms [101].

Gravity sedimentation

Despite the rudimental character of the process, sedimentation works for various types of microalgae is highly energy efficient [110]. Thus, when the end product has extremely low value, such as biofuels, gravity sedimentation should be selected for microalgal harvesting. Since microalgal density is a key to ensure the process efficiency, the reliability of this method is low. Microalgal settling rates of 0.1–2.6 cm h^{-1} result in a very slow sedimentation process that leads to the deterioration of most of the biomass during the settling time, limiting the application of this method for routine harvesting [97]. In this way, to fasten microalgal settling, it is common to apply a coagulation/flocculation step prior to gravity sedimentation [5,98]. The best results of microalgal harvesting using gravity sedimentation were achieved through lamella-type separators (recovery of 1.6% TSS) and sedimentation tanks (recovery of 3% TSS) attributable to microalgal autoflocculation [111]. The use of sedimentation tanks is viewed as a simple and inexpensive process, but the concentration achieved is very low without previous coagulation/flocculation. In the same way, microalgal concentration by lamella-type separators is low and unreliable, requiring further thickening [94].

Flotation

Flotation is often defined as “inverted” sedimentation where gas bubbles fed to the broth provide the lifting force needed for particle transport and separation. This process is commonly applied in wastewater treatment processes and is often preceded by coagulation/flocculation [112]. The success of flotation can be described as a product of two probabilities: (i) bubble-particle collision; and (ii) bubble-particle adhesion after a collision has occurred. In this way, it depends on the instability of the suspended particle, lower instability will result in higher air-particle contact, and on particle size, the smaller they are, the more likely they are to be lifted up by the bubbles [111]. Particles in suspension must be hydrophobic, in order to

attach to gas bubbles [113]. Flotation has been successfully applied in the separation of freshwater microalgae, such as *C. vulgaris*, and it is a promising low cost harvesting method at large scale [114]. On the other hand, flotation of marine microalgae may be compromised, as salinity is a key factor for bubble–cell adhesion [114]. Under high ionic strength, gas bubbles were reported to be larger and with tendency to rupture more easily. At controlled pH, an increase in ionic strength of the medium results in a decrease of flotation efficiency from 90–92% to 32% [114]. In this process, coagulation can be applied, by suppression of the electrical double layer, leading to floc formation [113]. Microalgal removal depends on recycling rate, air tank pressure, hydraulic retention time and particle floating rate, while the concentration of the produced slurry depends on skimmer velocity and relative positions towards the surface of the water [115].

Electrical based processes

Electrical approaches to microalgal harvesting are not largely disseminated. Nonetheless, these methods are versatile, as they are applicable to a wide variety of microalgal species, while being environmentally friendly (they do not require the addition of chemicals) [115]. As microalgal cells are negatively charged, when an electrical field is applied to the culture broth, the cells can be separated [115]. They can form precipitates on the electrodes (electrophoresis), as well as accumulate on the bottom of the vessel (electro-flocculation). Alternatively, electro-flotation mechanism could be applied: hydrogen bubbles are formed through water electrolysis [111]. The generation of these bubbles can be done at the anode and coupled with the electro-coagulation that occurs through the electrolytic oxidation that happens at the cathode [116]. This process is described as electro-coagulation-flotation [116].

Filtration

Filtration is mainly a dewatering means and it is normally applied following coagulation/flocculation to improve harvesting efficiency. Its application requires the maintenance of a pressure drop across the system to force fluid flow through a membrane. In this process, microalgal deposits on the filtration membrane tend to grow thicker throughout the process, increasing resistance and decreasing filtration flux upon a constant pressure drop [111]. This phenomenon (called fouling/clogging) represents the main draw- back

associated to filtration methods, increasing their operational costs [97]. Critical flux is defined as the lowest flux that creates irreversible deposit on the membrane. However, limiting flux represents the maximum stationary permeation flux that can be reached, for a given tangential velocity, by increasing trans-membrane pressure. Therefore, with the purpose of optimizing performance and minimize cleaning steps, it is necessary to work in the sub-critical zone. Nevertheless, even working at these conditions, gradual minor fouling can occur followed by a drastic increase that requires chemical cleaning. This phenomenon is further affected by the production of EPS, commonly secreted by microalgae when in stress conditions. These substances cause a gel-like layer in the filtration cake, increasing the resistance to flow, also requiring chemical cleaning to be eliminated [117]. Membranes must then be regularly cleaned to ensure sanitization and reusability. Filtration is only sustainable for harvesting long length microalgae or those forming large colonies [118]. Despite microalgal cells of very low densities can be harvested by this method (a major advantage), membrane filtration is not commonly applied in large scale processes [98].

Centrifugation

Centrifugation is the fastest harvesting method, but also the most expensive due to its high energy consumption, which limits its application to high-valued products, such as highly unsaturated fatty acids, pharmaceuticals and other commodities [97,98,110]. Centrifuges are able to harvest the great majority of microalgae [110]. However, there are evidences that the exposure of microalgal cells to high gravitational and shear forces results in cell structure damage [95]. Normally, centrifuges are set to maximize capture efficiency. However, cost-effective microalgal harvesting may not coincide with the maximum capture efficiency [119]. To achieve high harvesting efficiencies, longer retention times in the bowl are needed to enable their sedimentation, due to the small size of these cells. While high capture efficiency (slower flow rates) required more energy per volume of culture, lower recoveries were offset by the increase in the processed volume. This low energy conditions result in a decrease in overall cost per litter of produced oil [119].

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Chapter 3

Paper in review at

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Effect of light intensity and nutrients supply on biomass production, lipids accumulation and settleability characteristics of microalgae cultivated in urban wastewater

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ABSTRACT

Microalgae cultivation systems fed with wastewater as source of nutrients represents the sole suitable approach to produce microalgal biomass to be converted conveniently to biofuels. In order to optimize microalgae growth and their lipid content, the effect of light intensity and nutrients load in real wastewater was investigated through batch microalgal cultivation tests. A microalgal polyculture was used as inoculum and grown for 10 days in batch at different conditions of light intensity (i.e. 20, 50 and 100 µmol $s^{-1}m^{-2}$) and nutrients concentration in wastewater. Experimental results showed that biomass productivity decreased when nutrients concentration increased and increased when light intensity increased. The highest lipid mass content (29%) was found for the highest light intensity condition (100 µmol $s^{-1}m^{-2}$). Furthermore, microalgae settleability tests, conducted at the end of the cultivation time, resulted in the highest biomass recovery efficiency (72%) for the lowest light intensity and nutrients supply conditions.

Introduction

Microalgae are currently the most promising renewable feedstock for biodiesel production due to their more efficient photosynthetic process, higher growth rate and consequently faster biomass production compared to other energy crops [1–3]. Nevertheless, their use is limited by expensive operating costs [4] that can be summarized as follow: (i) cultivation system design and construction, (ii) nutrients supply, (iii) biomass harvesting operation. Recent studies [5,6] have stated that microalgae cultivation using wastewaters as nutrients source are currently the sole economically viable way to produce algal biomass for conversion to biofuels. Concerning the microalgae cultivation systems, the configuration that maximizes the biomass production efficiency is still object of studies as well as a highly efficient and economic harvesting method.

It is well-known that microalgal growth is affected by a combination of several operating parameters such as light intensity, photoperiod, temperature and nutrients availability in the growth medium [7,8]. Among them, light supply greatly affects not only the microalgal photosynthesis, cells composition and metabolic pathways, but also the economic efficiency of microalgal cultivation process [9,10]. Consequently, supply and efficient utilization of light energy have been the greatest scientific and technological challenge in research and development of microalgal commercial cultivation. Moreover, the concentration of nitrogen and phosphorus in the growth medium is considered to be a fundamental factor and has a direct influence on microalgal growth kinetics, which closely relates to nutrient removal and lipid accumulation [11]. In this context, effects of different culture medium and light intensity have been studied in synthetic growth medium for *Ankistrodesmus falcatus* [12], *Chlorella sp*. and *Monoraphidium sp* [10], *Scenedesmus obliquus* [13*]*, *Nannochloropsis sp*. [14]. Nutrients reduction under different light intensities has been also investigated for microalgae cultivation in biogas slurry [15]. Anyway, light intensity and nutrients supply variations for microalgae cultivation have never been studied using urban wastewater as growth medium.

Therefore, this work is focused on the evaluation of the effects of light intensities and nutrients supply on microalgal growth in urban wastewater, setting as targets of the process performance the following aspects: the microalgal biomass growth rate, the efficiency of nutrients removal from

growth medium, the settleability of microalgal biomass by autoflocculation and the amount of lipids accumulated in microalgae cells.

Materials and methods

Microalge inoculum cultivation

Microalgal inoculum used for cultivation tests was collected from the inner walls of the secondary clarifier of the urban wastewater treatment plant (WWTP) located in Pesche (Italy) and further grown in Bold Basal Medium (BBM, [16]) under controlled conditions of continuous and homogeneous light (Cool White Fluorescent Lamps, 20 µmol s^{-1} m^{-2}) as well as at the temperature of 25±2 °C. The medium is composed of the following elements: 250 mg L^{-1} $NaNO_3$, 25 mg L^{-1} $CaCl_2{\cdot}2H_2O$, 75 mg L^{-1} $MgSO_4{\cdot}7H_2O$, 75 mg L^{-1} K_2HPO_4, 175 mg L^{-1} KH_2PO_4, 25 mg L^{-1} NaCl, 11.4 mg L^{-1} H_3BO_3, alkaline EDTA solution (50 mg L^{-1} EDTA, 31 mg L^{-1} KOH), acidified Iron solution (5 mg L^{-1} $FeSO_4{\cdot}7H_2O$, 1 mg L^{-1} H_2SO_4), trace metals solution (8.8 mg L^{-1} $ZnSO_4{\cdot}7H_2O$, 1.4 mg L^{-1} $MnCl_2{\cdot}4H_2O$, 0.7 mg L^{-1} MoO_3, 1.6 mg L^{-1} $CuSO_4{\cdot}5H_2O$, 0.5 mg L^{-1} $Co(NO_3)_2{\cdot}6H_2O$. The cultivation was conducted in 1 L flask equipped with a magnetic stirring bar (150 rpm) which maintained the microalgal biomass in suspended condition. Images obtained from optical microscope (Primostar Zeiss, Axiocam ERc 5s) analysis showed that microalgal biomass was mainly composed of cyanobacteria (60%) and chlorophyte (30% *Chlorella* sp. and 20% *Scenedesmus* sp.), collectively named microalgae in this work.

Source of nutrients

With the aim of having different concentrations of nutrients, samples of real urban wastewater were collected from the inlet channel of the WWTP of Pesche (Italy) in 3 different times of the month. Before using the collected samples for experimental tests, they were left to settle for one night, and the resulting surnatants were used to feed microalgae in batch tests. Physical and chemical characteristics of the settled samples of wastewater, respectively named L (low), M (medium) and H (high) depending on their nutrients load, are reported in Table 1.

Table 1. – Pysicochemical characteristics of urban wastewater used as culture medium.

Parameter	***Low nutrients load (L)***	***Medium nutrients load (M)***	***High nutrients load (H)***
pH	7.0 ± 0.1	7.2 ± 0.3	7.4 ± 0.2
Dissolved oxygen (mg/L)	3.4 ± 1.2	4.7 ± 2.1	4.1 ± 1.8
TSS (mg/L)	70 ± 10	78 ± 6	83 ± 11
COD (mg O_2/L)	220 ± 10	240 ± 13	270 ± 8
Alkalinity (mg $CaCO_3$/L)	370 ± 20	351 ± 25	339 ± 17
NH_4^+ (mg/L)	9.8 ± 0.3	21.0 ± 1.2	31.4 ± 0.6
PO_4^{3-} (mg/L)	6.4 ± 0.2	7.2 ± 0.3	9.9 ± 0.6
NO_2^- (mg/L)	1.7 ± 0.2	1.2 ± 0.5	<1
NO_3^- (mg/L)	3.3 ± 0.5	3.2 ± 0.6	3.1 ± 0.8
N/P	4.1 ± 0.6	7.2 ± 1.3	7.6 ± 1.7

Experiments design and setup

In order to evaluate the effect of two parameters (i.e. light intensity and nutrients supply) involved in microalgal growth process, two different series of cultivation batch tests were conducted, each of them focused on the study of a sole parameter.

The first series was actually designed keeping constant the light intensity (i.e. 20 umol s^{-1} m^{-2}, that is the same value set for growing microalgal inoculum in BBM, under controlled conditions) and varying the amount of nutrients supplied with wastewater. Combining the value of light intensity with nutrients load in samples of wastewater (see Table 1), tests were labelled respectively as L20, M20 and H20.

The second series of tests was designed keeping constant the load of nutrients in samples from wastewater (i.e. corresponding to low load condition) and varying the light intensity (i.e. 20, 50 and 100 µmol $s^{-1}m^{-2}$). Using the same criterion for naming tests of the first series of experiments, they were labelled

respectively as L20, L50, L100. Experimental setup is summarized in Table 2.

Microalgal cultivation batch tests were conducted in triplicate in 500 mL glass bottles filled with samples of real wastewaters and equipped with a magnetic external stirrer, working continuously at 200 rpm. Microalgae inoculum was added to the samples up to set a volumetric ratio of 3% between inoculum and culture medium volume. An optical density of 0.1 abs was detected for all tests at the initial cultivation time.

Once inoculated, bottles were placed in a light incubator under controlled temperature condition (25 ± 2°C). In order to have homogeneous light conditions and regulate the light intensity, bulbs (PHILIPS Tornado 23WE27 fluorescent lamp; Philips Co., China) were mounted on both sides of the incubator. Cultivation time was set equal to 10 days for all tests.

Table 2. – Experimental Setup.

		Light intensity ***(µmol $s^{-1}m^{-2}$)***	***Nutrients load***
Series 1	*L20*	*20*	*Low*
	M20	*20*	*Medium*
	H20	*20*	*High*
Series 2	*L20*	*20*	*Low*
	L50	*50*	*Low*
	L100	*100*	*Low*

Analytical methods

Microalgal biomass growth evaluation

Microalgal biomass growth was evaluated measuring daily the optical density (OD550) of samples taken from each bottles at the wavelength of 550nm with an UV/Visible spectrophotometer (Shimadzu UV-1601). This specific wavelength is recommended for microalgae polyculture [17]. The relationship between microalgal dry cells weight (DCW, mg/L) and OD550 (abs) is shown in Equation 1:

$$DCW = 400.45\ OD550 + 6.9587;\ R^2 = 0.996 \quad (1)$$

The dry cells weight of microalgal biomass was determined using the method of suspended solid (SS) measurement [11]. The microalgal biomass productivity (P, mg/L/d) was calculated according to Equation 2:

$$P = \frac{DCW_t - DCW_o}{t - t_0} \quad (2)$$

where DCW_0 (mg/L) is the biomass concentration at time t_0 (d) and DCW_t (mg/L) is the biomass concentration at any time t(d) of the cultivation test following t_0(d).

Chemical analysis

Dissolved oxygen (DO) concentration and temperature (T) were determined using DO probe equipped with temperature sensor (YSI 550 DO). Values of pH were measured using a pH meter (HI 8424, Hanna). Both probes were calibrated daily with standard buffers. Light intensity (µmol/m^2/s) was measured by a digital lux meter (MS6612, RoHS).

Dissolved inorganic carbon (DIC) concentration in aqueous phase, as free CO_2, HCO_3^- and CO_3^{2-}, was calculated according to the standard methods [18]. Nutrients concentrations, as N-NH_4^+, N-NO_2^-, N-NO_3^-, P-PO_4^{3-} ions, were determined using Liquid Ion Chromatography (Dionex, ICS 1000).

Chemical Oxygen Demand (COD) was measured according to the standard methods [18], whereas total lipid content was determined by sulfo-phospho-vanillin method [19].

The removal rate R_i (mg/L/d) of the generic substrate i in the growth medium was calculated according to Equation 3.

$$R_i = \frac{S_{0,i} - S_i}{t - t_0} \quad (3)$$

where $S_{0,i}$ (mg/L) is the initial concentration of substrate i at time t_o (d), S_i (mg/L) is the corresponding substrate concentration at time t (d).

In order to quantify theoretically the fraction of ammonia that was stripped, free ammonia concentration was calculated according to Equation 4 [20]:

$$\frac{[NH_3]}{[TNH_3]} = \left(1 + \frac{10^{-pH}}{10^{-\left(0.09018 + \frac{2729.92}{T(K)}\right)}}\right)^{-1} \quad (4)$$

where $[NH_3]$ is the concentration of free ammonia, $[TNH_3]$ is the total ammonia concentration and T (K) is the temperature (kelvin).

Biomass settling and recovery

Settling tests were conducted at the end of the cultivation time (i.e. 10 days) in 500 mL glass cylinders under static hydraulic conditions for 30 minutes [21]. Optical density at 550 nm of wavelength was measured for liquid samples collected at the centre of each cylinder after 5, 20 and 30 minutes. The efficiency (η) of microalgal biomass recovery was estimated according to the following Equation 5:

$$\eta = \frac{OD\ 550^0 - OD\ 550^t}{OD\ 550^0}\% \quad (5)$$

where OD 550^0 (abs) is the OD at time zero and OD 550^t (abs) is OD measured at times 5, 20 and 30 minutes [22].

Results and discussion

Microalgal biomass growth rate

The typical growth of microalgae in batch condition, composed of four successive phases [16,23], was observed for all tests (Fig. 1): (phase 1) an initial period of physiological adaptation (lag phase) due to changes in operating cultivation conditions; (phase 2) an exponential growth phase when the microalgae, once adapted to the current operating conditions, begin to grow and multiply at constant rate; (phase 3) a stationary phase when microalgal biomass growth rate is next to zero as a result of nutrients depletion in the culture medium; and finally, (phase 4) a decline phase characterized by a decrease in microalgal biomass concentration as consequence of nutrients absence.

Microalgal biomass growth rates (P) reported in Table 3 were calculated applying Equation 2 for all tests in correspondence of the growth exponential phase (phase 2).

Results from the first series of tests (i.e. constant light intensity of 20 umol $s^{-1}m^{-2}$ and varying nutrients concentration) are displayed in Figure 1 and show the following findings: (i) the lag phase was longer when the nutrients concentration was lower, (ii) the highest microalgal biomass concentration in medium, equal to 211 ± 18 mg/L, was achieved for the lowest nutrients concentration and showed an average growth rate of 39.6 ± 1.4 mg/L/d.

Results from the second series of tests (i.e. constant growth medium characteristics and varying light intensity) are shown in Figure 1 and highlight the following outcomes: (i) the lag phase was longer when light intensity was set lower; (ii) growth decline phase started earlier when the light intensity was set higher; (iii) the highest microalgal biomass concentration of 227 ± 16 mg/L was achieved for the highest light intensity of 100 umol $s^{-1}m^{-2}$, showing an average growth rate of 58.7 ± 2.3 mg/L/d.

Biomass production generally performed better when light intensity increased in the range 20-100 umol $s^{-1}m^{-2}$, whereas performed worse when nutrients concentrations increased.

Although studies from the literature [11,24] found a positive direct dependence between microalgal growth rate and nutrients concentration in the culture medium, when concentration values are set distant from the inhibition threshold, a similar result was not obtained in the present work as no significant difference is noticeable from the first series of tests; at the most, an inverse tendency resulted. Actually, L. Xin et al. [11] and B. Wang et al. [24] tested *Scenedesmus sp.* and *N. Oleobundans sp.* respectively in medium characterized by an increase of nitrogen concentrations: both works show an increasing biomass growth rate when the initial nitrogen concentrations was set higher. Such divergent results are reasonably affected by different light sensitiveness characterizing the microalgae species [10]. Furthermore, the particular light condition set in first series of tests (i.e. 20 umol $s^{-1}m^{-2}$) limited the microalgal metabolism if these results are compared with those from the second series of tests.

Table 3. – Microalgal growth rate (P) calculated during microalgal exponential growth phase; dissolved inorganic carbon (DIC) removal rate calculated during microalgal exponential growth phase; maximum biomass concentration (DCW max) during cultivation time; total lipids mass (%fat/dried) in dry cell measured at the end of the cultivation time.

SERIES	***ID test***	***P (mg/L/d)***	***DIC (mg/L/d)***	***DCW max (mg/L)***	***% fat/dried***
1	L20	39.6 ± 1.4	22.7 ± 3.78	211 ± 18	23.4 ± 1.1
	M20	28.0 ± 1.8	15.7 ± 0. 73	187 ± 13	20.2 ± 0.7
	H20	18.5 ± 0.7	11.3 ± 1.44	159 ± 21	16.6 ± 1.4

	L20	39.6 ± 1.4	22.7 ± 3.78	211 ± 18	23.4 ± 1.1
2	L50	50.4 ± 1.6	29.2 ± 2.72	219 ± 19	27.6 ± 1.3
	L100	58.7 ± 2.3	32.6 ± 0.35	227 ± 16	29.4 ± 2.2

Microalgal growth trend in both series of tests are consistent with DIC and pH trends. It is well-known [25] that microalgal growth and their speciation in cultivation system depend on both abiotic (e.g. pH, temperature, light, salts concentrations) and biotic (e.g. interactions with other microorganisms as bacteria) factors, thus microalgae metabolism can be autotrophic or heterotrophic. In the first condition (i.e. autotrophic metabolism), microalgae consume DIC during the photosynthetic activity, whereas in the second condition (i.e. heterotrophic metabolism), they use organic carbon. In the present work, as COD in the culture medium was relatively low (220 – 270 mg/L) and tests were conducted under continuous and constant light conditions, the autotrophic metabolism was predominant.

DIC is the sum of carbon dioxide (CO_2), bicarbonate ions (HCO_3^-) and carbonate ions (CO_3^{2-}) concentration and the amounts of each species depend on pH and temperature. In the autotrophic metabolism condition microalgae preferentially uptake CO_2 through passive diffusion, rather than HCO_3^- which requires an active transport with higher energy consumption [26]. According to the pH values measured in the culture medium (Figures 1e and 1f), DIC is mostly present in the form of HCO_3^- and CO_3^{2-}, with negligible available CO_2. Trends of HCO_3^- and CO_3^{2-} concentration with time are shown in Figure 1c and 1d: initially DIC was mostly present as HCO_3^- in all culture media, while CO_3^{2-} was predominant next to the end of the cultivation time as consequence of pH increase. As expected, for all tests DIC consumption occurred mainly during the exponential growth phase showing values around zero at the end of such phase.

The decreasing trend of DIC concentration in growth medium with time is the result of two prevailing mechanisms: microalgae uptake and salt precipitation (as calcium and magnesium) for high pH. DIC consumption rate was calculated during the exponential growth phase using Equation 3 and the corresponding results are reported in table 2: from the first series of tests (i.e.

constant light intensity of 20 umol $s^{-1}m^{-2}$ and varying nutrients concentration) DIC consumption rate resulted lower when nutrients concentration increased, whereas from the second series of tests (i.e. constant growth medium characteristics and varying light intensity) it resulted higherwhen light intensity increased.

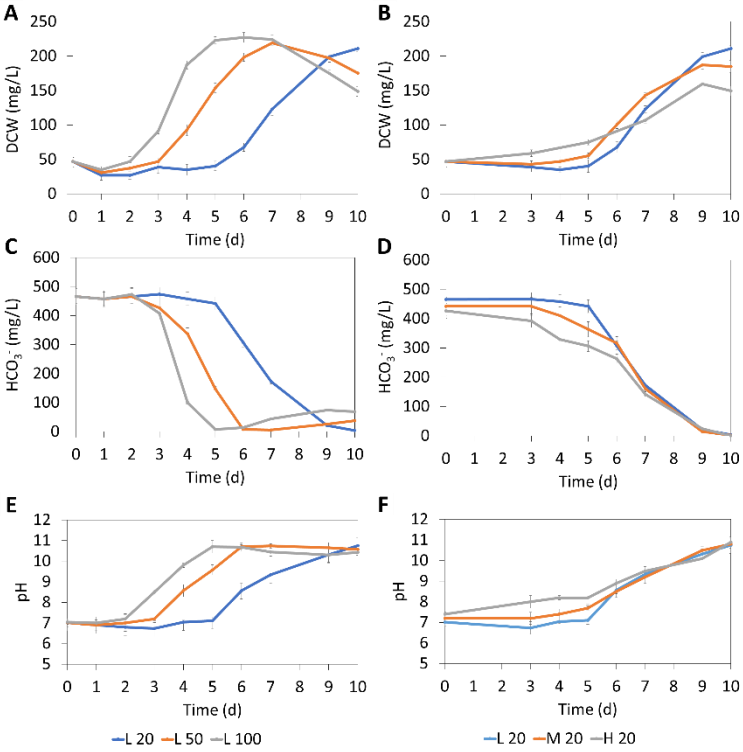

Figure 1. – Microalgal growth measured as dry cells weight (DCW); DIC measured as bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}); pH trend from the first series of tests (a,c,e) and second series of tests (b,d,e), respectively.

Microalgal growth affects pH in the culture medium. Actually, DIC consumption, as consequence of microalgal photosynthesis, was responsible for the increase of pH in the medium. Trend of pH is shown in Figures 1e and 1f: during the lag phase, pH remained almost stable at the starting value;

during the exponential growth phase pH increased significantly; stationary and decline growth phase kept the pH stable and caused a slight drop, respectively. As soon as strong alkaline conditions were reached in the culture medium (i.e pH > 10) microalgae stopped growing, therefore pH inhibited their growth more than DIC and nutrients depletion or light scarcity. Actually, high pH value can negatively affect microalgal photosynthetic activity in several ways: (i) DIC is mostly in the form of HCO_3^- and CO_3^{2-} with negligible available CO_2, thus creating conditions where the microalgal metabolisms is thermodynamically disadvantaged [27]; (ii) ammonium ion is mainly dissociated to free ammonia, which has been found to be responsible for reducing microalgal photosynthetic activity [28]; (iii) cell membrane transport processes are altered, thus hampering metabolic function and uptake of trace metals [29]. All these considerations are useful to understand the lower microalgae production obtained in this work compared to those mentioned before [11,24].

Nutrients removal

Nitrogen concentration as ammonium ($N\text{-}NH_4$), nitrite ($N\text{-}NO_2$) and nitrate ($N\text{-}NO_3$) in the growth medium was monitored during the whole cultivation time: resulting values are showed in Figure 2. In urban wastewater dissolved nitrogen is principally present as $N\text{-}NH_4$. In all microalgal cultivation tests, nitrogen uptake mainly occurred during the exponential growth phase. From the first series of tests (i.e. constant light intensity of 20 umol $s^{-1}m^{-2}$ and varying nutrients concentration), it was found that $N\text{-}NH_4$ removal rate increased when the initial $N\text{-}NH_4$ concentration increased, showing a maximum average rate of 5.4 ± 0.3 mg $N\text{-}NH_4$/L/d for the H20 test (i.e. growth medium with the highest nutrients load where initial $N\text{-}NH_4$ concentration was 30.2 ± 1.7 mgNH_4/L), whereas from the second series of tests (i.e. constant growth medium characteristics and varying light intensity) resulted that $N\text{-}NH_4$ removal rate was higher when the light intensity increased even if no significant differences were found. According to operating conditions tested, $N\text{-}NH_4$ removal rate mainly depended on nutrients availability rather than light intensities.

In microalgal culture media, such as urban wastewater, dissolved nitrogen removal mechanism involves biotic and abiotic processes: the biotic process is due to the mutual microalgae-bacteria activities, i.e. nitrification process

and direct uptake in microalgal cells [30]; the abiotic process consists in ammonia stripping related to the increase of pH during the photosynthetic activity [31].

Free ammonia produced during the exponential growth phase was calculated according to equation (4) in order to estimate the $N\text{-}NH_4^+$ fraction lost by ammonia volatilization from the culture medium. The corresponding analytical results are reported in table 4: comparing results obtained from the two series of tests, the amount of free ammonia produced during the exponential growth phase did not depend on the light intensity, but it was function of the initial nutrients concentration, increasing proportionally with this parameter. Therefore, the production of free ammonia is mainly affected by the nutrients load of the culture medium. Results showed that free ammonia volatilization contributed to the $N\text{-}NH_4^+$ removal by a value ranging from 70 to 80% and it was responsible for a less performing microalgal biomass growth.

Table 4. – $N\text{-}NH_4$ removal rate ($N\text{-}NH_4$ rate), $N\text{-}NH_4$ removed ($N\text{-}NH_4$ rem), free ammonia produced ($N\text{-}NH_3$ prod), percentage of $N\text{-}NH_4$ removed by volatilization during exponential growth phase ($N\text{-}NH_4$ vol), nitrite produced at the end of the exponential growth phase ($N\text{-}NO_2$ prod).

ID test	*$N\text{-}NH_4$ rate (mg/L/d)*	*$N\text{-}NH_4$rem (mg/L)*	*$N\text{-}NH_3$prod (mg/L)*	*$N\text{-}NH_4$vol (%)*	*$N\text{-}NO_2$prod (mg/L)*
L20	1.76 ± 0.41	7.04 ± 1.21	5.55 ± 0.62	78.9 ± 0.8	1.14 ± 0.12
M20	3.00 ± 0.83	15.0 ± 0.84	11.6 ± 1.13	77.4 ± 0.9	2.71 ± 0.23
H20	5.42 ± 0.27	27.1 ± 1.14	20.3 ± 0.77	74.8 ± 0.9	5.02 ± 0.77
L20	1.76 ± 0.41	7.04 ± 1.21	5.55 ± 0.62	78.9 ± 0.8	1.14 ± 0.12
L50	2.40 ± 0.16	7.19 ± 0.72	5.51 ± 0.39	76.7 ± 0.6	0.18 ± 0.09
L100	2.51 ± 0.23	7.54 ± 0.65	5.48 ± 1.64	72.7 ± 1.3	0.05 ± 0.02

Ammonium nitrification is a common process that takes place when sufficient amounts of DO are available in the culture medium. Therefore, oxygen produced from microalgae photosynthesis promotes the activity of

nitrifying bacteria that oxidize $N\text{-}NH_4^+$ to $N\text{-}NO_x$ [32]. In this work, an increasing trend of $N\text{-}NO_x$ concentration was observed during the microalgal exponential growth phase. Nitrites, rather than nitrates, were produced mostly in tests where light intensity was set at 20 umol $s^{-1}m^{-2}$ (i.e first series of tests), showing higher residual amounts of nitrites as the initial ammonium concentration was higher. Accumulation of nitrites in microalgae culture medium is quite frequent and its causes are still not clear [33]. The occurrence of this phenomenon can be explained according to two main assumptions as follow: (i) nitrifying bacteria contributed to oxidize ammonium to nitrite while nitrite conversion to nitrate was inhibited; (ii) nitrate produced from nitrification is partially assimilated into microalgal biomass. This latter assumption is more realistic since, as reported by Sanz-Luque et al. (2015), under adverse conditions for photosynthesis (i.e. low light intensity), nitrite production by nitrate reductase enzyme can be higher than nitrite reduction to ammonium by nitrite reductase enzyme [32]; as consequence, microalgal cells do not store the overproduced nitrites, which are excreted as an emergency strategy. This hypothesis is supported by results obtained monitoring ammonium removal under the highest light intensities (Figure 2), which enhanced the operating conditions for microalgal photosynthetic activity, resulting in a negligible nitrites accumulation. Data concerning nitrites accumulation, from both series of tests are reported in table 4: $N\text{-}NO_2$ accumulation was higher when nutrients load in culture medium increased and light intensity decreased. Finally, as suggested by Min et al. (2011) [34], nitrites accumulation at the end of the microalgal exponential phase can be attributed to the death of microalgae that fast release nitrite in the medium and other forms of nitrogen as a consequence of cell membrane breakage.

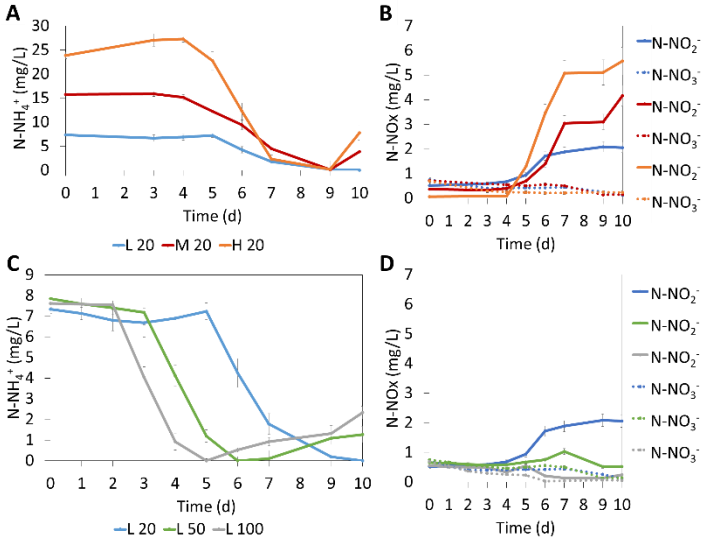

Figure 2. –Ammonium – nitrogen (N-NH_4^+) and oxidixed nitrogen (nitrite, N-NO_2^- and nitrate NO_3^-) variation during the cultivation time for light intensity increasing (a-c) and for nutrients load increasing (b-d).

Concerning phosphorus in wastewaters, orthophosphate usually amounts about 80% of the total phosphorus and the predominant form of orthophosphate is a function of the pH [31]. When pH is high, orthophosphate can be easily removed by precipitation of insoluble chemical complexes as calcium and magnesium salts. In this work, for all tests, a total removal of dissolved phosphorus (data not shown) was achieved at the end of the microalgal exponential growth phase in correspondence of the highest pH value. Therefore phosphate removal was achieved by the combination of biological assimilation into microalgal cells and abiotic precipitation.

Biomass settleability and recovery efficiencies

Sedimentation tests were conducted for all batch tests at the end of the cultivation time, when pH reached the highest values. In the scientific literature, several studies demonstrated that flocculation of microalgae can be naturally induced by increasing the pH of the growth medium. This phenomenon is commonly known with the name of 'autoflocculation' [22,35]. Microalgal biomass recovery efficiencies were calculated according

to Equation 5 and reported in Figure 3: the efficiency from the first series of tests (i.e. constant light intensity of 20 umol $s^{-1}m^{-2}$ and varying nutrients concentration), ranging from 52 ± 4% to 72 ± 3 % for 30 minutes long settlement process, resulted to be lower when the nutrients concentration in culture medium increased, whereas from the second series of tests (i.e. constant growth medium characteristics and varying light intensity) resulted to be the highest at the lowest light intensity and no significant difference in efficiency (i.e. less than 50%) was found between 50 and 100 umol $s^{-1}m^{-2}$. Vandamme et al. (2012) [22] obtained similar microalgal biomass recovery efficiencies (i.e. 75%) for *Chlorella* cultivation in an artificial medium at pH 11 and a higher value (96%) at pH 12.

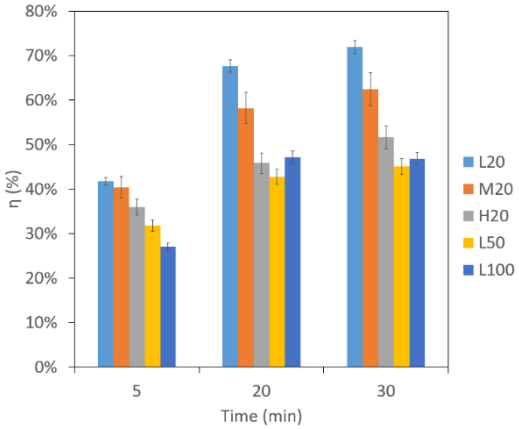

Figure 3. – Biomass recovery efficiencies evaluated at the end of the cultivation time.

The autoflocculation phenomenon is controversially discussed in the scientific literature: the mostly supported theory asserts that microalgal flocculation at high pH is promoted by chemical precipitation of calcium and/or magnesium salts [22,35]. In order to better understand the results obtained from biomass settling tests, concentrations of calcium and magnesium ions were monitored during the whole cultivation time and the results obtained are reported in Figure 4: variations in concentration of calcium and magnesium ions with time were clearly influenced by pH in the growth medium showing that high pH values induced salts precipitation. The percentage of calcium consumed resulted similar in all batch cultivation tests,

ranging from 70% and 80%; this result is likely correlated to the increasing trend of pH with time. Magnesium resulted to be not present as dissolved form when pH exceeded the value of 10.7, i.e. at day 6 for light intensities of 50 and 100 µmol $s^{-1}m^{-2}$ and showed low concentrations for the other conditions at the end of the cultivation time.

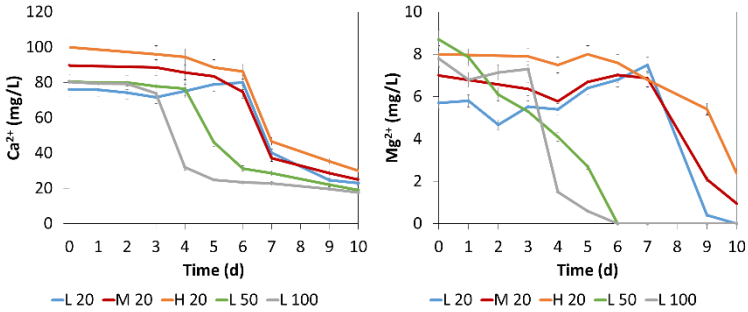

Figure 4. – Calcium (Ca^{2+}) and Magnesium (Mg^{2+}) depletion during the cultivation time.

Since no significant difference was observed in the formation of calcium and magnesium salts in all tests, the different efficiency in biomass settleability was investigated trough the optical microscope analysis of the settled microalgal biomass (Figure 5); results from the first series of tests showed different biomass physiological characteristics and a different state of aggregation. These images can be related to the growth curves shown in Figure 1b: at the end of the cultivation time, test L20 was in the last part of the exponential growth phase, M20 test was at stationary phase, whereas H_20 was at the beginning of decline growth phase. The differences found in settleability efficiency are therefore due to the specific microbial growth phase. Actually, at the end of the exponential phase, the settled biomass presented a compact aspect where microalgae and cyanobacteria resulted to be the dominant species, whereas next to the decline state, biomass showed a disaggregate aspect mainly constituted by organic matter, bacteria and few microalgae. The phenomenon of autoflocculation has been studied on a very small number of microalgal strains and has rarely been reported to date [36,37]. This phenomenon is really attractive because it is low cost, non-toxic to microalgal cells and avoids the use of chemical metal based flocculants,

thus making the growth medium totally recyclable and the settled biomass extremely pure for further use.

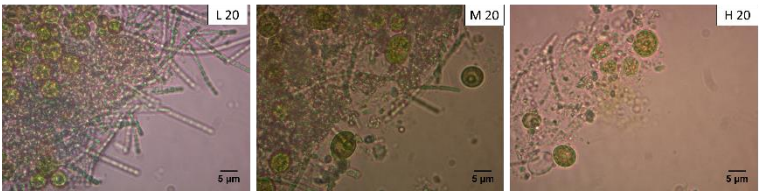

Figure 5. – Optical microscope image (40x) for the settled biomass at the end of the cultivation time for cultures under fixed light intensity of 20µmols^{-1}m^{-2} and with increasing nutrients concentrations in the medium.

Biomass lipid content

The microalgal growth rate affects the amount of lipids accumulated in cells as much as the nutrients availability in growth medium influences the lipids yield and their storage in microalgal biomass [11]. A rapid accumulation of lipids in microalgae occurs under stressful conditions and the rate typically increases with time for batch tests, in correspondence of more limiting nutrients conditions. Lipids content on dried microalgal biomass was measured at the end of all tests and the resulting values are reported in table 3.

The main outcomes can be summarized as follow:

(i) lipids content in microalgal biomass was lower when nutrients concentration in growth medium was higher (from first series of tests), showing an amount ranging from 17% to 23%. A similar trend was observed by Xin et al. (2010) [11] for *Scenedesmus sp.* in synthetic growth medium. This result is a consequence of the low initial nutrients concentration in the medium that caused, compared to operating conditions with higher nutrients amount, a faster nutrients depletion. This event, consequently, promoted an earlier lipids accumulation in microalgal biomass;

(ii) lipids amount in cells increased with light ranging from 23% to 29% (results from second series of tests). Similar results were reported by Q. He et al. for *Chlorella* sp. and *M. dybowskii* cultivated in synthetic medium and under continuous light conditions of 40, 200, 400 umol s^{-1}m^{-2}. Therefore,

under low light conditions, a limited amount of energy is supplied to microalgae, not enough to synthesize the energy stored in cells as lipids.

Conclusions

This work proves that light intensity and nutrients content in the growth medium affect the microalgal production efficiency and lipids accumulation in batch cultivation systems fed with urban wastewater: limiting the discussion to the operational conditions experimentally tested in this study, the highest light intensity and the lowest nutrients concentration in the growth medium produced the most performing and promising results. From an in-depth analysis of experimental activities, it was found that pH in the growth medium is the control parameter that most of others, governs the microalgal cultivation chain for the production of biomass used as biofuel source. Actually, in batch condition, pH varies progressively during cultivation time, showing an increasing trend as far as the photosynthesis process is well performed by microalgae. High pH values are positive because they induce the autoflocculation phenomenon in microalgae and consequently promote and favour their settling without using chemical flocculants. On the other hand, high pH values are inhibiting for the photosynthetic process, causing nitrite accumulation and consequently death of microalgae. Finally, the use of wastewater to feed batch microalgal cultivation systems is feasible and profitable since wastewater is a zero-cost substrate and microalgae can conveniently grow using wastewater as source of nutrients, especially under high light intensities. Additionally, autoflocculation process at high pH values makes economically sustainable the biomass harvesting, especially if it is conducted at the time of the exponential growth phase, avoiding cells lysis processes.

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Chapter 4

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Experimental study for the reduction of CO_2 emissions in wastewater treatment plant using microalgal cultivation

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ABSTRACT

Wastewater treatment plants (WWTPs) contribute to CO_2 emissions in atmosphere through direct (biological metabolism) and indirect (fuel combustion) oxidation of organic carbon. This detrimental effect of WWTPs operation can be mitigated by integrating the traditional treatment with a microalgae cultivation pond where CO_2 is fixed into autotrophic biomass and the positive side effect of removing nutrients also takes place. To test the feasibility of this modified WWTPs configuration, a pilot-scale 200 L raceway pond, operating outdoor, was designed and used for biomass cultivation in untreated urban wastewater. Nitrogen gas enriched with 20% CO_2, simulating the exhausted gas of biogas combustion, was supplied continuously during daytime at different flowrates. The dynamics of microalgae growth as well as inorganic carbon and nutrients uptake were studied during the pond start-up and semi-continuous feeding conditions. The absorbed bio-available CO_2 was monitored during daylight for different gas flowrates (0.2, 0.4 and 1.0 L/min) and for wastewater semi-continuous feeding conditions (0.8 L/h). The highest efficiency, equal to 83%, of bio-available CO_2 fixation was obtained for the lowest gas flowrate of 0.2 L/min., whereas the highest CO_2 removal rate of 24.6 mg/L/min was reached for the highest gas flowrate of 1.0 L/min. Furthermore, this operating condition resulted in the highest microalgae biomass productivity of 28.3 g/d/m^2. Nutrients removal was complete for each operating condition tested.

Introduction

Carbon dioxide (CO_2) accumulation in the atmosphere is nowadays one of the most serious environmental issues to be faced. According to the National Oceanic and Atmospheric administration (NOAA) data source, in September 2016, the concentration of CO_2 in the atmosphere has exceeded the threshold of 400 ppm, considered by most a point of no return. Recent studies have identified the urban wastewater treatment plants (WWTPs) as potential source of anthropogenic greenhouse gas (GHG) emissions and therefore a direct cause of climate change and air pollution [1–3]. The main source of CO_2 from WWTPs is the organic carbon present in wastewater that is either directly oxidized to CO_2 or incorporated into biomass by biological purification process. Such biomass is then partially converted into CH_4 and CO_2 in the anaerobic digestion phase of the sludge treatment line, and, finally, the CH_4 produced is oxidized in CO_2 through biogas combustion [4]. To limit the GHG emissions in atmosphere, extensive research has been carried out on CO_2 sequestration by chemical or physical sorption and membrane separation processes [5,6]. However, the application of these technologies is generally associated with capital as well as operational high costs and the generation of waste streams. As response, biological and eco-sustainable processes, as microalgae cultivation, are being considered attractive alternatives for CO_2 gas sequestration. Indeed, microalgae result 10-50 fold more efficient in CO_2 conversion compared to the terrestrial plants [7] and furthermore they can grow in urban wastewater [8]. Therefore, microalgae cultivation could be successfully integrated to the traditional treatments in municipal WWTPs for nutrients removal and CO_2 sequestration. The use of microalgae cultivation for the previous purposes does not represent a novelty in the international literature, where, however, only specific aspects have been fully analysed, whereas others, probably considered of less importance or not easily to be faced, have been neglected. Actually the recent scientific literature has been mostly focalized on studying the effectiveness of photobioreactors [9–12] fed with synthetic growth medium [13] or microalgae ponds fed with real wastewater, but already working in regime conditions [14–16], where CO_2 gas is sparged with for the specific purpose of regulating the pH in the system [17]. Therefore this work has been focused on studying the black-side of the microalgae cultivation systems used to remove pollutants and sequestrate CO_2 from WWTPs, in particular the effectiveness of microalgae in fixation CO_2 when the system is open, outdoor and consequently subject to variability of atmospheric conditions. In this configuration process start-up conditions are as important as regime

conditions and a trustful methodology capable to evaluate the effective CO_2 uptake by microalgae in an open space and at different atmospheric conditions is fundamental for a fully comprehension of the potentiality of microalgae in reducing CO_2 emissions from WWTPs at full scale. For this purpose, a native wastewater microalgae polyculture has been cultivated in a pilot-scale raceway pond using untreated urban wastewater as source of nutrients. The microalgae cultivation medium has been flushed at different flowrates (0.2, 0.4 and 1.0 L/min) with a gas mixture containing 20% CO_2 in volume in order to simulate the post combustion biogas emissions. The CO_2 uptake was constantly monitored during the whole experimental activity, start-up conditions included, by implementing an easy to use methodology that relates results of CO_2 uptake in presence as well as absence of microalgae in the raceway pond.

Materials and methods

Pilot scale raceway pond

The pilot raceway pond was placed outdoor, on the roof of the Department of Bioscience and Territory, University of Molise, Pesche (Isernia, Italy). It operated for 24 days with an average natural light intensity of 600 µmol/m^2/s and natural light/dark cycles. The raceway pond was composed of a single-loop open channel with semi-circular end-walls with 1 m^2 surface area and 0.2 m^3 volume. A four-blade paddle wheel coupled with a motor engine working at 6 rpm was used to perform the mixing of the culture medium and keep constant a mean surface velocity of 0.10 m/s.

The pond was equipped with a peristaltic pump (Cellai 302S, Italy) used to pump the influent in a section of the raceway pond located downstream the paddle wheel, thus ensuring a proper mixing of influent medium. The effluent from the raceway pond was collected by a superficial spillway. This type of outlet discharge system ensures the presence of a constant mass of microalgae culture in the pond without other techno-mechanical equipments. The gas addition system consisted of a gas cylinder filled with a 20% CO_2 and 80% N_2 gas mixture, a two stage gas regulator, a gas flow meter (Brooks Instruments, USA) capable to range the flowrate from 0.2 to 5.0 L/min and a 10 cm tubular gas diffuser. The point of gas sparging was located at the bottom of the raceway pond in the farthest section from the paddle wheels in order to avoid excessive turbulence and favour the gas solubilisation.

Microalgae inoculum

The microalgae inoculum used to inoculate the raceway pond was obtained from a native wastewater microalgae biomass. The biomass was collected from the effluent channel of the secondary clarifier of the municipal WWTP of Pesche (Isernia, Italy) and maintained in controlled conditions using Bold basal medium (BBM) [18] as growth substrate. The medium is composed of the following elements: 250 mg L^{-1} $NaNO_3$, 25 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 75 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 75 mg L^{-1} K_2HPO_4, 175 mg L^{-1} KH_2PO_4, 25 mg L^{-1} NaCl, 11.4 mg L^{-1} H_3BO_3, alkaline EDTA solution (50 mg L^{-1} EDTA, 31 mg L^{-1} KOH), acidified Iron solution (5 mg L^{-1} $FeSO_4 \cdot 7H_2O$, 1 mg L^{-1} H_2SO_4), trace metals solution (8.8 mg L^{-1} $ZnSO_4 \cdot 7H_2O$, 1.4 mg L^{-1} $MnCl_2 \cdot 4H_2O$, 0.7 mg L^{-1} MoO_3, 1.6 mg L^{-1} $CuSO_4 \cdot 5H_2O$, 0.5 mg L^{-1} $Co(NO_3)_2 \cdot 6H_2O$). BBM medium is adapted to generic freshwater microalgae, moreover, it does not include any organic carbon source, which would enhance the microalgae autotrophic metabolism. The cultivation was conducted in 1 L flask equipped with a magnetic stirring bar (150 rpm) to maintain the microalgae biomass in suspended condition. The culture was kept under a homogeneous and continuous light condition of 20 µmol/m^2/s (Cool White Fluorescent Lamps) at room temperature (25±2°C). Optical microscope analysis showed that microalgae biomass resulted composed of cyanobacteria, diatoms and microalgae (mostly *Scenedesmus* sp. and *Chlorella* sp.). The inoculum was added at the beginning of the experiment according to 3% of the total cultivation volume with a final optical density of 0.1 abs (measured at 550nm) for the culture growth medium.

Culture growth medium

Untreated urban wastewater, collected from the inlet channel of the WWTP located in Pesche (Isernia, Italy), was used as growth medium for the microalgal cultivation in the raceway pond. The wastewater was let to settle for 2 hours and 100 L of the corrisponding supernatant was half diluted with tap water for a total volume of 200 L and used for the microalgae cultivation start-up. The dilution was necessary to reduce the turbidity of the wastewater, thus favouring the light penetration and consequently reducing the initial microalgae growth lag-phase. Physical and chemical characteristics of undiluted wastewater are reported in Table 1.

Table 1. - Wastewater characteristics.

pH	**7.5 ± 0.2**
Dissolved oxygen (mg/L)	3.4 ± 1.2

TSS (mg/L)	70 ± 10
COD (mg O_2/L)	220 ± 10
Alkalinity (mg $CaCO_3$/L)	230 ± 20
NH_4^+ (mg/L)	35.8 ± 2.2
PO_4^{3-} (mg/L)	11.6 ± 1.2
NO_2^- (mg/L)	< 1
NO_3^- (mg/L)	< 1

Gas mixture enriched in CO_2 addiction

The specific gas composition used in this work (20% CO_2, 80% N_2) was related to the theoretical post combustion emissions for a condensed water vapour biogas composition of 50% CO_2 and 50% CH_4 in volume. Gas mixture was constantly sparged during daytime at fixed flowrate and pressure. The minimum gas flowrate of 0.2 L/min was applied for the microalgae cultivation start-up. Different flowrates were tested (0.2, 0.4 and 1.0 L/min) when the raceway pond was exercised in semi-continuous operating mode. For each experimental phase, the gas was added at the pressure of 2.2 bar.

Experimental setup

The experimental activity was conducted outdoor between May and July 2016 and consisted in three consecutive steps:

- *Step 1: microalgae cultivation start-up*.
  Microalgae culture was inoculated in the culture medium at time zero. Gas mixture was added at 0.2 L/min during daylight (9.30 a.m. – 17.30 p.m.). The cultivation was monitored for 13 days as long as nutrients present in wastewater were totally removed. Analysis of biomass growth, nutrients concentrations, dissolved oxygen (DO), pH and dissolved inorganic carbon were conducted on samples daily taken at 12 a.m..
- *Step 2: microalgae biomass enrichment*.
  30L of fresh undiluted wastewater was added twice to the raceway pond in order to increase biomass density, at day 13 and at day 17, respectively. Gas mixture was added at 0.2 L/min during daylight (9.30 a.m. – 17.30 p.m.). The cultivation was monitored until day 21 obtaining

the final total suspended solid (TSS) concentration of 210 mg/L. Same analysis of *Step 1* were conducted on samples daily taken at 12 a.m..

- *Step 3: microalgae biomass growth at regime conditions*
  Microalgae culture was continuously fed with undiluted urban wastewater and gas mixture during daylight (11.30 a.m. – 17.30 p.m.). The liquid was pumped at 0.8 L/h, corresponding to a hydraulic retention time (HRT) of 10 days. Gas mixture was added with three different flowrates: 0.2 – 0.4 and 1.0 L/min. Every time the flowrate was changed the microalgae cultivation system was preliminarily turned back to the same initial conditions: low nutrients concentration (< 1 mg/L) and 75 mg/L of TSS. The microalgae cultivation process was monitored in terms of biomass growth, pH and dissolve inorganic carbon during the continuous operating feeding mode of raceway pond (11.30 a.m. – 17.30 p.m.) each 30 minutes.

Analytical analysis

Biomass growth determination

Biomass growth was quantified measuring the optical density (OD) at the wavelength of 550nm using a UV/Visible spectrophotometer (Shimadzu UV-1601). This specific absorbance is recommended for microalgae polyculture [19]. TSS measures were conducted according to the standard methods [20]. Mixed liquor TSS were used as indicator for biomass concentration in the raceway pond in order to consider microalgae as well as microorganism [16]. OD was correlated to TSS measures, thus obtaining a linear correlation (TSS (mg/L) = 205.11·OD 550nm (abs)+0.3191; R^2 = 0.9886). The biomass productivity (P, mg $L^{-1}d^{-1}$) was calculated according to the following equation:

$$P = \frac{TSS_t - TSS_o}{t - t_0} \quad [1]$$

where TSS_0 (mg/L) is the biomass concentration at time t_0 (d) and TSS_t (mg/L)is the biomass concentration at any time t (d) subsequent to t_0.

Chemical analysis

DO and temperature (T) were determined using DO probe equipped with temperature sensor (YSI 550 DO). pH was measured using a pH meter (HI 8424, Hanna). Both probes were calibrated daily with standard buffers. Measures were performed at mid-depth of the culture medium. Light intensity (µmol/mq/s) was measured by a digital lux meter (MS6612, RoHS). Measures were performed at different times in a same point of the pond

surface. The average of daily measures was considered on the further calculations.

Nutrients concentrations, as ions N-NH_4^+, N-NO_2^-, N-NO_3^-, P-PO_4^{3-}, were determined using Liquid Ion Chromatography (Dionex, ICS 1000). The removal rate of the generic substrate *i*, R_i (mg/L/d) (where *i* indicates phosphate-P, ammonia-N, respectively), was calculated by Equation 2.

$$R_i = \frac{S_{0,i} - S_i}{t - t_0} \quad [2]$$

where $S_{0,i}$ (mg/L) is the initial concentration of substrate *i* at time t_o (d), S_i (mg/L) is the corresponding substrate concentration at time *t* (d). Chemical Oxygen Demand (COD) in undiluted wastewater was measured according to the standard methods [20].

Inorganic Carbon and free CO_2 measurement

The three forms of CO_2 that can co-exist simultaneously in aqueous phase (free CO_2, HCO_3^- and CO_3^{2-}) were quantified according to the standard methods [20]. For *Steps* 1 and *2*, the inorganic carbon variation during the microalgae cultivation process was evaluated as total CO_2 according to the stoichiometric balance. For *Step 3*, the free CO_2 removal during daylight was evaluated comparing the values of concentration obtained from microalgae cultivation tests to those obtained from blank tests.

Blank tests were conducted in the raceway pond, reproducing the same conditions investigated for microalgae cultivation tests with the only difference represented by the absence of microalgae. These tests were necessary to estimate the CO_2 volumetric mass transfer coefficient (K_La) of the gas mixture addition equipment. Therefore, the pond containing 200L of wastewater without microalgae was sparged with gas mixture at different flowrates (0.2, 0.4 and 1.0 L/min) for 240 min. The liquid velocity was set at 0.1 m/s and the dissolved CO_2 concentration in the culture medium was measured every 30 minutes. According to the two-film theory [21], the CO_2 dissolution in the medium was governed by the following equation:

$$\frac{dC}{dt} = k_L a(C^* - C) \quad [3]$$

where *dC/dt* is the volumetric transport rate of CO_2 in liquid (mg min^{-1} L^{-1}); K_La is the volumetric mass transfer coefficient of CO_2 (min^{-1}); C^* is the concentration of CO_2 in the culture medium in equilibrium with CO_2 content

in gas bubbles (mg L^{-1}); C is the CO_2 concentration in the culture medium (mg L^{-1}).

Integrating equations 3 and 4 with $C = C_0$ and t=0, the following equation results:

$$\ln \frac{C^* - C}{C - C_0} = -K_L a \cdot t \qquad [4]$$

A plot of the left hand side of this equation against time was used to calculate $K_L a$ (see figure 5).

In order to evaluate the amount of inorganic carbon effectively fixed by microalgae as free CO_2, the bio-available fraction of free CO_2 ($[CO_2]_{BA}$, mg/L), was estimated using the results obtained from blank tests. The inorganic carbon fraction effectively fixed by microalgae as free CO_2 ($[CO_2]_{fixed}$, mg/L) was evaluated subtracting the measured free CO_2 concentration from microalgae cultivation ($[CO_2]_{MA}$, mg/L) tests to the corresponding value estimated from blank tests, according to the following expression:

$$[CO_2]_{fixed}(t, f) = [CO_2]_{BA}(t, f) - [CO_2]_{MA}(t, f) \qquad [5]$$

Each term of equation 5 is function of both time (t, min) and gas flowrate (f, L/min). The equation was obtained considering the following mass balances:

$$[CO_2]_{in}(t, f) = [CO_2]_{BA}(t, f) + [CO_2]_{out}(t, f)) \qquad [6]$$

$$[CO_2]_{in}(t, f) = [CO_2]_{MA}(t, f) + [CO_2]_{fixed}(t, f) + [CO_2]_{out}(t, f) \qquad [7]$$

where equation 6 describes the CO_2 mass balance in blank tests, whereas equation 7 describes the mass balance in microalgae cultivation tests. Assuming that the amount of CO_2 added ($[CO_2]_{in}(t, f), mg/L$) and the amount of CO_2 dispersed in the atmosphere ($[CO_2]_{out}(t, f), mg/L$) are the same in blank as well as microalgae cultivation tests for a fixed gas mixture flowrate, equation 5 is obtained by the combination of equation 6 and 7.

The CO_2 fixation efficiency (η_f) was considered as the microalgae capability to absorb the bio-available CO_2, which represents the dissolved CO_2 in the culture medium. It was calculated as follow (equation 8):

$$\eta_f = \sum_{t=0}^{t=360\ min} \frac{[CO_2]_{BA} - [CO_2]_{MA}}{[CO_2]_{BA}} \% \qquad [8]$$

In order to evaluate also the capability of the wastewater microalgae cultivation to sequestrate part of the amount of CO_2 added, the CO_2 removal efficiency (η_r) was calculated as follow (equation 9):

$$\eta_r = \sum_{t=0}^{t=360\ min} \frac{[CO_2]_{in} - [CO_2]_{fixed}}{[CO_2]_{fixed}} \% \quad [9]$$

Finally, the maximum CO_2 consumption rate (r_{max}, mg/L/min) was calculated using the following equation 10:

$$r_{max} = \frac{\sum_{t0}^{t} [CO_2]_{fixed}(t) - [CO_2]_{fixed}(t_0)}{t - t_0} \quad [10]$$

where $[CO_2]_{fixed}(t)$ is the concentration (mg/L) of free CO_2 fixed at time t (min) and $[CO_2]_{fixed}(t_0)$ is at time zero.

Results and discussions

Step 1: microalgae cultivation start-up.

During *Step 1*, the gas mixture was added during the daylight in order to increase the inorganic carbon content of the medium and enhance the microalgae growth [17]. Carbon limitation has a negative impact on the microalgal growth in wastewater [22]. Park and Craggs [26] foundthat both wastewater treatment efficiency and freshwater microalgae production increased in raceway pond when CO_2 was added. The variation of the three forms of dissolved inorganic carbon is reported in figure 1. During *Step 1*, the pH increased from 7.0 to 8.8, as consequence, the main dissolved inorganic carbon form resulted in the bicarbonate ion (HCO_3^-) and the chemical balance moved towards carbonate (CO_3^{2-}) formation. Most of the microalgae species are not able to assimilate carbonate as source of inorganic carbon and its accumulation at high pH could inhibit biomass growth [18, 23]. This effect was not observed in the present work: the increase of pH , mainly caused by microalgae photosynthesis [24],was not inhibiting the biomass growth. Indeed, as represented in figure 2, microalgae growth showed 9 days of lag-phase, followed by an exponential growth phase.

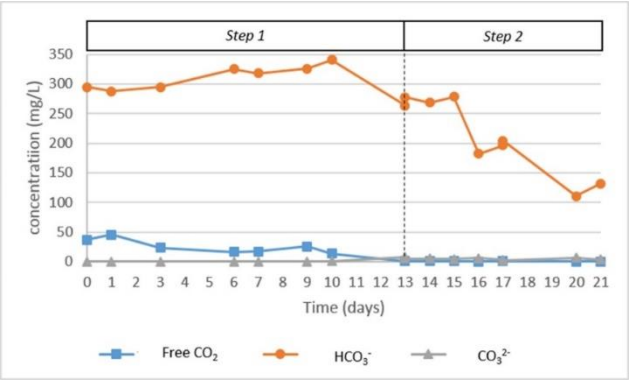

Figure 1. – Inorganic carbon speciation in the liquid culture during Steps 1 and 2

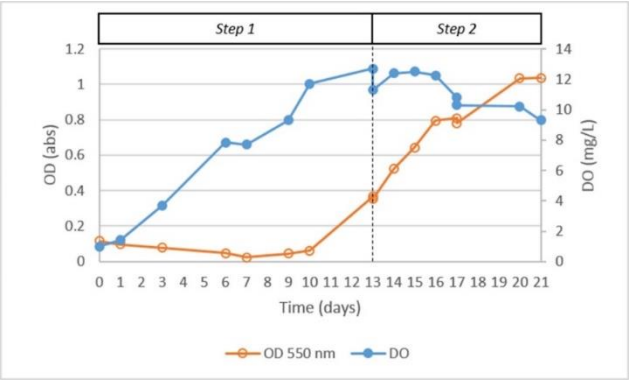

Figure 2. – Optical density (OD) at 550 nm and dissolved oxygen (DO) concentration during Steps 1 and 2

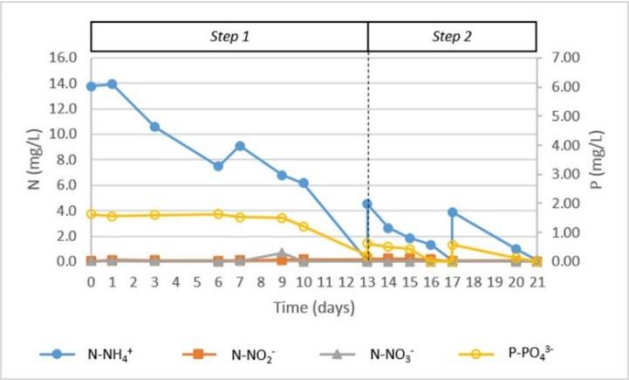

Figure 3. – Nitrogen and phosphorus concentration during Steps 1 and 2

Lag-phase.

The growth lag is the period of physiological adjustment for microalgae due to changes in nutrients or culture conditions [18]. The duration of this phase (9 days) was higher if compared to the study conducted by A.C. Eloka-Eboka et al. [25] where the lag phase was only 3 days long. This difference could be reasonably explained by the higher inoculation volume (i.e. 10% compared to 3% in this work) set as well as by the more suitable microalgae medium (broth agar as nutrient) used in their work. Indeed, in this work, the microalagae inoculum was cultivated in a medium without organic carbon and therefore required longer time to adapt to urban wastewater that is rich in organic compounds. During the lag-phase, a decrease of OD values was observed in the culture medium from day 0 to 7: the turbidity in the cultivation medium decreased and consequently better conditions for light penetration occurred. This effect, naturally occurred, favoured the microalgae photosynthesis and therefore promoted their growth. Similar cases of this phenomenon have been not reported in the literature, as the start-up condition of the microalgae pond has been not closely investigated.

During the lag phase, no significant change in pH was detected, as the value kept almost stable around 7.0. This result was expected since the microalgae activity, responsible for the pH increase, was not evident in this phase. On the contrary, DO concentration increased constantly during the lag-phase reaching a value of 9.3 mg/L at day 9 (figure 2), i.e. close to the saturation condition in pure water (9.17 mg/L at 20°C and 1atm). The DO concentration trend was the result of combined effects related to: (i) atmospheric oxygen

solubilisation, caused by the paddle-wheels turbulence conditions; (ii) microalgae oxygen production during the photosynthetic activity which occurred in the late lag phase; (iii) oxygen consumption due to heterotrophic bacteria metabolism.

Dissolved nutrients, i.e. nitrogen and phosphorus, are present in wastewater mainly as ammonia (NH_4^+) and phosphate (PO_4^{3-}) respectively. For freshwater microalgae, N and P potentially co-limit biomass growth when the N:P ratio is out of the range 10-30 [24]: when the ratio is above 30, P is the limiting factor, whereas below 10, N is the limiting factor. In this work, the N:P ratio at time zero was 19, therefore was optimal for microalgae growth. During the lag-phase, NH_4^+ concentrations (figure 3) showed a removal rate of 0.77 mgN-NH_4^+/L/d, calculated according to equation 2. As suggested by Park and Craggs (2011) [26], NH_4^+ removal was probably the result of a combination of biological and physical factors: (i) ammonia volatilization for high pH values (mostly for pH > 9); (ii) biological denitrification of nitrate under anoxic conditions in the pond at night. On the other hand PO_4^{3-} concentration remained constant in the culture medium (figure 3) since nor biological activity neither physico-chemical phenomena (high pH and high DO concentrations) capable to remove PO_4^{3-} took place in the culture medium [27].

Exponential growth phase.

The exponential growth phase followed the initial lag-phase and it was monitored in terms of OD. OD values moved from 0.05 abs to 0.35 abs, respectively from day 9 to day 13 (figure 2). The addition of CO_2 set the C:N ratio at the beginning of the exponential phase at 7.8, which resulted optimal for biomass growth since the typical C:N ratio in microalgae cell is between 6 and 15 [28]. Indeed, the complete nutrients removal occurred in 4 days: nitrogen and phosphorus were consumed with the rate of 1.9 mgN/L/d and 0.32 mgP/L/d respectively, according to equation 2. The pH of the culture medium never exceeded the value of 9 during the exponential phase and therefore physico-chemical phenomena as ammonia volatilization and phosphate precipitation did not affect significantly nutrients removal [29].

During the exponential phase, the pH increased from 7.3 to 8.6 as consequence of the microalgae photosynthetic activity. A relevant inorganic carbon consumption as well as oxygen production were also observed in this phase. Inorganic carbon was consumed as both bicarbonate and free CO_2 (figure 1) showing a consumption rate (P_{CO2}) of 15.8 mgCO_2/L/d for a TSS production rate ($P_{overall}$) of 16.4 mg/L/d. Both values were calculated

according to equation 2 and 1 respectively. The ratio between the two rates (P_{CO2}/ $P_{overall}$) was 0.96, which resulted lower compared to the theoretical ratio of 1.88 defined by Christi [30] and commonly used in the literature for the indirect determination of the CO_2 consumption rate [9,31]. The Oxygen concentration increased over the aqueous saturation value after day 9, reaching the amount of 12.3 mg/L at day 13 (figure 2).

Step 2: microalgae biomass enrichment.

Fresh wastewater addiction (30L) was conducted at day 13 and 17 in order to restore the nutrients concentration in the culture medium, thus enhancing the biomass production. Indeed, a linear biomass production, measured in terms of OD, was observed as consequence of wastewater addiction (figure. 2). These operating conditions of the raceway pond were used to calculate the ratio $P_{CO2}/P_{overall}$ mentioned before, which evaluates the carbon content in the algal cell (C_C, %w/w) according to the following equation reported by Anjos *et al. [32]*:

$$P_{CO_2} = C_c \times P_{overall} \frac{M_{CO_2}}{M_C} \quad [11]$$

where P_{CO2} is the CO_2 fixation rate (mg CO_2/L/d), $P_{overall}$ is the biomass growth rate (mg TSS/L/d), evaluated in the linear phase of microalgae growth; M_{CO2} (g/mole) and M_C (g/mole) represents the molar mass of CO_2 and elemental carbon, respectively. Furthermore, TSS content (mg/L) was plot against TIC (total inorganic carbon) content (mg/L) to estimate (figure 4) the carbon content of the biomass (C_c) (mg C/mg TSS). TIC content was calculated stoichiometrically by direct measures of carbonate, bicarbonate and free CO_2. The carbon content of the biomass resulted in 21.3%, which is lower than the typical value of 50% reported in the literature [7]. This result could be explained considering that microalgae can use both organic and inorganic carbon source and moreover mixotrophic conditions are enhanced when wastewater is used as culture medium [33]. As consequence, the indirect equation (11) for the evaluation of CO_2 fixation rate resulted not fully suitable for microalgae cultivation in wastewater.

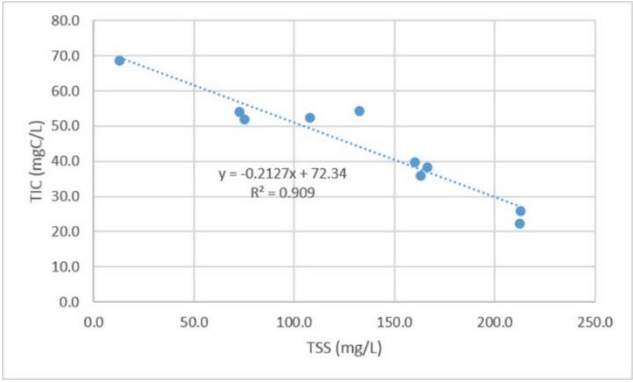

Figure 4. – Correlation between total inorganic carbon (TIC) and biomass concentration expressed as total suspended solids (TSS), evaluated during linear microalgal growth (Step 2).

DO concentration showed an increasing trend after the first supply of fresh wastewater (day 13) and remained constant after the second (day 17) feeding operation (figure 2). DO trend can be correlated to the biomass production trend (measured as OD), which showed the lowest values when the fresh wastewater was added for the second time likely because of the reduced photosynthetic microalgae activity as consequence of the reduced light penetration condition (OD 550nm > 1) [24]. Nitrogen and phosphorus were completely removed in 4 days after the supply of fresh wastewater showing a constant average rate of 1.1±0.1 mgN/L/d, and 0.15±0.01 mgP/L/d respectively (figure 3). During the first feeding operation, nutrients removal was mainly due to the biomass assimilation, since the pH in the culture medium resulted lower than 9. On the contrary, both direct and indirect removal mechanisms occurred in the second fresh wastewater supply.

Step 3: microalgae biomass growth at regime conditions.

CO_2 consumption is generally evaluated through a direct measure of CO_2 gas or measures of carbon content in microalgae biomass [7]. The use of these methods when the microalgae cultivation system is open and fed with wastewater, shows several problems. Indeed, when the system is not closed, it is possible to control the amount of gas added, but results difficult to monitor the amount of gas dispersed in the atmosphere. Moreover, when

wastewater is used as culture medium, carbon content measured for microalgae biomass could be affected by metabolism of different species, heterotrophic as well as autotrophic, therefore measurements of carbon content, reasonably could not be totally proportional to the inorganic carbon consumption, as demonstrated in the previous subsection. In order to overcome these problems, in this work, the comparison between CO_2 solubility conditions at different times in the culture medium in presence as well as in absence (blank tests) of microalgae was used to calculate the inorganic carbon fixed by microalgae metabolism. Dissolved inorganic carbon was evaluated as function of pH and alkalinity according to the standard methods. The same procedure was also applied by Bhakta *et al.* [23] to monitor CO_2 consumption in microalgal photobioreactors. Inorganic carbon concentration, as free CO_2 in the culture medium changed as consequence of CO_2 gas supply. More in details, the solubilised CO_2 concentration followed a logarithmic law [21] until to reach an equilibrium value which is function of the specific operating conditions (i.e. flowrate) and characteristics of culture medium. The trend of free CO_2 concentration in blank tests (raceway pond not inoculated with microalgae) is reported in figure 5 for each tested gas flowrates. Approximately, the free CO_2 concentration in the culture medium (C) increased linearly with time up to reach the equilibrium value (C*). The linear trend of the curves in figure 5 was used to calculate the CO_2 volumetric mass transfer coefficient according to equation 4 and the corresponding results are reported in table 2. Under the same CO_2 gas concentration, K_La was proportional to the gas sparging rate. Similar results were obtained by S. Li *et al.* [34] for the calculation of K_La of CO_2 in a closed raceway pond. As showed in Figure 5, for a fixed gas sparging time (240 min), the equilibrium concentration C* was reached for all the tested gas flowrates and are reported in table 2: increasing the gas flowrates, C* increased proportionally and the equilibrium conditions were reached earlier when the gas flowrate was higher.

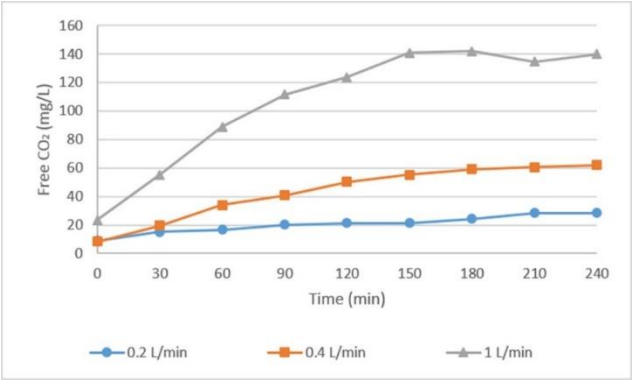

Figure 5. – Free CO_2 in time measured during blank tests (pond without microalgae) for the gas flowrates of 0.2, 0.4, 1.0 L/min.

Table 1. - Blank tests results for different gas flowrates: C is the CO_2 equilibrium concentration, C is the free CO_2 variation law as function of time and initial CO_2 concentration (C_0), R^2 is the error associated to the linear laws, K_La is the CO_2 volumetric mass transfer coefficient.*

Gas flowrate (mL/min)	**C* (mg/L)**	**C (t, C_0) (mg/L)**	**R^2**	**K_La**
0.2	28.3 ± 0.1	$0.0783 \cdot t + C_0$	0.9367	0.00655
0.4	60.6 ± 1.14	$0.2861 \cdot t + C_0$	0.9589	0.01576
1	139.3 ± 2.8	$0.7751 \cdot t + C_0$	0.9659	0.01676

For the same gas sparging conditions, the CO_2 concentration at the equilibrium resulted the same in tests inoculated by microalgae as well as in tests without inoculum (blank tests), whereas CO_2 concentration trend resulted different. These results proved that the free CO_2 concentration at equilibrium does not depend on the microalgae activity, whereas the consumption of free CO_2, as expected, is affected by the presence of microalgae. Moreover, increasing the gas mixture flowrate, free CO_2 concentration increased in the culture medium while the bicarbonate concentration remained constant. As consequence, the free CO_2 added was

not totally utilized by microalgae for the photosynthetic activity but it was progressively accumulated in the culture medium up to reach a concentration approximately constant (figure 6). Similar results were obtained by Suvida Gupta *et al.*, which observed the inorganic carbon accumulation in closed microalgae cultivation systems. However they found that the inorganic carbon consumption occurred in different proportions with respect to the different operating conditions, as consequence, the inorganic carbon accumulation did not mean that it was not consumed during the microalgae cultivation [33].

In this work, the free CO_2 measured during microalgae cultivation ($[CO_2]_{MA}$) was compared to the bio-available fraction of free CO_2 ($[CO_2]_{BA}$) for each tested gas flowrates (figure 6). The inorganic carbon fraction fixed by microalgae as free CO_2 ($[CO_2]_{fixed}$), during the 6 hours of gas addition, was evaluated according to equation 5 and results are reported in figure 7. The free CO_2 fixed by microalgae photosynthetic activity could be described as the result of three successive steps depending on the free CO_2 concentration ($[CO_2]_{MA}$) measured in the culture medium:

1) Linear increasing trend: $[CO_2]_{MA}$< 60% C*
2) Stationary trend: 60% C* < $[CO_2]_{MA}$ < C*
3) Decline trend: $[CO_2]_{MA}$= C*.

Microalgae capability to absorb CO_2 can be reasonably correlated to the free CO_2 concentrations ($[CO_2]_{MA}$) reached in any specific operating conditions. Microalgae growth during the regime conditions of the raceway pond was affected by the flowrate of gas mixture added. Microalgae concentration remained constant or even decreased during stationary (figure 8) and decline trend of CO_2 fixation (figure 7). On the contrary, microalgae concentration increased during the first 180 min (figure 6), corresponding to the highest light intensity (figure 9). Indeed, light actually plays a central role in microalgae productivity, providing the photon energy required in photosynthetic process to convert dissolved inorganic nutrients into organic molecules [22, 34]. Oxygen is produced in the process of photosynthesis and eliminated from the microalgae culture as byproduct: actually an increase in the microalgae growth rate is generally proportional to an increase of oxygen production [36]. Therefore, a strict relation between solar irradiance, oxygen production and biomass growth is usually found: high OD concentration is in accordance with high luminosity value and maximum biomass production (figures 8 and 9). A decreasing trend of DO concentration was observed after 180 min (figure 8). These findings are in agreement with the general

decreasing rate of microalgae photosynthetic activity in the system due to the high free CO_2 concentration in the culture medium and low light intensity measured after 180 min. These results are supported by the international literature that states that microalgae can tolerate free CO_2 concentration only up to a certain level. Above this threshold free CO2 concentration is detrimental for the growth of the cells [37].

Considering all tests, the CO_2 maximum removal rate was evaluated, according to equation 10, for the linear trend of fixed CO_2 (figure 7) which resulted longer for lower gas flowrates (90 min for 1L/min, 120 min for 0.4 L/min, 300 for 0.2 L/min). Results are reported in table 3 and are comparable to those published by A.C. Eloka-Eboka *et al.* (2017), which studied the CO_2 removal in microalgal photobioreactors. Higher values were calculated for higher flowrates obtaining the highest removal rate of 24.6 mg/l/min for the gas flowrate of 1.0 L/min. These conditions corresponded to the maximum value of CO_2 concentration fixed (40.5±1.7 mg/L). Such operating condition resulted in the highest biomass productivity of 28.3±0.1 g/d/m^2, evaluated according to equation 1 for the first 180min. Lower gas flowrates showed similar results in terms of both CO_2 fixation and biomass production (see Table 3).

Results concerning the free CO_2 fixation efficiencies are summarized in Table 3. The best operating condition for the highest CO_2 fixation in microalgae raceway pond fed with wastewater was achieved with the lowest gas flowrate of 0.2 L/min. Higher gas flowrates induced lower CO_2 fixation efficiencies. These results are in agreement with those obtained for microalgal photobiorectors, where the biomass productivity increased when the percentage of CO_2 in the gas mixture increased up to a certain value beyond that the microalgae growth and the CO_2 fixation efficiencies decreased [37]. Microalgae mixed cultures for CO_2 sequestration were tested in closed photobiorectors with higher CO_2 removal percentage (60%) compared to this work [13]. This difference could be explained by the more favourable microalgae environmental conditions applied in that study in terms of culture medium and cultivation system. Moreover, the applied biomass yield of that study (~ 5 g/L) was higher compared to that considered in this work (75 mg/L).

Finally, nutrients content was measured in the fresh wastewater as well as in the liquid effluent from the microalgal pond. A complete nutrients removal (figure 10) was achieved in all experimental conditions. pH in the system showed values under 9 during daylight, hence, the direct nutrient uptake

performed by microalgae was more significant than the indirect removal by abiotic factors (i.e. ammonia volatilization and phosphate precipitation). This result shows the feasibility of incresing the influent flowrate, thus promoting a higher microalgae growth.

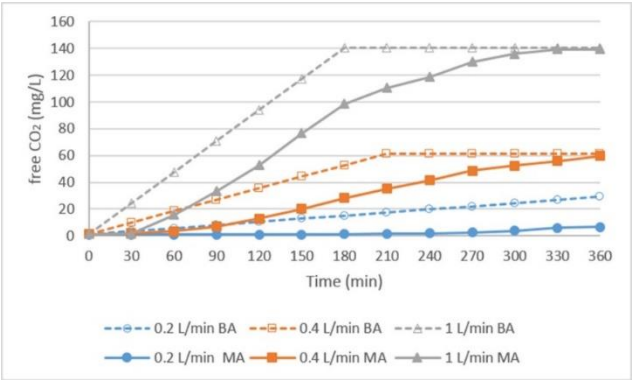

Figure 6. – Free CO_2 (mg/L) measured during microalgae cultivation (MA) and bio-available CO_2 calculated through blank tests laws (BA) for 360 min of gas addiction at 0.2, 0.4, 1.0 L/min as gas flowrates.

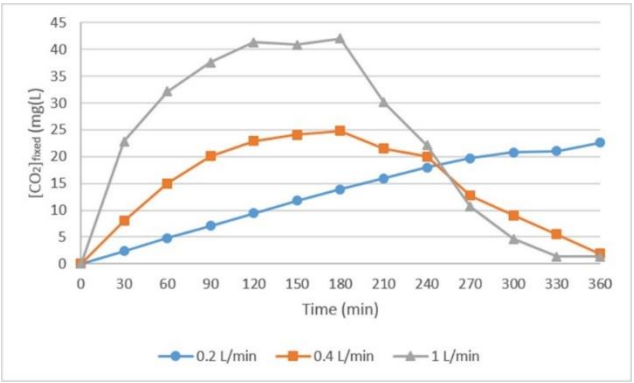

Figure 7. – CO_2 (mg/L) fixed by microalgae culture during 360min of gas addiction for gas flowrates of 0.2, 0.4, 1.0 L/min.

Table 2. – Maximum values of CO_2 (mg/L) fixed by microalgae cultivation, CO_2 microalgae fixation efficiency (η_f), CO_2 cultivation pond removal efficiency (η_r) and maximum biomass production of the system for the tested gas flowrates.

Gas flowrate (mL/min)	**$[CO_2]_{fixed, max}$ (mg/L)**	**η_f (%)**	**η_r (%)**	**P_{max} ($g/d/m^2$)**	**r_{max} (mg/L/min)**
0.2	21.0 ± 1.1	86	9	14.6 ± 0.1	11.3
0.4	22.2 ± 1.85	35	5	15.0 ± 0.1	13.7
1	40.5 ± 1.7	23	3	28.3 ± 0.1	24.6

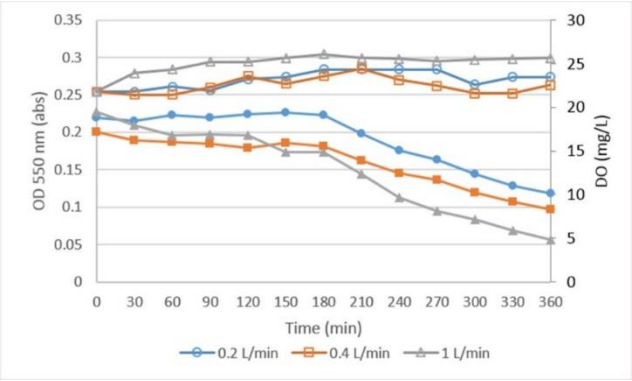

Figure 8. – Trend of Optical Density (OD) at 550nm (filled markers) and Dissolved Oxygen, DO (empty markers) during Step 3.

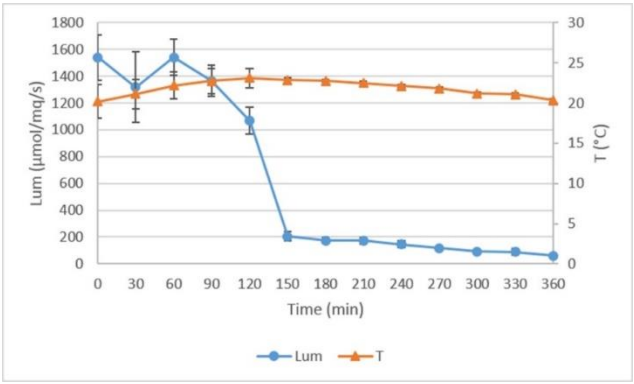

Figure 9. – Luminosity and temperature trend during Step 3.

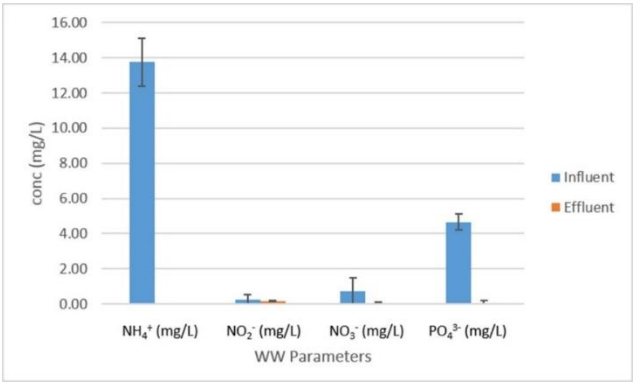

Figure 10.- Nutrients content in the influent and effluent stream of the microalgal pond.

Conclusions

This study showed that microalgae cultivation in outdoor raceway pond, fed with urban wastewater is characterized by a longer initial lag-phase (9 days) than microalgae cultivation performed in photobioreactors and growth in synthetic medium. This difference is caused by higher turbidity level in wastewater than synthetic medium and worse luminosity conditions in outdoor naturally lightened systems than in artificially lightened ones. Despite the adverse conditions, microalgae are nevertheless endowed with capacity to improve the environmental conditions, performing a self-flocculation process able to increase the light permeability in the system and

consequently promote their growth. Microalgae cultivation therefore shows capacity of self-adaptation and this aspect is of great interest in WWTPs where the water turbidity can change frequently as it is affected by rainfall and variable daily polluting loads. After the initial adaptation period, the microalgae growth is exponential until the complete nutrients removal. Therefore, nutrients are limiting for the microalgae growth. Actually, successive addictions of fresh wastewater rich in nutrients promoted the biomass growth that showed a linear growth trend. A relevant aspect for microalgae growth is represented by CO_2 gas addiction that provides the system with inorganic carbon and simultaneously limits the pH increase, avoiding the occurrence of strict basic conditions that would inhibit the microalgae activity. Particular attention has to be payed when CO_2 gas sparging systems are designed and used since this study proved that when in culture medium free CO_2 equilibrium concentration is reached, the CO_2 fixation into microalgae becomes limited and the biomass growth is inhibited. Actually the lowest gas flowrate tested (i.e. 0.2 L/min) enhanced both bio-available CO_2 fixation during daylight and CO_2 water solubility, resulting the most efficient condition for the microalgae cultivation pond fed with real wastewater. An efficient and easy-to-use method to calculate the effective CO_2 fixed by microalgae during daylight in open systems was also developed in this work. Furthermore this study found that microalgae biomass production showed a rate proportional to the total inorganic carbon consumption with a ratio lower than that reported in the international literature proving that microalgae community can also utilize organic carbon and therefore contribute to decrease the chemical oxygen demand (COD) concentration in urban wastewater.

All the previous considerations demonstrate that microalgae cultivation pond can be successfully integrated to the traditional processes used in municipal WWTPs enhancing nutrients removal and contributing to CO_2 sequestration. For this purpose, a treatment with microalgae can be reasonably located after the secondary settlement tank of WWTPs where the effluent present low both turbidity as well as results still rich in nutrients. Furthermore, the microalgae pond can be fed by the exhaust gas coming from the biogas combustion process using gas-dissolving systems similar to those used in activated sludge tanks for oxygen supply. This upgrade in WWTPs can promote the carbon recycle, thus limiting the global emissions of greenhouse gases. Finally, microalgae produced from this upgraded WWTPs represent valuable and low cost raw material for many applications in biofuels and biopolymers production.

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Chapter 5

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Combined yeasts and microalgae cultivation in pilot scale raceway pond for urban wastewater treatment and potential biodiesel production.

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Keywords: biofuel, disinfection, microalgae, raceway pond, wastewater, oleaginous yeast.

ABSTRACT

A mixed culture of oleaginous yeast *Lipomyces starkeyi* and wastewater native microalgae (mostly *Scenedesmus* sp. and *Chlorella* sp.) was performed to enhance lipid and biomass production from urban wastewaters. A 400 L raceway pond, operating outdoor, was designed and used for biomass cultivation. Microalgae and yeasts were inoculated into the cultivation pond with a 2:1 inoculum ratio. Their concentrations were monitored for 14 continuous days of batch cultivation. Microalgae growth presented a 3 days long initial lag phase, while yeast growth occurred in the first days. Yeasts activity during microalgae lag phase enhanced microalgal biomass productivity, corresponding to 31.4 mgTSS m^{-2} d^{-1}. Yeast growth resulted limited by low concentrations in wastewater of easily assimilated organic substrates. Organic carbon was absorbed in the first three days with 3.7 $mgC \cdot L^{-1} \cdot d^{-1}$ of removal rate. The complete nutrients removal occurred during microalgae linear growth with 2.9 $mgN \cdot L^{-1} \cdot d^{-1}$and 0.96 $mgP \cdot L^{-1} \cdot d^{-1}$ of removal rates. Microalgal photosynthetic activity induced high pH and DO values resulting in a natural bactericidal and antifungal action. A 15% fat/dry weight was measured at the end of the cultivation time. FAME analysis indicated that lipids resulted mainly composed by arachidic acid.

Introduction

The current demand for alternative energy sources to meet the growing global energy needs continues to rise. Non-renewable energy sources, such as oil, are projected to be mostly depleted in less than 50 years. Moreover, the extensive use of petroleum, coal and natural gas caused a number of environmental concerns, i.e. the climate change, resulting from the global warming effects. For this reasons, alternative sources of fuels that are renewable, economical, and less harmful to the environment needs to be widely implemented. One such alternative is the use of biodiesel, whose production is based on the transesterification of long chain triglycerides from renewable sources using methanol [1]. Biodiesel contributes no net carbon dioxide or sulfur to the atmosphere and emits less gaseous pollutants than normal diesel [2].

Biofuels produced from plants have the potential to replace a portion of fossil fuel consumption with a renewable alternative. However, the use of food crops for biodiesel and other renewable fuels may be an uneconomical long term solution [3,4]. As feasible solution, microbial oils, produced by oleaginous microorganisms, can be used as potential alternative feedstock for biodiesel production, due to their high growth rate, non-use of arable agriculture land and fatty acid profiles similar to those derived from vegetable oils [5,6]. Moreover, oleaginous microorganisms are able to use wastes as source of nutrients, which makes their cultivation economically sustainable and environmentally friendly [1]. Oleaginous microorganisms, including bacteria, yeasts, molds and algae are defined as microbial species with microbial lipid content higher than 20% [7].

Yeasts can use a vast variety of organic materials accumulating high amount of lipids, up to 70% of their dry weight [8]. Microalgae are considered as attractive source for biodiesel production due to their high lipid content, photosynthesis efficiency and CO_2 reduction ability [9]. Recent studies showed that the combined cultivation of microalgae and yeasts could significantly enhance biomass and lipid production [6,10]. In mixed cultures, microalgae produce the oxygen used by yeasts respiration, yeasts provide the CO_2 consumed by microalgal photosynthesis and both carry out the lipid production.

The use of combined yeast and microalgal cultures is still at its early stage and the data available in literature are lacking. The principal aim of studies based on this topic and available in the literature is to investigate the

possibility to obtain higher lipid accumulation in mixed yeasts-microalgae culture. They were conducted at lab-scale conditions using synthetic growth medium [1,6,10] or enriched urban wastewater [11,12]. The studies confirmed the possibility to obtain a synergistic effect in the combined yeasts-algae cultivations with different growth substrate and using different oleaginous species. In this work, the combined yeast-microalgae cultivation is conducted in a 400 L raceway pond, operating outdoor and using raw urban wastewater as growth substrate. A native wastewater microalgal culture was used as inoculum in order to reduce the initial time of adaptation to the medium [13]. Lipomyces starkeyi was added as oleaginous yeast since it shows characteristics of high interest, as the ability to accumulate lipids, high flexibility in carbon source utilization and culture conditions, and a fatty acid composition highly similar to vegetable oils [5]. Several physiological studies, relating to growth and lipid production by Lipomyces starkeyi, was reported in literature but urban wastewater was never tested as a growth medium for this microorganism [14–16].

The aim of this work is to investigate the synergistic effect of mixed yeasts-microalgae cultures to enhance the microbial lipids accumulation using urban wastewater as a growth substrate. The mixed biomass growth was monitored and the dissolved nutrients concentrations were measured during the cultivation period. A microbial evaluation was also conducted in order to understand the evolution in time of the microbial community in the water pond. Indeed, in the last decade, microalgae were found to produce antibiotics: a large number of microalgal extracts and extracellular products showed antibacterial, antifungal, antiprotozoal and antiplasmodial functions. The antimicrobial activity of microalgae was attributed to several chemical compounds, as indoles, terpenes, acetogenins, phenols, fatty acids and volatile halogenated hydrocarbons [31,32].

Materials and methods

Strains

Lipomyces starkeyi DBVPG 6193 was used as oleaginous yeast, purchased from the Culture Collection of the Dipartimento di Biologia Vegetale of the Perugia University (Italy). The strain was maintained at 5 °C on a YPD solid medium with the following composition (g/L): yeast extract (10), peptone (20), D-glucose (20), agar (20). Prior to fermentation, yeast was grown in a

100 mL Erlenmeyer flasks with an initial volume of 50 mL which contained (g/L): KH_2PO_4 (3.0), Na_2HPO_4 (1.0), yeast extract (5.0), glucose (10.0), peptone (5.0). The pre-culture broth was sterilized at 121 °C for 21 min prior inoculation. Cultures for lipid production were inoculated with 5% v/v of the pre-culture media. The incubation of the pre-culture was carried out at 30 °C, 160 rpm for 48 hours (Minitron HT Infors, Switzerland).

The microalgae polyculture was obtained from a native wastewater biomass. It was collected from the effluent channel of a secondary clarifier located in the urban wastewater treatment plant of Isernia (Italy). Afterwards, the biomass was maintained in laboratory controlled conditions using Bold basal medium [17] as growth substrate. The cultivation was conducted in 1 L flask equipped with a magnetic stirring bar (150 rpm) which maintained the biomass in suspended conditions. The culture was kept under a homogeneous and continuous light of 1500 Lux (Cool White Fluorescent Lamps) with the environmental temperature of (25±2) °C. Optical microscope analysis showed that biomass resulted composed by cyanobacteria, diatoms and microalgae (mostly *Scenedesmus* sp. and *Chlorella* sp.).

Culture media and conditions

The culture media consisted in raw urban wastewater, collected in the entrance of the wastewater treatment plant located in Pesche (Isernia, Italy). The raw wastewater was half diluted with tap water for a total cultivation volume of 150 L. The dilution was performed in order to reduce odours emissions and liquid turbidity, resulting in a better light penetration. Indeed, light penetrability is one of the principal parameter that influence the microalgal photosynthesis and it is principally affected by pond depth, microalgal and suspended particulate concentrations in the medium [18]. Physical and chemical characteristics of the raw wastewater are reported in Table 1. Pre-cultured wastewater native microalgae were added for the 3% v/v and pre-cultured *Lipomyces starkeyi* was added for the 1.5% v/v. The cultivation was carried out for 14 days in batch mode.

Tab. 3. - Wastewater characterization

Urban Wastewater physic-chemical characteristics	
pH	7.5 ± 0.1
Dissolved oxygen (mg/L)	3.4 ± 1.4

TSS (mg/L)	310 ± 13
COD (mg O_2/L)	480 ± 19
NH_4^+ (mg/L)	18.0 ± 0.6
PO_4^{3-} (mg/L)	2.1 ± 0.4
NO_2^- (mg/L)	< 0.1
NO_3^- (mg/L)	< 0.1

The experimental activity was conducted in outdoor conditions in July 2016, with an average natural light intensity of 600 µmol m^{-2} s^{-1} and natural light/dark cycles. The pilot scale raceway pond was installed on the roof of the Department of Bioscience and Territory, University of Molise, Pesche (Isernia, Italy). The pond is composed of a single-loop open channel with semi-circular end-walls with 1 m^2 surface area and 0.4 m^3 as total volume. A four-blade paddle wheel, coupled with a motor engine working at 6 rpm, was used to mix the culture media and keep constant a mean surface velocity of 0.10 m/s.

Analytical methods

Total suspended solids (TSS) were considered as indicator of the total biomass concentration in the raceway pond [19,20]. TSS measurements were conducted every day according to Standard Methods (2012).

Dissolved oxygen (DO) and pH values were determined using a DO meter (YSI 550 DO) and a pH meter (HI 8424, Hanna) respectively. The biomass concentration was monitored with a Shimadzu UV 1601 spectrophotometer (Japan) and measuring the turbidity of liquid samples at 600 nm and 680 nm. These readings result proportional to yeasts and microalgae concentrations respectively, according to the scientific literature [5,22].

Yeasts and microalgae cells growth was monitored by cells counting. Moreover, chlorophyll a (Chl a) measurements were used as a further indicator of microalgal growth. A fluorometer (AquaFluorTM; Handheld Fluorometer/Turbidimeter; Turner Designs) was used to measure the content of *in vivo* chlorophyll a in raw samples.

Biomass production, for both yeasts and microalgae, was evaluated according to a first order Monod law (Equation 1); as consequence, the biomass production rate (μ, d^{-1}) was calculated according to Equation 2, where X is the biomass concentration (cells/mL) and t is the cultivation time (day).

$$\frac{dX}{dt} = \mu X \quad [1]$$

$$\mu = \frac{[\ln(X_t/X_0)]}{(t-t_0)} \quad [2]$$

The biomass productivity (P, mg $L^{-1}d^{-1}$) was calculated according to the following Equation 3:

$$P = \frac{TSS_t - TSS_o}{t-t_0} \quad [3]$$

where TSS_0 (mg/L) is the biomass concentration at time t_0 (d) and TSS_t (mg/L) is the biomass concentration at any time t (d) subsequent to t_0.

Dissolved nutrients quantities were determined using Liquid Ion Chromatography (Dionex, ICS 1000) as NH_4^+, NO_2^-, NO_3^-, PO_4^{3-} concentrations. Chemical Oxygen Demand (COD) measurements were conducted for raw samples and surnatant after centrifugation (3000 rpm, 10 minutes), according to Standards Methods (2012). The removal rate of relevant substrates, R_i (mg $L^{-1}d^{-1}$, i = phosphate-P, ammonia-N, organic carbon-C), was calculated applying Equation 4.

$$R_i = \frac{S_{0,i} - S_i}{t_0 - t} \quad [4]$$

Where $S_{0,i}$ (mg/L) is the initial concentration of substrate i and S_i (mg/L) is the corresponding substrate concentration at time t (days).

Concentrations of organic acids, alcohols and carbohydrates were measured by HPLC (LC2010, Shimadzu, Japan) with a refractive index detector (RID-20A, Shimadzu, Japan). Samples were first centrifuged at 12,000 rpm for 15 min and then supernatants were filtered with 0.2 μm syringe filters. HPLC analysis were performed at a flow rate of 0.7 mL/min on an supelcogel, 300 x 7.8 mm (Supelco) column at a temperature of 60 °C. H_3PO_4 at 4 mM was used as the mobile phase. Total lipid content was determined by sulfo-phospho-vanillin method [23]. For lipid composition analysis, the extraction was performed with a method adapted to Bligh and Dyer protocol [24]. The

samples were stirred in a $CHCl_3/CH_3OH$ mixture (2:1 w/v) over 24 hours, and the oleaginous biomass was filtered off and washed with additional $CHCl_3$. The solvent was then removed by evaporation under N_2 stream. The extracted lipids were subjected to a transesterification reaction in a stirred container at 60 °C for 10 min, using NaOH (1% w/v) as catalysts and using methanol as reagent. The samples were dried by N_2 stream and subsequently 1 mL of heptane was added for the analysis. The fatty acid compositions of the FAME were analyzed using a gas chromatography (GC). The GC (GC-MS 2010, Shimadzu, Japan) was equipped with a flame ionization detector and an Omegawax 250 (Supelco) column (30 m x 0.25 mm I.D., 0.25 µm). Helium was used as a carrier gas (flow rate: 30 mL/min). The samples were initially dissolved in 1 mL of heptane and 1 µL of this solution was injected to the column. The temperature of the column was kept at 50 °C for 2 min, then heated to 220 °C at a rate of 4 °C/min, and finally kept constant for 2 minutes. Methyl decanoate was used as the internal standard. The peaks of each methyl ester were identified by comparing the retention time with the peak of the pure standard compound.

Microbial Evaluation

Samples were imaged using a standard microscope equipped with a 40x objective (Nikon eclipse 80i). Samples, collected at 0 day, 1 day, 7 days and 14 days, were incubated over night (O.N.) at 37°C on 300 rpm rotating agitator. The day after, 200 µL of each sample were inoculated into 10 mL of "Brain Heart Infusion" (BHI) (OXOID – CM1135) liquid growth medium at 37 °C O.N. as before. The microbiological growth curve was evaluated via optical density (O.D.) at 600 nm using a spectrophotometric method (Eppendorf BioPhotometer UV/vis Spectrophotometer mod. 6131) and compared to the respective controls.

Then, 10 µL of each samples were spread on BHI (Brain Heart Infusion Agar), MSA (Mannitol salt agar–bioMérieux - 43671), MCK (Mac Conkey agar – bioMérieux - 43141), SAB (Sabouraud glucose agar – bioMérieux - PO5001A) and SAB CG (Sabouraud supplemented with Chloramphenicol and Gentamicin agar – bioMérieux – 46651).

The plates were then incubated at 37 °C and observed after 48 hours. All data are representative of biological triplicates.

Results and discussion

Biomass growth

Microalgae and yeast were both added to the cultivation water pond at time zero and their growth was monitored for 14 days (Figures 1 a, b). The optical density (OD) readings at the two different wavelengths of 600 nm and 680 nm showed the same variation trend during the cultivation time; as consequence, the higher values (OD 680 nm) were reported in Figure 1a in order to show the mixed biomass growth in time. In the same figure (1a), the Chl-a concentrations were also reported in order to monitor the microalgal biomass production during the cultivation time. For the first 3 days, Chl-a concentrations resulted near to zero, corresponding to the initial microalgal lag-phase; then Chl-a values increased during the microalgal exponential growth phase. OD values corresponded to 0.3 abs for the first two days and decreased until day 6: the initial density could be linked to the yeast presence, whose decline conditions started after 3 days, contributing to explain the decreasing OD values. Moreover, the decreasing OD trend could be also associated to the microalgal autoflocculation, which occurred in this period, just before the microalgal linear growth phase. This phenomenon was already observed in a previous study [25] and was considered as a microalgae physiological adaptation: the autoflocculation clarifies the culture liquid promoting microalgal growth through higher light penetrability. From day 6 (Fig 1a), OD values and Chl-a concentrations followed a similar growth trend, certifying the correlation between culture liquid density and microalgal concentration. However, in order to better understand the evolution in time of microalgae and yeast, their concentrations (cells/mL) were reported in logarithm scale in Figure 1b.

Yeast growth occurred in the first day, while microalgal production occurred after an initial lag-phase. This result could be explained by the different metabolism of the two oleaginous microorganisms since microalgae growth is slower than yeast's growth [10]. During the linear growth phase (days 3-9), the microalgal consortium showed the specific growth rate of 0.36 d^{-1}, calculated according to Equation 2. After 9 days of batch cultivation, microalgae growth reached a stationary phase, while yeast was not detected until the end of the test. These results could be principally explained by the high pH values reached after day 9 (Figure 3). At the end of the cultivation period, microalgae concentration resulted of $1.4 \cdot 10^7$ cells/mL and was mainly represented by *Scenedesmus* sp., as showed by microscopical analysis (Figure 2). A similar results was obtained by Park and Craggs (2010) [26];

indeed, *Scenedesmus sp*. and *Chorella sp*. are particularly tolerant to the wastewaters conditions [27].

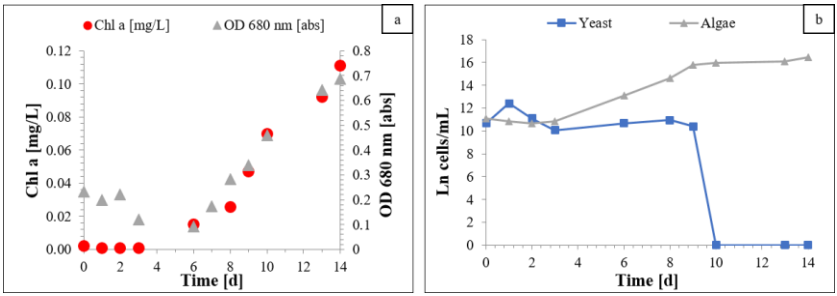

Fig. 1. –Biomass growth in time: a) Chlorophyll-a concentrations and ODs 680 nm in time; b) yeasts and microalgae cells concentrations in time.

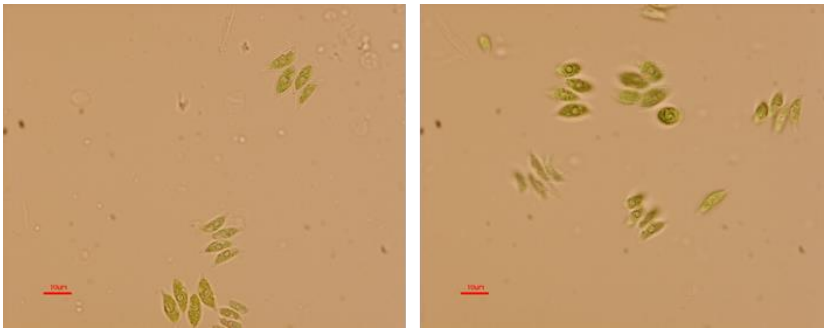

Fig. 2. - Optical microscope microalgal pictures, day 14th.

Dissolved Oxygen (DO) concentrations and pH measures were monitored during the cultivation time (Figure 3) since these parameters are crucial for microalgae and yeast growth [6,11]. Yeasts heterotrophic activity is responsible of oxygen consumption and CO_2 production [14], while microalgal photosynthetic activity induces DO and pH increasing [19]. At time zero, the inoculum addition caused pH and DO reductions. During the first 3 days, low values of DO concentrations and pH were measured, corresponding to 1 mg/L and 7.6 respectively. These values could be related to the yeast presence, whose heterotrophic metabolism limited the DO concentrations rise, that generally occur during the microalgal pond start-up [25]. On the contrary, the microalgal photosynthetic activity was responsible

of DO and pH increasing noticed after day 3. Finally, at day 9, pH values and DO concentrations showed little variations since the stationary phase of biomass growth was reached.

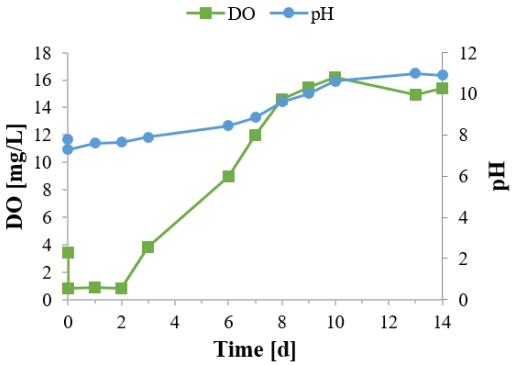

Fig. 3. - Culture dissolved oxygen (DO) and pH variation with time.

High pH and DO values, corresponding to 10.9 and 15.4 mg/L respectively, were obtained at the end of the cultivation period. These conditions could explain the almost complete absence of fungus and bacteria found at the end of the cultivation period (Figures 4A, B). The presence of these species in the samples was evaluated by a double approach: i) spectrophotometric absorbance (Figure 4A) and ii) colony forming unit on agar plates (Figure 4B). Turbidimetric data resulted comparable to the microbial growth observed on the agar plates. A remarkable decrease in microorganisms growth was observed for a corresponding increase of microalgal viability, therefore a microbicidal effect of the pond cultivation can be hypothesized. Similar results were obtained by [28] for sunlight disinfection in a high rate pond, but this topic still need further investigations.

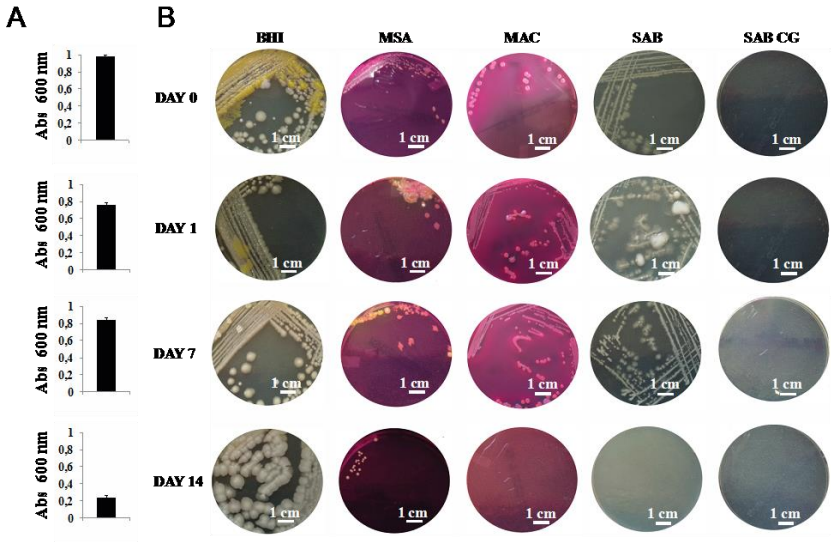

Fig. 4. - A. Spectrophotometric absorbance at 600 nm of the respective samples after O, N growth in BHI media. P-value < 0,05. B. Growth of raw (day 0) and treated sample (day 1, 7 and 14) on specific and selected media agar plates.

Total Suspended Solids (TSS) variation was monitored during the cultivation time and results were reported in Figure 5. The inoculum addition at time zero caused the TSS increasing from 160 to 215 mg/L. A decreasing trend was monitored during first 6 days of cultivation, followed by an increasing trend until the end of the test. Initial TSS reduction was related to both yeast decline and microalgae autoflocculation, as explained before for the ODs decreasing values, obtained for the same period. On the other side, TSS increasing from day 6 was principally linked to microalgae biomass production, since other microorganism growth resulted inhibited by the high monitored values of pH and DO. Microalgal productivity was calculated according to Equation 3 and corresponded to 31.4 mgTSS m^{-2} d^{-1}. This value is comparable or even higher than the ones obtained for microalgae cultivation in wastewaters. Metamoros *et al.* (2015) monitored a TSS productivity of 24 mg/L and 13 mg/L for HRTs of 4 and 8 days respectively. Garcìa *et al.* (2006) registered a TSS productivity of 12.7 mg/L and 14.8 mg/L for HRTs of 4 and 7 days respectively. Moreover, a previous study, conducted in similar experimental conditions but using only the microalgal

inoculum, showed a biomass productivity of 11.7 mgTSS m^{-2} d^{-1} [25] and an initial lag phase of 9 days. The last comparison evidenced that yeast activity during the initial microalgal lag-phase could be able to enhance biomass production. This result could be explained by the CO_2 gas enrichment of the culture liquid, which occurred during the microalgae lag-phase because of yeast respiration.

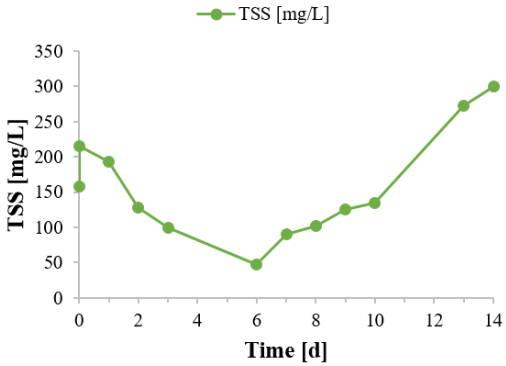

Fig. 5: Raceway pond TSS variation in time

Nutrients removal

The organic carbon content was evaluated for both raw and clarified samples; measures were conducted during the cultivation time and results were reported in Figure 6. As clarified samples were considered the culture liquid without the suspended biomass, which is also constituted by organic carbon; as consequence, the COD measures for raw samples resulted higher than the ones obtained for the clarified samples. The initial inoculum addition at time zero led to the COD increasing from 240 mgC/L to 455 mgC/L because of the high organic carbon concentration in yeast inoculum. Organic carbon was principally utilized during the first 3 days with a removal rate of 3.7 $mgC \cdot L^{-1} \cdot d^{-1}$, calculated according to Equation 4 for clarified samples. Its removal could be principally linked to both bacteria and yeast heterotrophic metabolism. On the other side, the deep COD decreasing, observed for raw samples after day 1, could be associated to the decline conditions of yeast. After the initial 3 days, a carbon accumulation and stabilization occurred in the clarified medium, possibly related to the absence of heterotrophic activities. At the same time, COD increasing values (from 100 to 500 mg/L)

were measured for raw samples starting from day 6. These results could be explained by the microalgal biomass production, which occurred in this time. On the contrary, limited yeast metabolic activity was strictly related to low concentration of easily assimilated organic substrates (fermentable sugars), as indicated by HPLC analysis.

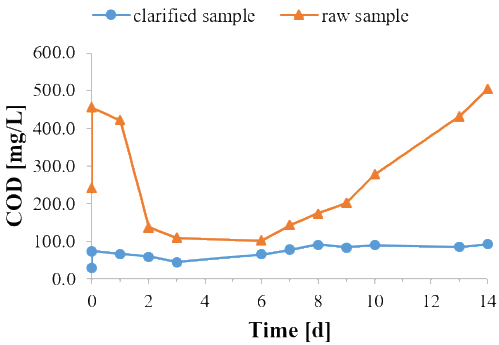

Fig. 6. - *Time monitoring of organic carbon in the cultivation media for raw and clarified samples (surnatant of centrifuged samples).*

Dissolved nitrogen (NH_4^+, NO_3^-, NO_2^-) and phosphorus (PO_4^{3-}) were monitored during the cultivation time and results were reported in Figure 7. Dissolved oxidized nitrogen (NO_2^-, NO_3^-) maintained concentrations lower than 1 mg/L during the cultivation period, so their variation was not reported in Figure 7. The inoculum addition at time zero induced phosphate increment from 0.9 to 12.2 mg/L and ammonium reduction from 8.9 to 4 mg/L, probably due to its complexation with ionic species contained into the inoculum medium. As showed in Figure 7, phosphate concentrations decreased during the microbial heterotrophic activity (day 0-2), then increased to reach the initial value of 12.2 mg/L at day 6. This variation could be associated to the yeast cell viability decline, which induced the release of accumulated phosphorus. At the same time, for the first 2 days, ammonium concentrations were maintained at 4 mg/L and then increased towards the initial value. Also in this case, ammonium variations could be associated to the yeast activity. These considerations could be confirmed seeing at results obtained in the previous experimentation, conducted without yeast inoculum: during the microalgal lag-phase, phosphate concentrations remained constant while ammonium quantities decreased because of heterotrophic bacteria activity

[25]. As consequence, since ammonium was not consumed in this case, nitrifying bacteria metabolism could be inhibited by yeast competition.
At day 6, corresponding to microalgae spreading, the depletion of both ammonium and phosphate occurred because of the combined effect of microalgal nutrients absorption and pH rise [29]. Nutrients removal rates resulted in 2.9 mgN·L^{-1}·d^{-1} and 0.96 mg mgP·L^{-1}·d^{-1}. They were evaluated until the microalgal decline conditions, applying Equation 4. At the end of the cultivation period, both ammonium and phosphates were depleted. Cheirsilp *et al*. (2011) valued COD and nitrate removal rates for industrial wastes by *Rhodotorula glutinis* and *Chlorella vulgaris* mixed cultures, which resulted higher than the ones obtained using not mixed cultures of yeasts or microalgae.

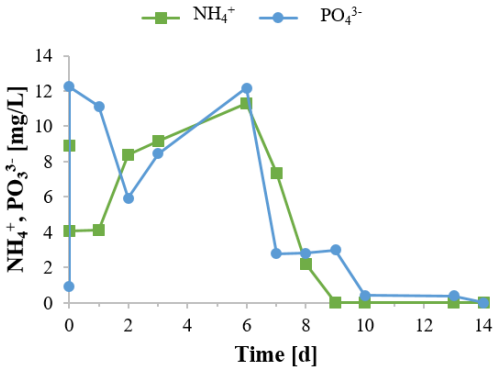

Fig. 7. - Time monitoring of dissolved ammonia and phosphates in the cultivation media.

Lipids accumulation

Biomass lipids accumulation was monitored during the cultivation period and results were reported in Figure 8 as lipids/dry weight. At time zero, the inoculum addition led to the lipids concentrations increasing from 5.2 to 8.8 mg/L (from 3.3% to 4.1% as lipids/dry weight). The first peak of 7% lipids/dry weight in the graph was registered at day two, possibly due to yeast lipid accumulation. The second and highest peak of 15% lipids/dry weight was measured at the end of the cultivation time and could be principally related to the microalgae lipid content. Indeed, similar lipids percentages were obtained by Sacristán de Alva et al. (2013) for *Scenedesmus acutus* growth in wastewaters with low nutrients content (7.3 mg/L of

orthophosphate, 27.7 mg/L of organic nitrogen and ammonia). Moreover, the higher lipids content, measured at the end of the cultivation period, agrees with previous studies [30], which highlighted that high lipid accumulation occurs when the cells are under physiological stress conditions (as nitrogen depletion) or if they are at a stationary growth state. The composition of lipid extracted from mixed yeast/microalga culture resulted principally in long-chain fatty acids with 16 and 20 carbon including palmitic acid (3%), and arachidic acid (97%).

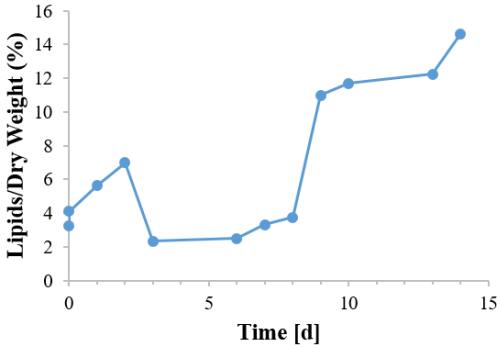

Fig. 8. - Biomass lipid accumulation in time

Conclusions

The preliminary results obtained for the combined yeasts-microalgae biomass, cultivated outdoor, using urban wastewater as growth substrate, highlighted that yeasts and microalgae showed different growth phases. Indeed, when the mixed biomass was inoculated at the same time, yeast growth started just after the inoculation, while microalgal growth occurred only after a lag-phase. Moreover, yeast growth resulted poor developed because of the low concentration of fast-assimilated organic carbon in urban wastewater. These findings suggest that lipid accumulation, for the tested experimental conditions, could be enhanced adding the yeast inoculum during the microalgal exponential growth and increasing the easily assimilated organic substrates to urban wastewater or using dairies wastewaters. The first solution would led to the synergistic growth of the combined species, while the second solution would enhance yeasts growth.

On the other side, yeast activity during the initial microalgal lag-phase seemed to enhance the microalgal biomass production.

The complete nutrients removal resulted feasible in the combined yeast-microalgae cultivation, even if it could be principally associated to microalgal activity.

Finally, the disinfection capability, related to the high pH and DO values (induced by microalgal photosynthetic activity) or linked to microalgal metabolites, resulted of relevant interest for further investigations.

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Chapter 6

Paper I

Bioflocculation of filamentous cyanobacteria, microalgae and their mixture in synthetic medium and urban wastewater for low-cost biomass harvesting.

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Keywords: bioflocculation, cyanobacteria, microalgae, nutrients removal, biomass harvesting

ABSTRACT

Cyanobacteria and microalgae represent a significant feedstock for the production of added value products and biofuels. However, high cost of production of biomass associated with harvesting technologies is one of the major bottlenecks for commercialization of algae-based industrial products. Recent studies identified bioflocculation as a promising process for low-cost and environmental sustainable biomass harvesting technique. In the present work, bioflocculation has been studied for three different inocula: wastewater born filamentous cyanobacteria, microalgae and their combination. Their cultivation has been conducted in batch mode, using synthetic medium and urban wastewater as growth liquor, comparing nutrients removal and bioflocculation behavior along time. Main results showed that flocculation and settling naturally occurs in case of filamentous cyanobacteria cultivated in synthetic medium, while biomass settling was prevented in wastewater medium. In each case, microalgae bioflocculation was limited; however, when cultivated with cyanobacteria, their flocculation was enhanced.

Introduction

Microalgae and cyanobacteria attract a lot of interest as new biomass feedstock for the production of food, feed, fuels, and chemical building blocks [1–3]. There are several aspects of microalgal and cyanobacterial production that capture the interest of researchers and entrepreneurs around the world. These interests include: (1) they are able to perform oxygenic photosynthesis using water as an electron donor, (2) they can grow to high densities and have high per-acre productivity compared to typical terrestrial oil-seed crops, (3) they are non-food based feedstock resources, (4) they can utilize otherwise non-productive, non-arable land, (5) they utilize wide variety of water sources (fresh, brackish, seawater and wastewater), and (6) they produce both biofuels and valuable co-products [4]. Despite these interests, microalgae and cyanobacteria upscaling production is especially limited with a focus here on the harvesting step.

Harvesting the microalgal biomass is challenging given the small size of the cells (5–20 μm), which confers low settleability to the biomass, and the relatively low biomass concentration in the culture medium (0.5–5 g/L) [5,6]. At this moment, there is no microalgal harvesting method that is both economically viable and efficient [7]. In commercial systems, microalgae are currently harvested using centrifugation; however, it is too expensive because of the high investment costs and high energy demand, especially for low-value applications [8]. In the context of wastewater treatment, only low-cost techniques capable of managing large volumes of water and biomass can be applied, such as coagulation–flocculation followed by solid/liquid separation; unfortunately these processes lead to sludge volumes increment and could contaminate down-stream products, thus restricting biomass valorisation [9]. Actually, microalgae cultivation in wastewater is considered the only economically viable way to produce algal biomass for conversion to biofuels with minimum environmental impact [10]. Lowering harvesting costs is thus considered a key factor for the development of sustainable full-scale production of microalgae and cyanobacteria biomass.

In this context, an attractive alternative is represented by the natural flocculation of the biomass, consisting in autoflocculation and bioflocculation processes. Autoflocculation occurs at high pH levels, caused by consumption of dissolved carbon dioxide during photosynthesis: increasing pH causes precipitates of calcium and phosphate, which will be positively charged; algae cells serve as a solid support for the precipitant and charge neutralization is accomplished [1]. Anyway, autoflocculation may not

be possible in all waters; moreover, high pH values could compromise microalgae further valorization [2]. Bioflocculation represents an attractive solution for biomass harvesting since it is low cost, low energy, non-toxic to microorganisms and does not require the use of flocculants, enabling simple medium reuse [3]. Bioflocculation refers to the naturally induced flocculation due to the secreted biopolymers by the microbial cells [4,5]. Recent researches studied the bioflocculation process for algal-bacteria, algal-fungal or algal-algal interactions [6], but cyanobacteria-microalgae interactions remained unexploited. However, in case of wastewater microalgal cultures, high settling efficiencies have been observed when filamentous cyanobacteria are prominent in the microalgal community [7]; this finding suggests a possible bioflocculation process derived from microalgae-cyanobacteria interaction.

For this purpose, this experimental study investigated the potential flocculation of filamentous cyanobacteria, microalgae and their mixed culture in both synthetic medium and urban wastewater. Batch cultivation tests of 10 days were conducted monitoring biomass growth, nutrients removal and biomass flocculation tendency along time. Biomass flocculation was assessed maintaining each culture in steady state conditions and an innovative method relying on image analysis was developed in order to quantify the flocculation efficiency. Finally, microscope observations were carried out to understand the microorganisms interactions and the evolution of their characteristics along time.

Materials and methods

Microbial inocula

Three different microbial inocula were used for this study: filamentous cyanobacteria (F), microalgae (M) and a combination of both (F&M).

Cyanobacteria inoculum was obtained from a native wastewater microbial polyculture, which was collected, as biofilm state, on the effluent channel of the secondary clarifier located in the urban wastewater plant of Isernia (Italy) and further cultivated in modified Bold Basal Medium (BBM) under controlled conditions. The medium was composed of the following elements: 250 mg L^{-1} $NaNO_3$, 25 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 75 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 75 mg L^{-1} K_2HPO_4, 175 mg L^{-1} KH_2PO_4, 25 mg L^{-1} NaCl, 11.4 mg L^{-1} H_3BO_3, alkaline EDTA solution (50 mg L^{-1} EDTA, 31 mg L^{-1} KOH), acidified Iron

solution (5 mg L^{-1} $FeSO_4 \cdot 7H_2O$, 1 mg L^{-1} H_2SO_4), trace metals solution (8.8 mg L^{-1} $ZnSO_4 \cdot 7H_2O$, 1.4 mg L^{-1} $MnCl_2 \cdot 4H_2O$, 0.7 mg L^{-1} MoO_3, 1.6 mg L^{-1} $CuSO_4 \cdot 5H_2O$, 0.5 mg L^{-1} $Co(NO_3)_2 \cdot 6H_2O$, 8.4 mg L^{-1} $NaHCO_3$, 4.77 g L^{-1} HEPES buffer [8]. Inoculum cultivation was conducted in 500 mL glass flasks, with 200 mL of medium, mixed thanks to a shaking table operating at 150 rpm. The culture was kept under homogeneous and continuous light intensity of 100 ± 10 µmol $m^{-2}s^{-1}$ (cool white fluorescent lamps) with constant temperature of 25±1 °C. A standardized procedure [9] was applied to control biomass growth, consisting in the reinoculation each three days in new medium at exponential phase condition. Microscopic observations showed that this inoculum was mainly characterized by filamentous cyanobacteria, identified as *Pseudanabaena* sp. and *Leptolyngbya* sp. (Figure 1, left) by their morphological features. In the described cultivation process, cyanobacteria inoculum showed a natural flocculation tendency.

Microalgal inoculum was collected from a microalgal raceway pond (22 m^3; 60 m^2) performing outdoor at Laboratoire de Biotechnologie de l'Environnement (LBE) located in Narbonne (France). The pond was continuously fed with a synthetic medium characterized by a chemical composition similar to the urban wastewater [10]. The inoculum was further cultivated in modified BBM under the same operating conditions of the cyanobacteria inoculum. Microscopic observations showed that this inoculum was mainly composed by *Chlorella* and *Scenedesmus* microalgal species (Figure 1, right).

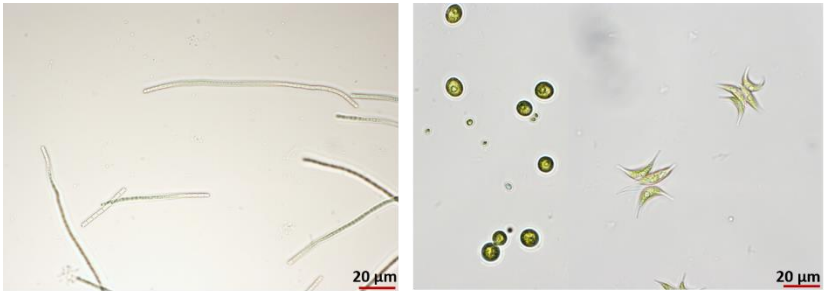

Figure 2. – Filamentous cyanobacteria (Pseudanabaena sp., Leptolyngbya sp.), left. Microalgae (Chlorella sp., Scenedesmus sp.), right.

Experimental setup

The three different inocula were cultivated in 150 mL of medium in 250 mL glass flasks, stoppered with cotton batting, placed on a shaking table operating at 150 rpm. The inocula were cultivated in batch conditions for 10 days (until nutrients depletion), under homogeneous and fixed light intensity of 100 ± 10 µmol $m^{-2}s^{-1}$ and at the constant temperature of 25 ± 1 °C. Two different media were used for this study: synthetic medium (modified BBM) and urban wastewater. The wastewater was collected at the entrance of the urban wastewater treatment plant of Narbonne (France), decanted and further filtered at 2.7 µm of porosity (Whatman glass-fiber filter, grade 50). After filtration, the wastewater medium was enriched with 4.77 g L^{-1} of HEPES buffer in order to buffer the pH drift during the batch experiment.

Each inocula was centrifuged for 20 minutes at 18500 rpm and the pellets were resuspended in 700 mL of the synthetic growth medium (modified BBM). Two stock solutions were obtained: F (filamentous cyanobacteria) and M (microalgae), both characterized by an optical density of 0.5 at 620 nm and 660 nm respectively. Same quantities of the two stock solutions were homogeneously mixed to obtain the combined culture of cyanobacteria and microalgae (F&M). The produced stock solutions (F, M, F&M) were used to make triplicate of each culture. The same operation was applied for the filtered wastewater medium obtaining the three cultures of cyanobacteria (Fw), microalgae (Mw) and their combination (F&Mw). In this case, the optical densities measured for the cultures were 0.1 at the specific wavelength as indicated in the analytical methods. The higher density related to the cultures in wastewater was principally caused by the higher turbidity of the medium itself.

Analytical methods

In case of cyanobacteria cultivation (F), biomass growth was monitored by optical density reading at 620 nm as a proxy for phycocyanin content, which is proper of cyanobacteria biomass [11]. In case of microalgae cultivation (M), biomass growth was monitored by optical reading at 660 nm [8] and by total chlorophyll a measurements [12]. Biomass growth for mixed cultures (F&M) was monitored by optical density reading at both 620 and 660 nm. Biomass growth was monitored every day taking the sample in suspended condition during the cultivation. The dry cells weight (DCW) of biomass was

determined using the method of suspended solid measurement [13]. The biomass productivity (P, mg $L^{-1}d^{-1}$) was calculated according to Equation 1:

$$P = \frac{DCW_t - DCW_o}{t - t_0} \quad (1)$$

where DCW_0 (mg/L) is the biomass concentration at time t_0 (d) and DCW_t (mg/L) is the suspended solids concentration at any time t(d) of the cultivation test following t_0(d).

Dissolved nutrients concentrations were quantified using an ion chromatography system (ICS 3000 Dionex, USA). In particular, nitrogen (N) as nitrate (NO_3^-), nitrite (NO_2^-) and ammonia (NH_4^+) and phosphorus (P) as phosphate (PO_4^{3-}) were monitored. The removal rate R_i (mg $L^{-1}d^{-1}$) of the generic substrate i (N- NH_4^+, N- NO_3^-, P- PO_4^{3-}) in the growth medium was calculated according to Equation 2.

$$R_i = \frac{S_{0,i} - S_i}{t - t_0} \quad (2)$$

where $S_{0,i}$ (mg/L) is the initial concentration of substrate i at time t_0 (d), S_i (mg/L) is the corresponding substrate concentration at time t (d).

Stoichiometry of nutrients consumption was evaluated applying the following Equation 3.

$$R\left(\frac{N}{P}\right) = \frac{N_{t0} - N_t}{P_{t0} - P_t} \quad (3)$$

where N_{t0} and P_{t0} are the nitrogen (as N- NH_4^+ or N- NO_3^-) and the phosphorus (as P- PO_4^{3-}) concentrations (mg/L) respectively at time zero, while N_t and P_t are the nutrients concentrations (mg/L) at a given time (t, day).

Biomass flocculation evaluation

Biomass flocculation was assessed maintaining each culture in static conditions. The flocculation was performed in 100 mL glass tubes for a working volume of 50 mL of each culture during the cultivation time. The tubes were located in fixed positions into a box, where static and light-controlled conditions were maintained. Biomass flocculation was monitored taking photos at fixed times and for fixed relative position between camera (Canon EOS 7D model) and glass tubes. The photos elaboration conduced to the evaluation of the “clarified area”, consisting in the zones within the liquid

cultures where there is a concentration of biomass lower than the one estimated at time zero. The clarified area formation was strictly related to biomass flocculation, so it can be considered as an indicator of flocculation intensity.

The photos elaboration was conducted using Image J software. Photos were first homogenized in order to obtain the same light of background. After which, for each glass tube, a matrix was extracted in order to convert the culture's area in numbers: each pixel was associated to a specific number depending on its colour (Supplementary materials - section A). Applying this operation to not inoculated media at time zero, the blank value was defined for each medium and used for the normalization of matrices. Finally, matrices were analysed in order to evaluate the "clarified area", which corresponded to the number of pixels characterized by values that differ at most 60% from the blank value. The indicated percentage was obtained considering the blank variability for the culture at time zero.

Microbial composition of the flocculated biomass was studied analysing samples with an optical microscope (Olympus BX53F) and images were taken using a camera (micro Olympus, DP 80). Biomass characterisation was conducted by morphological features and comparisons with literature databases.

Results

Biomass growth and nutrients removal

Biomass growth is reported in Figure 2 in terms of optical density variations for each tested condition. In case of mixed inocula (FM and FMw cultures), optical densities at the two wavelengths of 620 and 660 nm showed similar values. The different microbial species showed higher biomass production if cultivated in wastewater medium (Fw, Mw, F&Mw) compared to BBM medium (F, M, F&M), as indicated by the higher optical densities reached by wastewater cultures during cultivation time. For both cultivation media, the microbial growth showed the same trend until day 4, indicating that growth condition in this period resulted not influenced by different microbial species or growth media characteristics, but only by environmental parameters such as light intensity and temperature since they are fixed for all the tested conditions. In case of BBM cultures, after day 4, microalgal cultures (M) showed optical density increasing until day 10, while cyanobacteria cultures

(F) and mixed cultures (F&M) showed an optical density decrease after day 5 and a decline state was observed for cyanobacteria cultures at the end of the cultivation time. Using BBM modified medium, a lower biomass concentration was measured in case of cyanobacteria cultures compared to the microalgal cultures. In case of wastewater, biomass growth resulted similar for the different microbial species; moreover, the optical density increasing was monitored during the entire cultivation time suggesting no strong limitation during the batch duration.

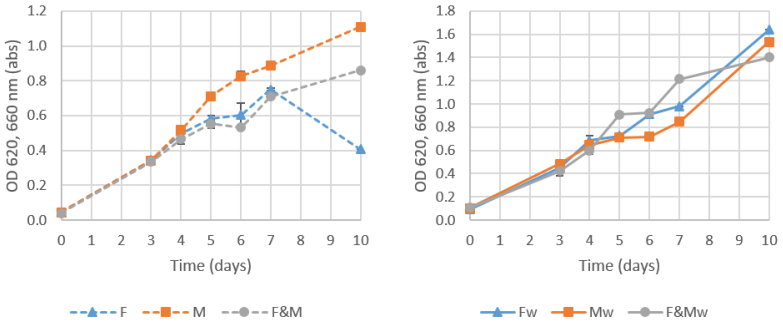

Figure 3. – Biomass growth during the cultivation time for cultures in BBM (cyanobacteria – F, microalgae – M, mixed cyanobacteria and microalgae – F&M) and in wastewater (Fw, Mw, F&Mw).

Measurements for chorophyll a concentrations were conducted in case of microalgal cultures in order to better differentiate microalgae growth in the different growth media; results are reported in Figure 3. Wastewater medium induced higher chlorophyll a concentrations compared to BBM medium, corresponding to a higher biomass production. In case of BBM medium, a chlorophyll a decrease was observed after day 6, indicating that limiting condition for biomass growth occurred in the cultivation liquid. In case of wastewater medium, a stationary state was observed after 7 days.

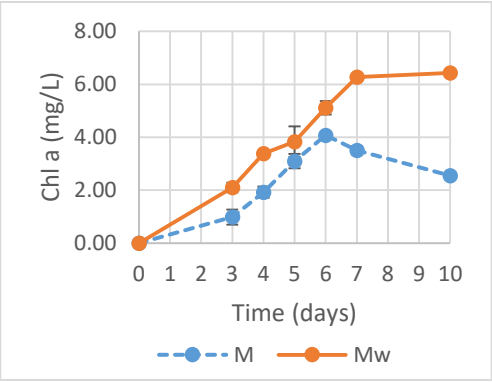

Figure 4. – Chlorophyll a variation in time for microalgae cultivation in BBM (M) and in wastewater (Mw).

Biomass productivity (P) was finally measured as dry weight variation in time, applying equation 1, between time 0 until day 7 for cultures in BBM and till day 10 for cultures in wastewater in order to obtain a positive growth rate. Results are summarized in Table 1 for each tested conditions. Inocula cultivated in wastewater (Fw, Mw, F&Mw) showed higher biomass productivity compared to the ones cultivated in the synthetic medium (F, M, F&M). Anyway, for both cultivation media, the combined cyanobacteria and microalgae cultures (F&M, F&Mw) reached the highest biomass productivities, corresponding to 97.6 ± 4.9 mg/L/d and 129 ± 7 mg/L/d in BBM and in wastewater, respectively. The two different cyanobacteria and microalgae inocula showed similar biomass production rates in BBM (66.6 ± 3.3 mg/L/g for F, 64.2 ± 3.2 mg/L/d for M) and in wastewater (119 ± 6 mg/L/d for F, 114 ± 6 mg/L/d for M).

Table 4. – Biomass productivity (P), nutrients concentrations at time zero, nutrients removal rate and N/P ratio in BBM and in wastewater for the different inocula.

Sample	**F**	**M**	**F&M**	**Fw**	**Mw**	**F&Mw**
P $_{overall}$ (mg/L/d)	66.6 ± 3.3	64.2 ± 3.2	97.6 ± 4.9	119 ± 6	114 ± 6	129 ± 7
N-NH_4^+ t=0 (mg/L)	-			56.2 ± 0.6		
R(N-NH_4^+) (mg/L/d)	-	-	-	5.55 ± 0.4	5.48 ± 0.7	5.49 ± 0.5
N-NO_3^- t=0 (mg/L)	29.1 ± 0.2			-		

R(N-NO_3^-) (mg/L/d)	5.76 ± 0.2	5.48 ± 0.3	5.65 ± 0.2	-	-	-
P-PO_4^{3-} t=0 (mg/L)	52.6 ± 0.4			5.9 ± 0.3		
R(P-PO_4^{3-}) (mg/L/d)	4.43 ± 0.6	1.67 ± 0.3	1.96 ± 0.6	1.00 ± 0.1	1.45 ± 0.1	1.14 ± 0.2
R(P-PO_4^{3-}) (%)	25%	14%	14%	96%	100%	100%
N/P	1.2 ± 0.6			21.3 ± 0.9		
R(N/P) (mg N/mg/P)	1.59 ± 0.3	4.50 ± 0.5	5.54 ± 0.4	5.72 ± 0.6	4.58 ± 0.5	5.31 ± 0.7

Dissolved nutrients concentrations were monitored during the cultivation time. In this study, nutrients removal could be mainly associated to their biotical absorption since the pH of both cultivation media was maintained at the value of 7.2 ± 0.3 thanks to the HEPES buffer. Indeed, high pH values would not be reached, so abiotic loses though ammonia volatilization and phosphorus precipitation would not be considered as removal mechanisms [14,15].

Nitrogen reduced form (ammonium, NH_4^+) was mainly present in wastewater with the initial concentration of 56.2 ± 0.6 mg N-NH_4^+/L, while nitrogen oxidized form (nitrate, NO_3^-) was mainly present in the synthetic medium with the initial concentration of 29.1 ± 0.2 mg N-NO_3^-/L (Table 1). The variation in time of both nitrogen-dissolved species are showed in Figure 4 for each tested inocula. Nitrogen removal rate resulted similar for the different inocula and for the different cultivation media, corresponding to 5.5 mg N/L/d. Su et al. measured similar nitrogen removal rate for mixed microalgae cultures in wastewater with an initial ammonia concentration of 48.9±1 mg N/L [16]. In our study, in modified BBM medium, nitrogen was depleted after 5 days of cultivation, while in wastewater it was completely absorbed at the end of the cultivation period (10 days). These data contribute to explain the biomass growth trend observed for the two different cultivation media (Figures 2, 3): in BBM, the nitrogen lack found after day 5, induced the microbial growth decreasing; in wastewater, the ammonium availability until day 10, allowed the microbial increasing trend during the entire cultivation period. Moreover, the similar nitrogen removal rates, could also explain the similar biomass productivity observed for the different species (Table 1); anyway, the higher nitrogen availability in wastewater, induced a higher biomass productivity. These results are in agreement with the study conducted by Xin et al. on *Scenedesmus sp*., who observed a positive

correlation between nitrogen availability and biomass growth with higher biomass production for higher nitrogen initial concentrations [13].

As reported in the scientific literature, microalgae and cyanobacteria are able to assimilate different nitrogen sources including ammonium, nitrate, nitrite and urea [17,18]. Anyway, ammonium is the preferred nitrogen source, since less energy is required for its uptake. Ruiz-Marin et al. reported that the microalgae *C. vulgaris and S. obliquus* showed preferences for ammonium to any other form of nitrogen present in wastewater [19]. This study showed that, when the cultivation media is composed by one specific nitrogen form, its depletion occurs at the same removal rate for any oxydized or reduced nitrogen form, for any microalgal or cyanobacteria inoculum and for any initial nitrogen concentration. Explanations for this behaviour could be different. Environmental cultivation conditions such as light availability and temperature could be considered the main influencing factors in nitrogen removal process; moreover, the pH buffered conditions, maintained for the growth medium, could also contribute to explain the same nitrogen removal trend for ammonium and nitrate. Indeed, ammonium depletion induced medium acidification, while nitrate depletion induced medium alkalinisation [18]; in this study, the buffered pH prevented these effects.

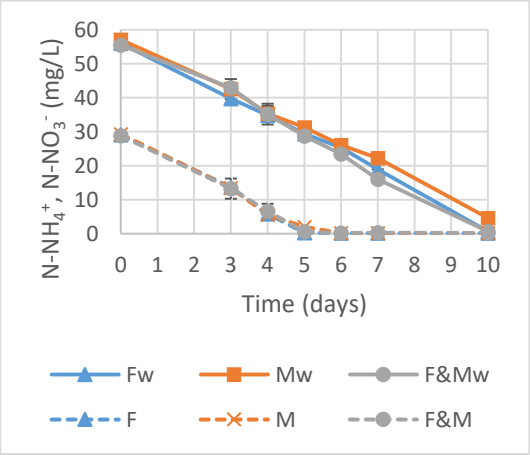

Figure 5. – Nitrogen as nitrate (N-NO_3^-) and as ammonia (N-NH_4^+) during the cultivation time for cultures in BBM (cyanobacteria – F, microalgae – M, mixed cyanobacteria and microalgae – F&M) and in wastewater (Fw, Mw, F&Mw).

Dissolved phosphorus was monitored during the cultivation time for each tested condition as orthophosphate ion; results are showed in Figure 4. In this study, in case of BBM cultures, phosphorus removal occurred during the first 6 days, after which part of it was released, obtaining the higher phosphorus removal percentage of 25% (Table 1) for cyanobacteria cultures (F). In case of wastewater cultures, phosphorus removal mainly occurred during the first 4 days and resulted completely absorbed at the end of the cultivation time. Phosphorus removal rates are reported in Table 1 for each tested condition: higher values were measured for BBM cultures and the highest removal rate of 4.43 ± 0.6 mg $P\text{-}PO_4^{3-}$/L/d was measured for cyanobacteria inoculum, while similar rates were obtained for cultures in wastewater. The different results could be principally explained seeing at the different growth state observed for BBM and wastewater cultures in time. Indeed, the decreasing growth trend, measured for BBM cultures after the nitrogen depletion, indicates stressful condition for biomass growth, which induced phosphorus release. On the contrary, the linear growth trend monitored for wastewater cultures led to the complete phosphorus uptake. As reported in the scientific literature, many microalgae can uptake much more phosphorus than required for survival under unfavourable growth conditions and usually stored these phosphorus in the form of polyphosphate (Poly-P) in cells [20]. Finally, phosphorus assimilation rate was elevated with increasing initial phosphorus concentrations. Similar results were reported by Zhu et al. studying the phosphorus assimilation by *Chlorella sp.* in case of nitrogen deficiency and for different initial phosphorus concentrations [21]. They observed that phosphorous uptake occurs mainly in the first two or three days for the different concentrations; moreover, the total phosphorous removal occurred for the lowest initial phosphorus concentration (5.3 mg/L), while phosphorus was not completely removed for higher initial concentrations (155, 310 mgP/L). Similar results were also obtained for nitrogen (as nitrate) and phosphorus removal by Liu and Vyverman for *Pseudanabaena* sp. cultivated in synthetic medium with N/P ratio of 1; they also measured higher specific growth rate for the higher N/P ratio of 20 [9]. Anyway, their study reported a lower biomass productivity (21.8 ± 0.7 mg/L/d) compared to this study, but their value was referred to a photoperiod of 12 hours, while light was continuously supplied in this study. In a similar way, Cai et al. reported nitrogen and phosphorus removal by various genera of microalgae and cyanobacteria in the axenic batch processes of different waste streams showing generally the total nitrogen removal but a lower capability in case of phosphorus [22].

Finally, in table 1 the stoichiometry of nutrients consumption, R(N/P), is reported applying equation 3 from time 0 to day 5 for each tested condition, since nitrogen was depleted at this time for BBM cultures. When the rate R (N/P) is 1, nitrogen and phosphorus consumption is equivalent; for values lower than 1, phosphorus removal is favoured, while for values higher than 1, nitrogen removal is higher than phosphorus. Results generally showed higher nitrogen removal compared to phosphorus, according to the biomass stoichiometry [23]. However, higher phosphorus uptake was registered for cyanobacteria cultures in BBM, indicating their higher luxury uptake tendency compared to microalgae, which was probably favoured in BBM because of the higher phosphorus availability in this medium compared to wastewater. This result agree with the study conducted by Beuckels et al., which demonstrated that the N concentration in the biomass not only depends on the N supply in the medium, but also on the P supply, so models for nutrient uptake by microalgae based on a fixed Redfield stoichiometry are not reliable for estimating the capacity of microalgae to remove N and P from wastewater [24].

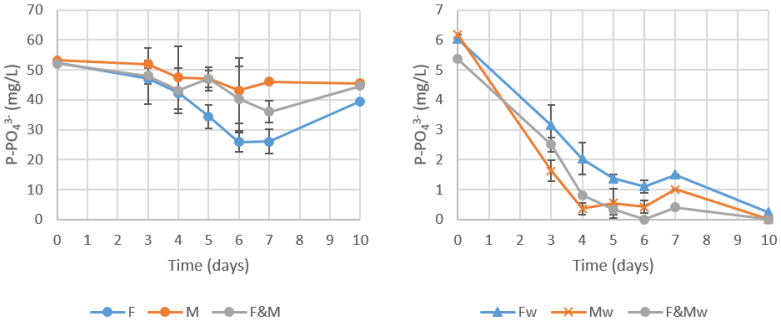

Figure 6. –Phosphorus as phosphate (P-PO_4^{3-}) during the cultivation time for cultures in BBM (cyanobacteria – F, microalgae – M, mixed cyanobacteria and microalgae – F&M) and in wastewater (Fw, Mw, F&Mw).

Biomass flocculation

Biomass flocculation was investigated through settling tests, which were conducted every day of the cultivation period for each tested condition.

Photos of the different cultures at time zero and after 50 minutes are reported in the supplementary materials in sections B and C for cultures in BBM and in wastewater respectively.

Bioflocculation in synthetic medium

In case of cultures in BBM, biomass flocculation was observed for cyanobacteria cultures (F) from day 4. The microbial aggregation showed progressively better settling proprierties until day 7, leaving a well-clarified supernatant. The sedimentation ability of flocculated biomass could be clearly correlated to the flocs size increasing from day 4 to day 7, as showed by microscope observations (Figure 6). Cyanobacteria concentration increment seemed to enhance the formation of bigger flocs, which were able to settle with progressively higher velocity, in fact higher percentages of clarified area were observed and measured from day 4 to day 7 (Supplementary materials – section B).

Figure 7. – Microscope pictures of cyanobacteria cultures in BBM at day 4, 6, 7 from left to right.

Microalgae (M) and mixed cultures (F&M) in BBM did not showed any flocculating ability at day 4 (Supplementary materials – section B), as consequence, the third replicate of cyanobacteria sample and the one of microalgae sample were mixed in order to obtain the cultures "E" with duplicate. At day 4, both inocula showed the specific optical density of 0.5 abs. This operation was aimed to favour the interaction between suspended microalgae and filamentous cyanobacteria exploiting the cyanobacteria flocculating ability, which seemed to be enhanced during the biomass exponential growth phase (E). Microscope observations for "F&M" cultures at day 4 and for "E" cultures at day 5 (Figure 7) confirmed this assumption: microalgae showed little interactions with cyanobacteria aggregation in F&M cultures, while they were included in cyanobacteria flocs seeing at E cultures. These results also allowed to E cultures to exhibit a better setting ability compared to F cultures as showed in Figure 7. Finally, at day 10, the different cultures reached the decline state, as indicated by the yellowish colour of the cultivation liquids (supplementary materials, section B).

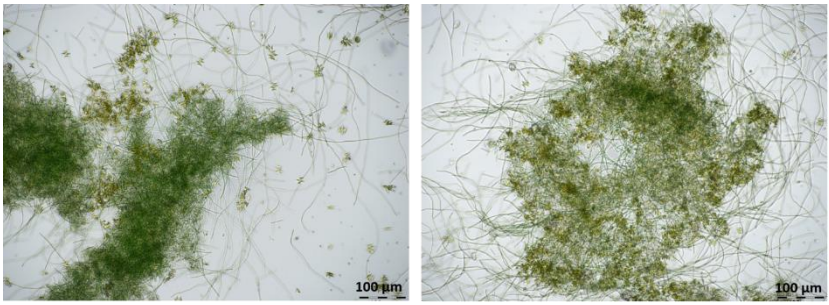

Figure 8. – Mixed cyanobacteria and microalgae cultures in BBM (F&M) at day 4 (left) and enriched mixed cultures in BBM (E) at day 5 (right).

Bioflocculation in wastewater

In case of wastewater cultures, biomass flocculation was observed already at day 3 (supplementary materials, section C) for both cyanobacteria (Fw) and mixed inocula (F&Mw) while it was not evident for microalgae inoculum (Mw). This finding, compared to the results obtained for BBM medium, could be explained by the faster biomass growth observed in wastewater medium for the different inocula, which induced a better flocculation tendency for both cyanobacteria and mixed cultures. Mixed cultures showed a good interaction between cyanobacteria and microalgae already after 3 days of cultivation, which favoured the bioflocculation. As well as BBM cultures, also in this case, the "Ew" cultures were obtained mixing the third replicate of cyanobacteria and microalgae cultures at day 3, when their specific optical density resulted of 0.5 abs. Anyway, a similar behaviour was observed for "F&Mw" and "Ew" cultures in terms of both flocculation tendency and cyanobacteria-microalgae interaction.

Compared to BBM cyanobacteria cultures, in case of wastewater medium, the cyanobacteria aggregation did not settled in time. This result could be explained seeing at the microscope observation of cyanobacteria biomass (Figure 8): in wastewater medium, cyanobacteria inoculum formed longer filaments compared to the ones observed for BBM cultures. The long cyanobacteria filaments did not aggregate in flocs structure, but they formed clouded structure which remained in suspended conditions. This behaviour could be caused by colloids particles in wastewater that could negatively interact with biomass sedimentation. Indeed, as reported by Semerjian and Ayoub, wastewater colloidal suspensions consist of negatively charged particles; when particles are similarly charged, the resulting repulsive forces

tend to stabilize the suspension and prevent particle agglomeration. Moreover, the surface of the microalgal cells is negatively charged because of the ionized functional groups on the cell wall, as consequence, electrostatic repulsion between cells prevents them from coming together and spontaneously adhering to each other by van der Waals forces [25].

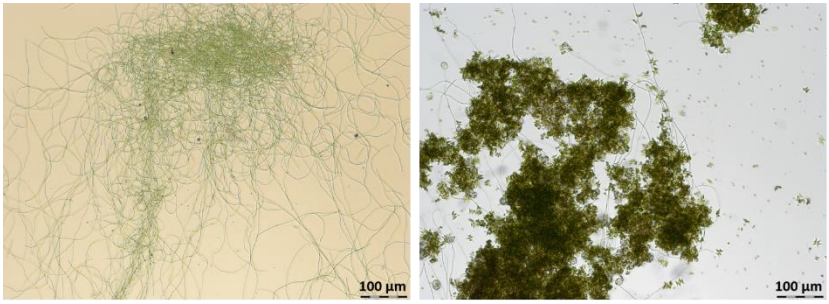

Figure 8. – Microscope pictures of cyanobacteria aggregation in wastewater at day 3 (left) and microalgae aggregation in wastewater at day 7 (right).

In this context, a cationic flocculant need to be used to neutralize the surface charge on the cells and facilitate the spontaneous formation of flocs. Coagulants that have been traditionally used in water and wastewater treatment are salts of aluminium or iron, but these substances have a limited application in microalgal systems because they can contaminate down-stream products, thus restricting biomass valorisation [26]; moreover, in case of wastewater applications, they increase the sludge production [27]. To overcome these disadvantages, chitosan biopolymer could be applied to wastewater treatment. Chitosan is a natural, biodegradable, non-toxic, polycationic polymer, whose flocculating action has been studied for different microalgal species; in case of microalgae bacteria consortia, the chitosan dosage results in 240 mg/L for 90% of biomass recovery [26]. The same chitosan addition (240 mg/L) was conducted at day 6 for the each tested culture: chitosan flocculation resulted efficient for microalgae cultures (80% of clarified area) but applying the same dosage to cyanobacteria and mixed cultures, even biomass flocculation was prevented (Supplementary Materials – section C). Lama et al. previously studied the chitosan effect on *Pseudanabaena* sp. flocculation evaluating a minimum chitosan concentration of 80 mg/L for the cyanobacteria cultivated in synthetic medium; the cationic polymer chitosan induces flocculation through a bridging mechanism [28]. The non-flocculating effect found for chitosan in

this study could be explained by the interference of colloids, which are present in wastewater medium and probably negatively interacted with filamentous cyanobacteria flocculation.

Microalgae cultures in wastewater exhibited the auto-flocculation phenomenon at days 7 and 10, as showed by microscope observations (Figure 8). Auto-flocculation refers to the cell aggregation and adhesion of cells to each other in liquid culture, due to special cell surface properties or some other factors; it can occur naturally in certain microalgae as response to some environmental stress [4]. Microalgae flocs were able to settle in 50 minutes, as showed by photos (supplementary materials, section C), anyway, the supernatant was not clarified as the one observed for cyanobacteria cultures.

Cultures in wastewater, differently from the ones in BBM, did not reached the decline conditions at day 10 but increased their biomass concentrations as showed by the greener colour intensity; anyway, the biomass increment did not still favoured its sedimentation.

Quantification of bioflocculation

As described in the materials and methods section, a specific method was developed to quantify the biomass flocculation intensity: flocculation was monitored in time through photos that were further elaborated in order to quantify the "clarified area" within each sample. More specifically, results will be analysed for cultures after 50 minutes of static conditions since not significant variations were observed after this time for the biomass flocculating state. The clarified area percentage is reported in Figure 9 and is able to numerically reproduce what is visible on the photos. Results for microalgae cultures are not reported since the clarified area was not appreciable for them. Higher clarification in liquid cultures were observed in case of cyanobacteria cultivated in BBM medium, which showed better flocculation and sedimentation tendency during the exponential growth phase (day 4-7) obtaining the 90% of clarified area. In BBM cultures, flocculation was also exhibited by mixed cultures (E) but only at day 5. In case of wastewater cultures, flocculation was observed for both cyanobacteria and mixed cultures: the flocculation intensity generally decreased in time for the different cultures. Differently from BBM cultures, in wastewater, the biomass increasing did not favoured its flocculation and settling; this behaviour could be related to the presence of colloids, as explained above, whose superficial charge did not allow the settling of the formed biomass aggregation.

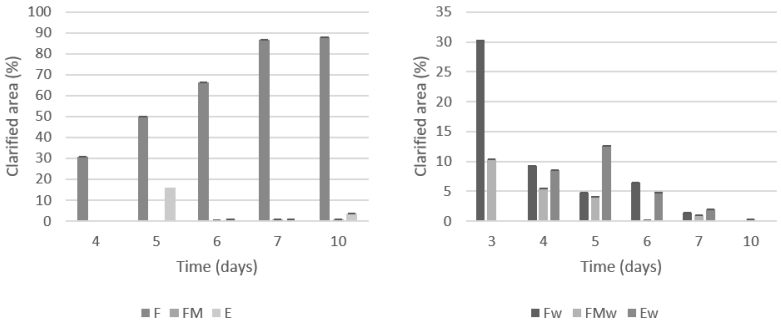

Figure 9. – Percentages of clarified area in time for cultures in BBM (F, F&M, E) and in wastewater (Fw, F&Mw, Ew) for cyanobacteria (F, Fw), mixed cyanobacteria and microalgae at day zero (F&M, F&Mw), mixed cyanobacteria and microalgae at day 4 (E, Ew).

Several studies have demonstrated that flocculation of microalgae can also be induced by increasing the medium pH, a phenomenon that is often referred to as 'autoflocculation'. It has been suggested that flocculation at high pH is caused by chemical precipitation of calcium and/or magnesium salts or by precipitation of calcium phosphate [29–31]. In this study, the pH of both cultivation media was controlled by the HEPES buffer, which stabilized its value at 7.2 ± 0.3. As consequence, biomass flocculation could not be explained by pH high values.

Conclusions

This experimental study showed that cyanobacteria and microalgae growth is influenced by similar parameters, such as nitrogen availability and environmental factors (light and temperature). The combined cultivation of microalgae and cyanobacteria enhanced the biomass production. Bioflocculation was clearly observed in case of cyanobacteria cultures, while resulted limited for microalgae inoculum. The combination of the two inocula favoured microalgae flocculation because of microalgae interactions with filamentous cyanobacteria, that could be caused by electrostatic forces or by physical connections. The natural biomass flocculation led to its settling in case of synthetic growth medium, while this effect was probably prevented by colloidal particles in case of wastewater medium. As consequence, biomass harvesting by natural flocculation resulted feasible in synthetic medium and for filamentous cyanobacteria, obtaining higher biomass

recovery during the exponential growth phase, for higher biomass concentrations in the cultures.

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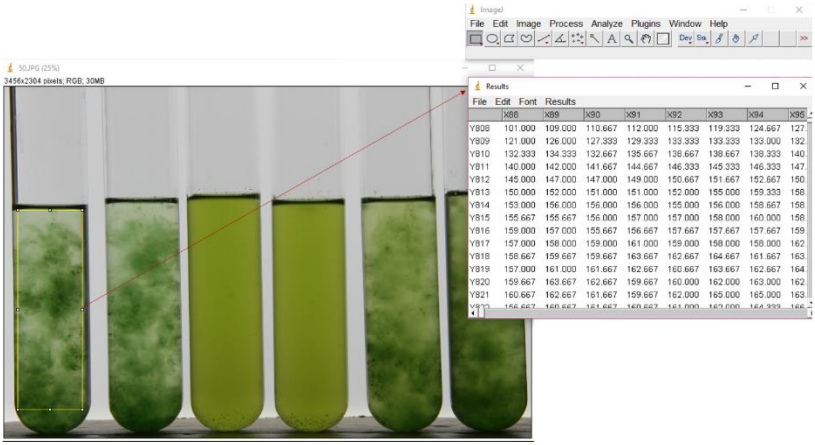
Supplementary materials

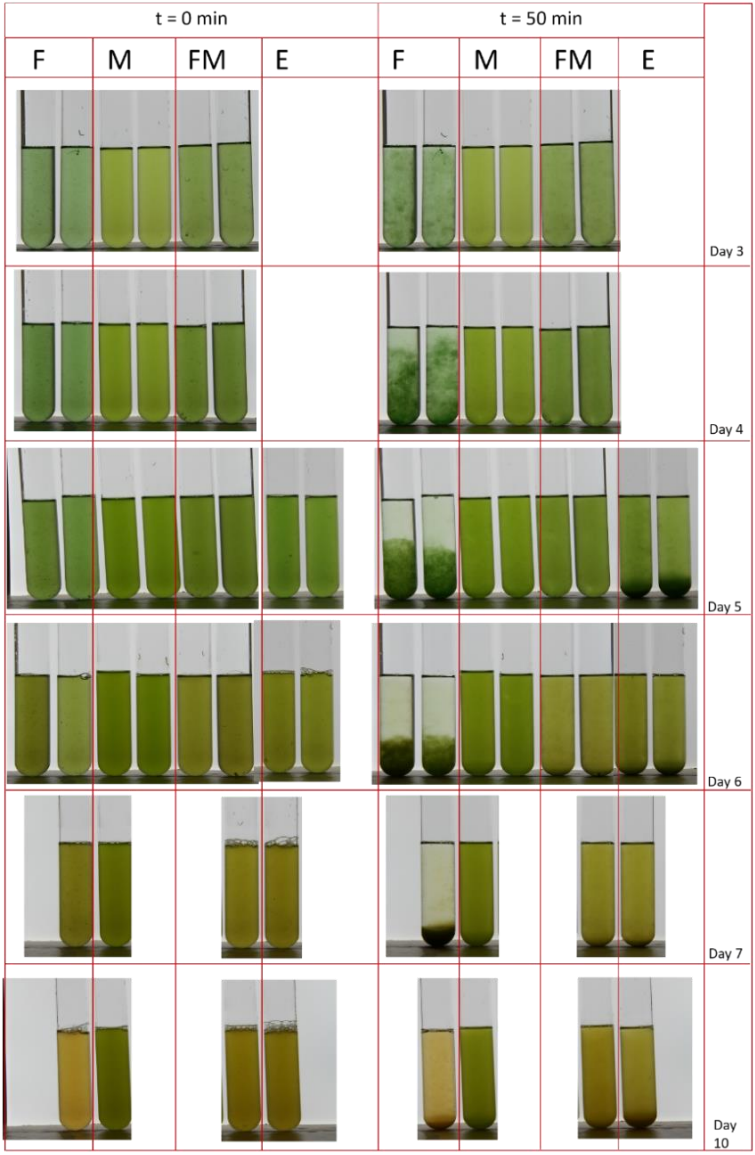
A) Photos elaboration: matrices creation

Elaboration was conducted using the software Image J. For each tube, a yellow rectangle was drawn in order to isolate the culture's area. The bottom of the glass tube was not included in the elaboration since it is a sedimentation zone, so the clarified area could be neglected for this zone. The yellow rectangle was transformed from image to results obtaining a matrix whose each number corresponds to one pixel in a way proportional to its colour.

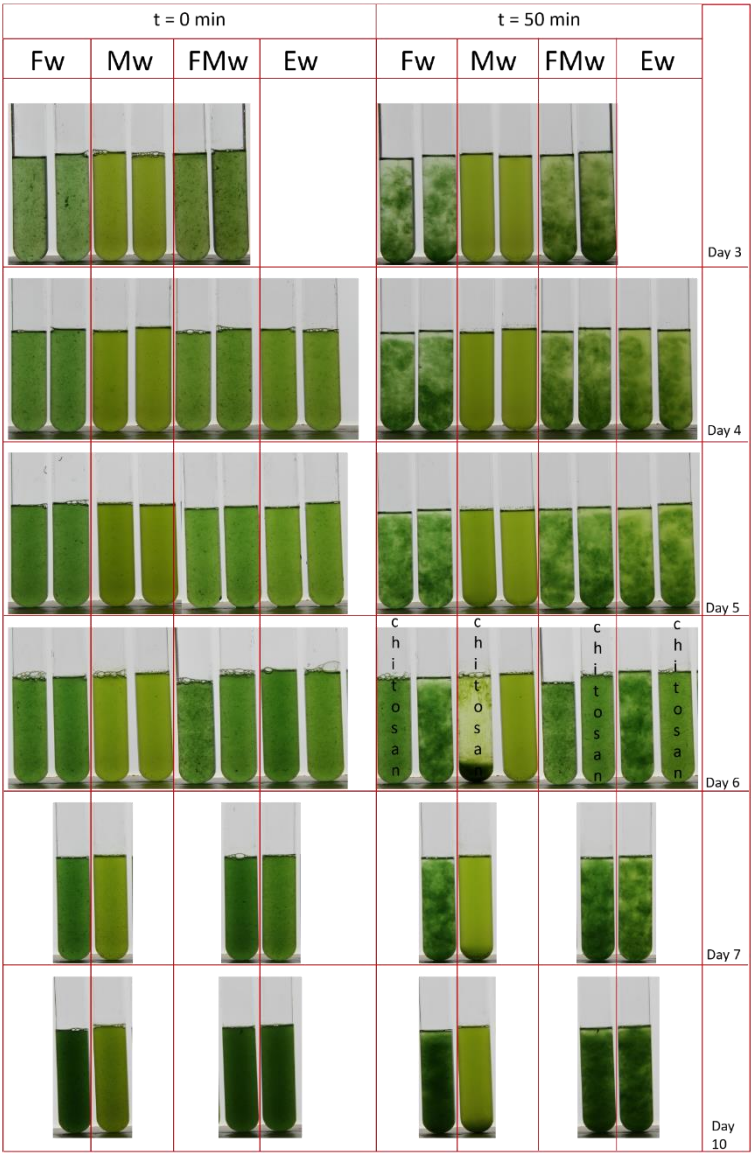


	X86	X89	X90	X91	X92	X93	X94	X95
Y808	101.000	109.000	110.667	112.000	115.333	119.333	124.667	127.
Y809	121.000	126.000	127.333	129.333	133.333	133.333	133.000	132.
Y810	132.333	134.333	132.667	135.667	136.667	138.667	138.333	140.
Y811	140.000	142.000	141.667	144.667	146.333	145.333	146.333	147.
Y812	145.000	147.000	147.000	149.000	150.667	151.667	152.667	150.
Y813	150.000	152.000	151.000	151.000	152.000	155.000	159.333	158.
Y814	153.000	156.000	156.000	156.000	155.000	156.000	158.667	158.
Y815	155.667	155.667	156.000	157.000	157.000	158.000	160.000	158.
Y816	159.000	157.000	155.667	156.667	157.667	157.667	157.667	159.
Y817	157.000	158.000	159.000	161.000	159.000	158.000	158.000	162.
Y818	158.667	159.667	159.667	163.667	162.667	164.667	161.667	163.
Y819	157.000	161.000	161.667	162.667	160.667	163.667	162.667	164.
Y820	159.667	163.667	162.667	159.667	160.000	162.000	163.000	162.
Y821	160.667	162.667	161.667	159.667	162.000	165.000	165.000	163.

B) Bioflocculation for cultures in BBM



C) Bioflocculation for cultures in ww



Paper II

Bioflocculation of wastewater native filamentous cyanobacteria for low-cost biomass harvesting

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Keywords: bioflocculation, cyanobacteria, wastewater, sedimentation model

ABSTRACT
Cyanobacteria and microalgae represent a significant feedstock for biofuel production. However, the principal obstacle for their large-scale application consists in biomass harvesting. Recent studies identified bioflocculation as a promising process for low-cost and environmental sustainable biomass harvesting technique. Unfortunately, bioflocculation process remains poorly understood, so its application is limited. In the present work, bioflocculation has been studied for filamentous cyanobacteria. Native wastewater filamentous cyanobacteria showed a natural flocculation tendency when cultivated in synthetic medium under controlled conditions of light and temperature. Bioflocculation characteristics have been analysed for two different biomass mixing systems, i.e. air bubbling and shaking table, and for different initial biomass concentrations. Flocs formation and biomass settling were monitored during batch cultures. Results showed that the two cultivation systems caused a different bioflocculant behaviour. Air bubbles promoted the formation of small and dense flocs, while oscillatory movements favoured bigger (14 mm^2 VS 4 mm^2) but more loose structures. As consequence, better biomass settleability has been obtained with air bubbling. Differences resulted principally explainable by a biomass speciation which occurred for the two biomass mixing systems. Initial biomass concentrations also influenced biomass settleability. Higher settling rates have been measured for higher initial concentrations. Finally, results are promising for biomass harvesting since the 70% of biomass resulted recoverable for natural sedimention.

Introduction

The global consumption of fossil-based fuels is continuously increasing, while the planetary reservoir is significantly depleting. Increased fuel consumption causes environmental pollution, risks for human health and global warming. Thus, a need arises to address the current energy and environmental issues to produce biofuels. Of all biomass sources, microalgae and cyanobacteria received considerable renewed attention to become a feedstock for large-scale biofuel production [1]. They have higher photosynthetic efficiency and biomass productivity compared to terrestrial crops and they are able to grow on wastewater [2]. Indeed, wastewater treatment algal pond are presently the only economically viable way to produce algal biomass for conversion to biofuels with minimum environmental impact [3]. Despite recent intense efforts to make algal-based biofuel economically viable with fossil fuels, there are still several obstacles to overcome. One of the major obstacle for large scale applications is biomass harvesting, accounting for 20–30% of the total costs of microalgal biomass cultivation [4]. Principal solutions for biomass harvesting are centrifugation and flocculation but they cannot be applied at large scale [5]. Indeed, centrifugation is a high-energy consuming process and chemical flocculation leads to the secondary pollution of the liquid effluent. An attractive alternative is represented by the natural flocculation of the biomass. This process is known as bioflocculation and represents an attractive solution for biomass harvesting since it is low cost, low energy, non-toxic to microorganisms and does not require the use of flocculants, enabling simple medium reuse [6,7]. Bioflocculation refers to the naturally induced flocculation due to the secreted biopolymers by the microbial cells [8]. Recent researches studied the bioflocculation process for algal-bacteria, algal-fungal or algal-algal interactions [6], but cyanobacteria flocculation remains little exploited. In case of wastewater microalgal cultures, high flocculation efficiencies have been observed when filamentous cyanobacteria are prominent in the microalgal community [9]. Laboratory studies demonstrated that gliding and colliding of cyanobacteria filaments produce reticulates and associated structures on solid surfaces [10]. These findings suggested that cyanobacteria natural flocculation could be possible. Starting from these observations, the present work studied the bioflocculation of wastewater native filamentous cyanobacteria in order to estimate their potential application in large scale wastewater treatment systems. Shepard & Sumner experimented that the geometry of cyanobacteria structures, their morphology and the time required for macroscopic organization depend on

cells density [10]. Moreover, studies on cells aggregation [11,12] demonstrated that the level of physical mixing influences the extent of aggregations. As consequence, the present study has been conducted in a synthetic growth medium testing different mixing conditions and different initial biomass concentration. Biomass growth and productivity were analysed for the two cultivation systems. In this work, biomass sedimentation profiles appeared to be different from classical sludge settling profiles [13]. A dedicated experimental setup and sedimentation model were thus developed to follow cyanobacteria settling. Flocs formation for filamentous cyanobacteria in synthetic growth medium were also clarified through microscope analysis.

Materials and methods

Inoculum cultivation

Microalgae biofilm was collected in the secondary clarifier of the urban wastewater plant located in Isernia (Italy) and further cultivated in modified Bold Basal Medium (BBM) under controlled conditions. The medium is composed of the following elements: 250 mg L^{-1} $NaNO_3$, 25 mg L^{-1} $CaCl_2{\cdot}2H_2O$, 75 mg L^{-1} $MgSO_4{\cdot}7H_2O$, 75 mg L^{-1} K_2HPO_4, 175 mg L^{-1} KH_2PO_4, 25 mg L^{-1} NaCl, 11.4 mg L^{-1} H_3BO_3, alkaline EDTA solution (50 mg L^{-1} EDTA, 31 mg L^{-1} KOH), acidified Iron solution (5 mg L^{-1} $FeSO_4{\cdot}7H_2O$, 1 mg L^{-1} H_2SO_4), trace metals solution (8.8 mg L^{-1} $ZnSO_4{\cdot}7H_2O$, 1.4 mg L^{-1} $MnCl_2{\cdot}4H_2O$, 0.7 mg L^{-1} MoO_3, 1.6 mg L^{-1} $CuSO_4{\cdot}5H_2O$, 0.5 mg L^{-1} $Co(NO_3)_2{\cdot}6H_2O$, 8.4 mg L^{-1} $NaHCO_3$, 4.77 g L^{-1} HEPES buffer. Inoculum cultivation was conducted in 500 mL glass flasks, with 200 mL of medium, located on a shaking table operating at 150 rpm. The culture was kept under homogeneous and continuous light intensity of 100 µmol/m^2/s (cool white fluorescent lamps) with constant temperature of 25 °C. An experimented standard procedure [14] was applied to control biomass growth, consisting in the reinoculation in new medium at exponential phase condition (3 days after inoculation).

Experimental setup

Two different cultivation systems were tested: a shaking table and a multicultivator with air bubbling. Cultivation on shaking table was performed using 250 mL glass flasks, containing 100 mL of culture medium and covered by a cotton stopper, which favoured air exchange. The shaking table operated at 150 rpm in order to mix microalgal biomass with oscillatory movements. Light was continuously supplied by cool white fluorescent lamps at 100 µmol

$s^{-1}m^{-2}$ as light intensity. Room temperature was maintained in the range of 25±2°C. Multi-cultivator (MC 1000 – OD, PSI, Czech Republic) consisted of 8 cultivation tubes where 80 ml of culture medium was maintained under controlled temperature, light and aeration conditions. The cultivation tubes were immersed in temperature controlled water bath of 25°C. Each tube was illuminated by an array of LEDs that generate incident irradiance of 100 µmol $s^{-1}m^{-2}$. In Multi-cultivator microalgal biomass was maintained in suspended conditions by air bubbling system. Cultivation was carried out for 9 days in batch conditions for both systems. The modified BBM used for the cultivation of the inoculum was adopted in the experimental tests. Inoculum was centrifuged for 20 minutes at 18500 rpm and the pellets were resuspended in the cultivation medium. Three stock solutions with different initial biomass concentrations (IBC) of 10^4, 10^5, 10^6 cells/mL were prepared at time zero. Each solution was divided for shaking table and multi-cultivator cultures. Tests were conducted in duplicate for multi-cultivator and in triplicate for shaking table.

Methods

Biomass growth and nutrients removal

Biomass growth was monitored by optical density readings at 620 nm as wavelength following phycocyanin content, which is proper of cyanobacteria biomass [15]. Total suspended solids (TSS) were determined following the standard methods procedure [16] in order to measure the dry weight of the produced biomass in time. A linear correlation was found for TSS and optical density readings: TSS (mg/L) = 1022.2*OD620nm + 34.466 (R^2 = 0.9998). Biomass growth was monitored every weekday taking the sample in suspended condition during the cultivation.

Dissolved nutrients concentrations were measured by liquid ion chromatography analysis (ICS 3000 Dionex, USA). In particular, nitrogen (N) as nitrate (NO_3^-), nitrite (NO_2^-) and ammonia (NH_4^+) and phosphorus (P) as phosphate (PO_4^{3-}) were monitored.

Biomass settling and recovery

Settling tests were performed in 100 mL glass tubes and conducted in duplicate for each tested condition. The tubes contained 80 mL of sample and were located in fixed positions into a box, where static and light-controlled conditions were maintained. Settling velocity and dynamics were evaluated taking photos at fixed times and for fixed relative position between camera (Canon EOS 7D model) and glass tubes. Results of settling tests will be

reported for 50 minutes of observation since no significant variations were monitored after this period. Acquired pictures were further analysed using the ImageJ software (NIH, USA).
The pictures elaboration consisted in the conversion of each pixel of the photos in numerical values that are proportional to the grey colour intensity. This method allowed to estimate a correlation between OD readings and grey values at time zero (Equation 1) and to analyse the biomass settling dynamics.

$$OD620nm(abs) = -0{,}0054*grey + 0{,}977;\ R^2 = 0{,}9267 \quad (1)$$

Equation 1 allows an indirect estimation of the biomass concentration into the cultures through pictures elaboration (Supplementary materials, section A).

Biomass recovery (BR) during settling test was determined comparing the OD readings at 620 nm at time zero ($OD\ 620^0$) and after 50 minutes ($OD\ 620^{50}$) for samples taken at the centred half height of each tube [7]:

$$BR\ (\%) = \frac{OD\ 620^0 - OD\ 620^{50}}{OD\ 620^0}\% \quad (2)$$

Biomass settling in time was studies through photos elaboration. More specifically, a rectangle was drawn along each tube, containing the sample, and the variation in time of grey values was studied (Supplementary materials, section B). Grey values were correlated to optical densities through Equation 1; at the same time, optical densities were correlated to cells concentrations in order to show the results in terms of particles settling. Cells concentrations were evaluated, as cells/mL, using the Malassez chamber for samples with cells in suspension. The measured concentrations were correlated to the respectively optical density values according to the following experimental correlation:

$$C = (1.0 \cdot 10^7\ OD\ 620(abs) + 2.9 \cdot 10^5)\ (cells/mL) \quad (3)$$

Cells concentrations profiles were used for modelling the sedimentation process. First and last centimetres of profiles were not taken into account for the modelling to avoid side effects due to the meniscus on the top of the liquid and to tube curvature at the bottom.

Sedimentation modelling

Particles sedimentation is assumed to be governed by steady gravitational drift and diffusion, and was modelled by Mason-Weaver equation with two populations of particles:

$$\frac{dC}{dt} = f_1\left(D_1\frac{d^2C}{dz^2} + v_1\frac{dC}{dz}\right) + f_2\left(D_2\frac{d^2C}{dz^2} + v_2\frac{dC}{dz}\right) \quad (4)$$

Where f_i corresponds to concentrations ratio C_i/C of biomass i over total biomass. D_i and v_i are the diffusion coefficient and the settling velocity of biomass i respectively.

At steady state, concentration profile was then expressed as function of height z:

$$C(z) = C_0\left(f_1 e^{-\frac{z}{D_1/v_1}} + f_2 e^{-\frac{z}{D_2/v_2}}\right) \quad (5)$$

C_0 was estimated from homogeneous concentration profiles a t = 0. Parameters f_i and D_i/v_i were evaluated on concentration profiles after 50 min settling. Population 1 corresponds to "suspended cells" that settles only slightly while population 2 correspond to "settling cells" that settle efficiently (see figure 1 in supplementary material, section C). Parameters for population 1 were thus estimated by linear regression on ln(C) on the upper part (z > 3 cm) of the profiles where $C_2 \approx 0$. Parameters for population 2 were then estimated on the residues by linear regression on $\ln(C-C_1)$ on the lower part of the curve (z < 3 cm).

For experiments with high settling fractions ($f_2 > 0.2$), sedimentation profiles after 5 min were modelled with COMSOL Multiphysics® using parameters C_0, f_1, f_2 and ratio D_1/v_1 and D_2/v_2 estimated above with parametric sweeps over settling velocities v_1 and v_2. Parameters tested were 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} m/s for v_1 and from $0.1\cdot10^{-4}$ to $5\cdot10^{-4}$ m/s every $0.1\cdot10^{-4}$ m/s for v_2. Calculations were carried out on a 1D grid constituted of 0.01 cm elements on the whole height of liquid column measured in the experiment (H = 13.6±0.9 cm), assuming null flux boundary in the upper and lower bounds of the grid. Best fit of predicted sedimentation profile to experimental sedimentation profile at 5 min was selected using minimum residual sum of squares (RSS) criterion:

$$RSS = \sum\left(C_{measured} - C_{predicted}\right)^2 \quad (6)$$

This allowed identifying v_1, D_1, v_2 and D_2 parameters for each case and simulating complete dynamics as shown in Figure 2 in supplementary material, section C.

Biomass flocculation

Biomass flocculation was studied in terms of flocs size, shape and microbial composition. After sedimentation, a subsample (1 mL) of the particulate phase was taken and analysed using a stereo zoom microscope (Leica Microsystems, M 205 FA) and images were taken using a camera (Leica Microsystems, camera DFC 495). Particle size analysis was conducted using ImageJ (NIH, USA). The original images were transformed to 8 bit and thresholded. After transformation of the image, particles with diameter higher than 1 mm^2 were isolated and their area was calculated. Analysis were conducted on maximum and average flocs area. Microbial composition of the settled biomass was determined using an optical microscope (Olympus BX53F) and images were taken using a camera (micro Olympus, DP 80). Biomass characterisation was conducted by morphological features and comparisons with literature databases.

Results and discussions

Inoculum characterization

The inoculum was mainly dominated by two strains of cyanobacteria, identified as *Pseudanabaena* sp. and *Leptolyngbya* sp. by their morphological features (Fig. 1). *Pseudanabaena* sp. is a filamentous cyanobacterium. Filaments (trichomes) can grow solitary or agglomerated in very fine, mucilaginous mats, straight or slightly waved or arcuate. Trichomes are composed from cylindrical cells, usually with slight constrictions at the distinct cross walls. Cells are cylindrical, always longer than wide (0.8 – 3 µm). *Leptolyngbya* sp. is also a filamentous cyanobacterium. Filaments are composed of single trichomes (chains of cells) that are straight to wavy and lack conspicuous motility. They can grow solitary or coiled into clusters and fine mats, arcuate, waved or intensely coiled [17]. The combination of the two filamentous cyanobacteria showed tendency of forming suspended flocs in the liquid culture. Flocs remained in suspended condition during the cultivation but could easily settle if the culture was maintained in static condition.

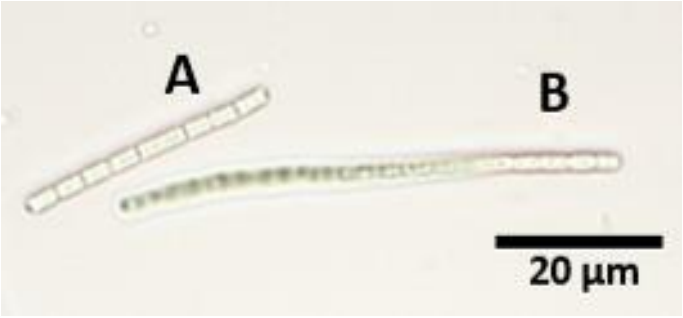

Figure 1. – Optical microscope pictures of the inoculum: Pseudanabaena sp. (A) and Leptolyngbya sp. (B).

Biomass growth

For both cultivation systems, biomass growth showed the typical microbial growth phases [18]: lag, exponential, stationary and decline (Figure 2). Biomass growth trend resulted similar for shaking table and multi-cultivator systems for each IBC, so biomass mixing conditions was not determining for biomass growth-trend and production. Anyway, decreasing IBC caused a longer initial lag-phase, as consequence, the higher biomass concentration favors a more rapidly biomass adaptation and growth. At the same way, the decline phase occurred later for lower initial biomass concentrations and when the cultures density was near to 1 abs for each tested condition. This result could be explained by light limitation which occurred for high biomass density or by nutrients depletion in the cultures.

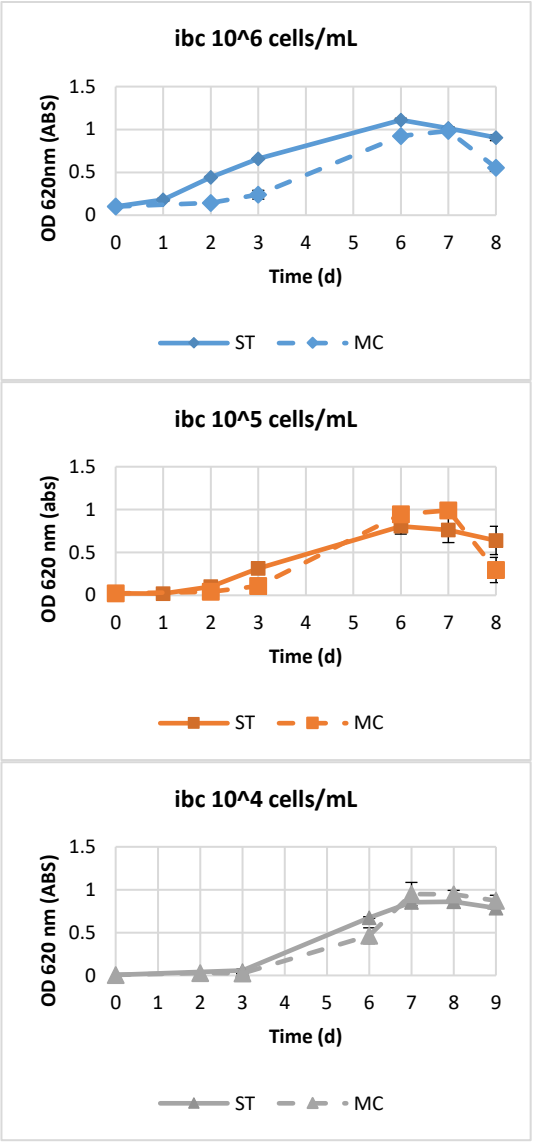

Figure 2. – Biomass concentration in time for different initial biomass concentrations (IBC) and for the two cultivating systems: shaking table (ST) and multi-cultivator (MC).

Biomass settling and recovery

Settling tests were performed in 100 ml glass tubes. Results were very different from classical zone-settling behaviour reported for sludge [19–21] with no apparent interface between a clarified supernatant and settling particles. Sedimentation for filamentous cyanobacteria is different from activated sludge principally because cyanobacteria show higher dimensions compared to the heterotrophic activated sludge bacteria [22]. Moreover, the two cyanobacteria species involved in this study show different morphological characteristics, which could influence flocs forming and settling as consequence. A dedicated experimental setup and model were developed to follow cyanobacteria settling. Sedimentation profiles were evaluated through image analysis and were modelled taking into account steady gravitational drift and diffusion with two populations of cells. Population 1 corresponds to "suspended cells" that settles only slightly, while population 2 corresponds to "settling cells" that settle efficiently. Both populations are characterized by their settling velocities (v_1 and v_2), their diffusion coefficient (D_1 and D_2) and by their proportion in the initial biomass (f_1 and f_2). Measured and modelled concentrations profiles after homogenization (t = 0 min) and after settling (t = 50 min) are shown on Figure and Figure for ST and MC cultivation systems respectively.

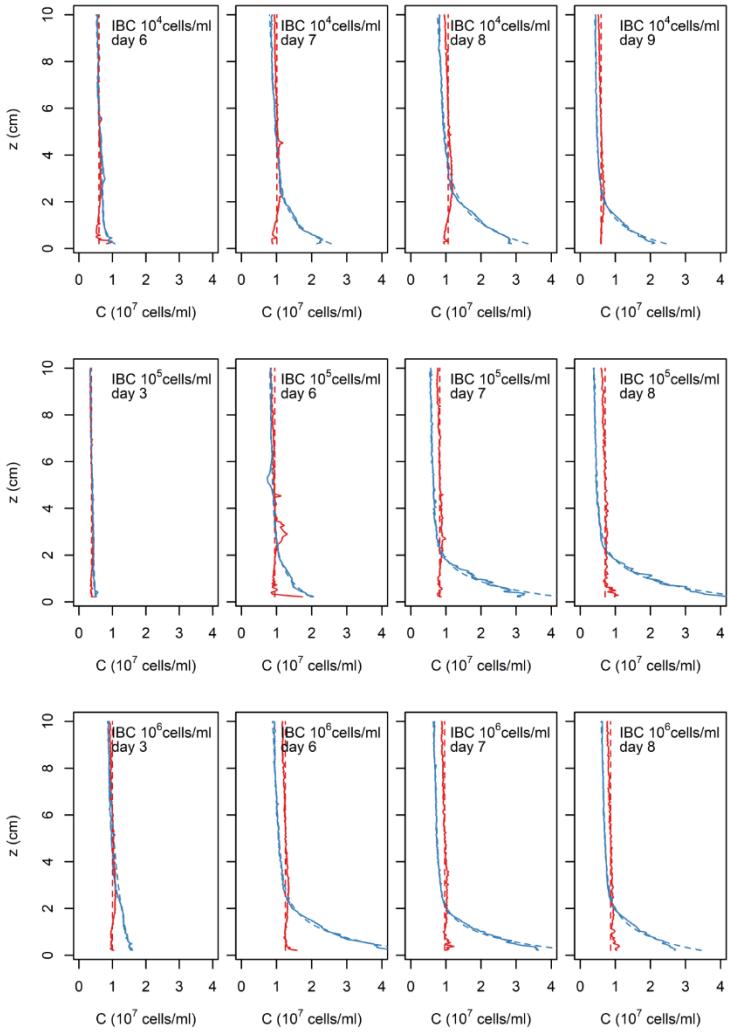

Figure 3. – Measured and modelled cells concentrations profiles for different experiments with ST system. Experiments with Initial Biomass Concentrations (IBC) of 10^4, 10^5 and 10^6 cells/ml are shown on lines 1, 2 and 3 respectively, while different days of cultivation are shown on the different columns. IBC and day of cultivation are indicated on the graphs. Concentration profiles after homogenization (t=0 min) and after 50 min settling are plotted in red and blue respectively. Measured and modelled profiles are shown in plain lines and dashed lines respectively.

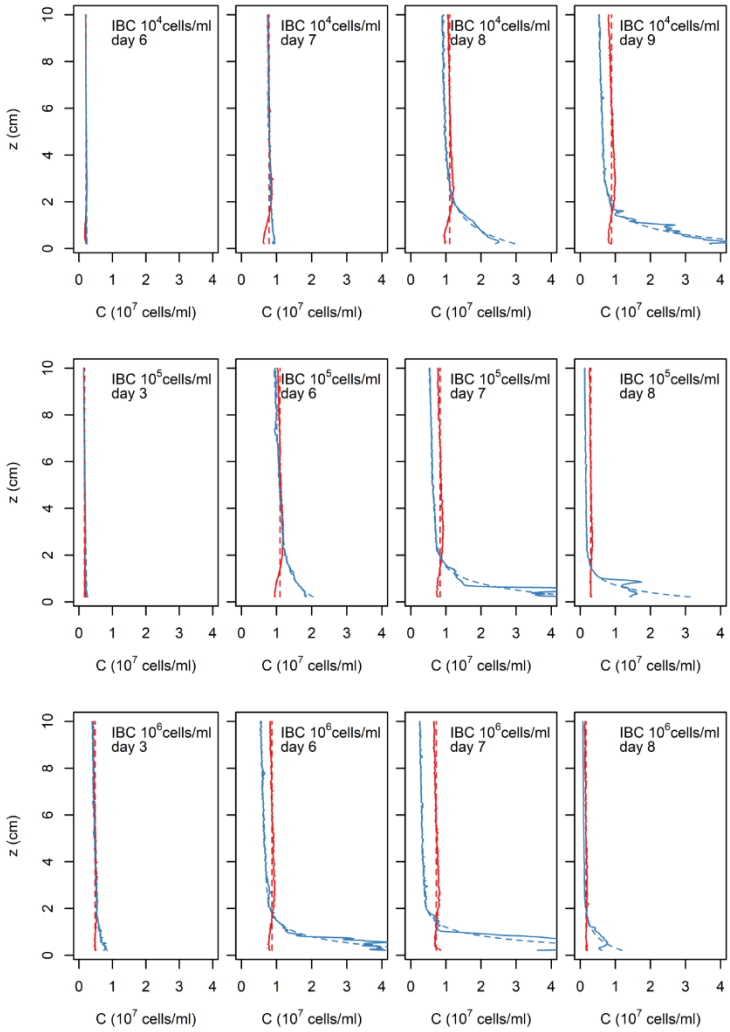

Figure 4. – Measured and modelled cells concentrations profiles for different experiments with MC system. Experiments with Initial Biomass Concentrations (IBC) of 10^4, 10^5 and 10^6 cells/ml are shown on lines 1, 2 and 3 respectively, while different days of cultivation are shown on the different columns. IBC and day of cultivation are indicated on the graphs. Concentration profiles after homogenization (t=0 min) and after 50 min settling are plotted in red and blue respectively. Measured and modelled profiles are shown in plain lines and dashed lines respectively.

Model fitting to the final settling profiles allowed evaluating D/v ratio and relative proportions for both populations (see Table 1 from supplementary material, section C). D/v ratio was 32±14 cm for population 1, and 0.58±0.14 cm for population 2. As expected these values indicate low settling for population 1 with $D_1/v_1 > H$ (tube height) corresponding to a settling dominated by diffusion and a profile only slightly different from the initial homogenous distribution (see Figure 1 from supplementary material, section C). On the contrary, population 2 settles efficiently with $D_2/v_2 << H$ corresponding to a settling dominated by gravitational drift (see Figure 1 from supplementary material, section C). Interestingly population 2 in ST and MC systems appears to have different characteristics with D_2/v_2 values of 0.70±0.06 cm and 0.45±0.06 cm respectively. This indicates that MC system allows denser settling than ST system.

From the modelled profile, it can be estimated that 95% of cells from population 2 are found on a distance $3D_2/v_2$ from the bottom of the tube, i.e. 2.1 cm for ST system and 1.3 cm for MC system in the mean. Biomass fraction corresponding to population 2 can thus be considered to be recoverable biomass than can be harvested for further processing. This estimated fraction of recoverable biomass is shown on Figure 5A as a function of time for the different experiments. Fraction of recoverable biomass was also evaluated with OD measurements after 50 minutes of settling tests according to equation (2) and is shown on Figure 5B.

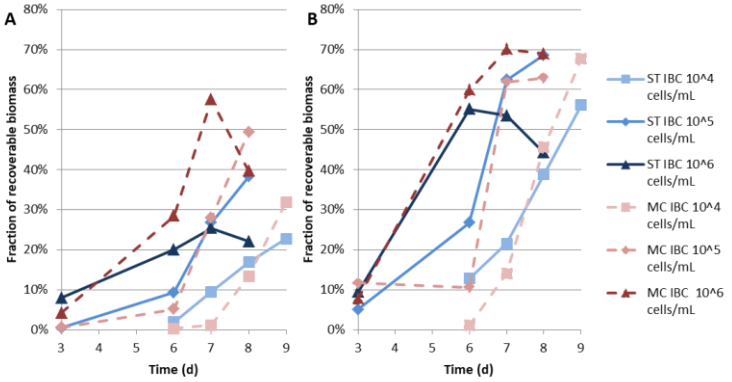

*Figure 5. – Fraction of recoverable biomass estimated through modelling (**A**) and calculated from OD measurements (**B**) as function of cultivation time for ST system (blue plain lines) and MC system (red dashed lines) with various Initial Biomass Concentrations (IBC).*

Fraction of recoverable biomass evaluated using the two different methods gives different estimations for the maximal recoverable fraction: 57% estimated through modelling and 70% estimated through direct OD measurements. However, their variations trends over time for the different experiments are similar. Indeed, recoverable fraction increases with time for every experiment except for experiments with the highest IBC (10^6 cells/ml) for which recoverable fraction decreases in the last days. Moreover, MC systems appear to allow better biomass recovery than ST systems in the end of cultivations. Finally, the highest recovery is obtained for MC system with highest IBC (10^6 cells/ml) on day 7. Interestingly this day appears to be the end of the stationary phase of this cultivation (see Figure).

To more precisely assess settling performances, settling velocities were evaluated through simulation of sedimentation dynamics for experiments with recoverable fraction above 20%. Sedimentation dynamics are illustrated in supplementary material (Figure 2 in supplementary material, section C). Evaluated settling velocities are comprised between $0.5 \cdot 10^{-4}$ and $3.7 \cdot 10^{-4}$ m/s (see supplementary material, section C, Table 2), with highest velocities obtained for IBC 10^6 cells/ml at day 7 for MC system. ST system gives slower kinetics with $2.4 \cdot 10^{-4}$ m/s maximal velocity obtained for IBC 10^5 cells/ml at day 8. The estimated settling velocities are within the velocities range estimated by Francois at al. for activated sludge settling [23].

As main result, IBC and culture mixing mode influenced biomass settling. More specifically, air bubbling and high initial biomass concentration promoted biomass settling. Physiological growth state appeared to be the principal factor influencing biomass recovery, with low sedimentation during the exponential growth phase, while the stationary state promoted the natural flocculation and sedimentation.

Biomass flocculation

Flocs formation in time was monitored by stereomicroscope analysis (Figure 6). In case of shaking table cultures with the IBC of 10^6cells/mL, flocs formation occurred already after one day of cultivation. From days 1 to 3, flocs sizes increased for shaking table cultures but their shape resulted progressively more loose. For the same IBC (10^6 cells/mL), in case of multi-cultivator cultures, flocs became visible only after three days of cultivation. Multi-cultivator flocs appeared smaller but denser than shaking table flocs. This result could be explained by the different shear stress conditions of the

biomass in the two cultivation systems. More specifically, air bubbles interacted directly with biomass flocs inhibiting their formation in the first 3 days. The same shear stress effect limited flocs sizes promoting a dense and round shape. On the contrary, oscillatory movements promoted interactions within the suspended biomass. As consequence, for shaking table cultures, flocs formation occurred already after the first days of cultivation because of the agglomeration of the inoculated biomass.

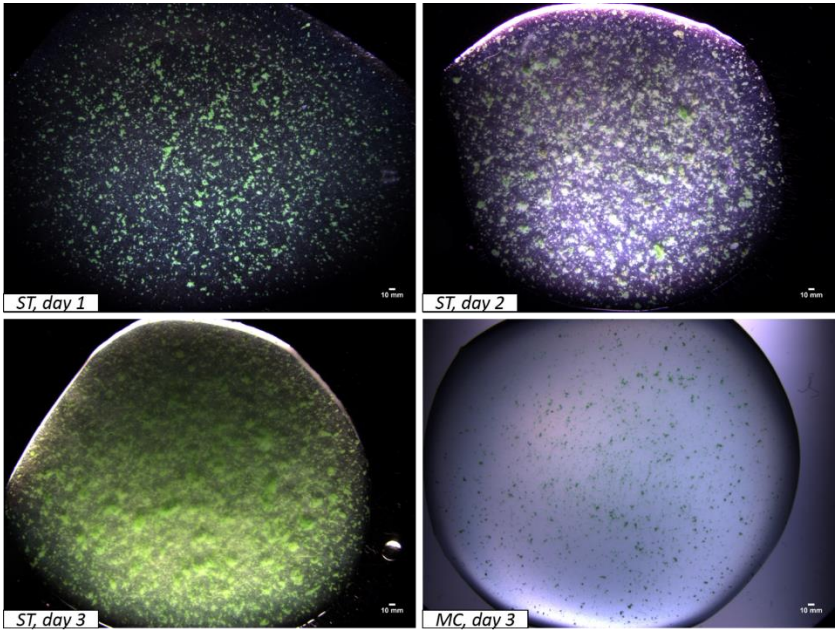

Figure 6. – Stereomicroscope pictures (e. t. ¼ sec) in time for shaking table (ST) and multicultivator (MC) samples with the initial biomass concentration of 10^6 cells/mL.

Flocs dimensions analysis is reported in table 1. In case of oscillatory movement (ST), flocs size were assessable at day 1, 2, 3 for cultures with the IBC of 10^6, 10^5, 10^4 cells/mL respectively. During the first 3 days, higher average flocs dimensions were progressively measured for higher IBC. This result implies that the new biomass tended to flocs aggregate since biomass growth favored flocs size increasing. At day 7, average flocs sizes reached the same value of (8.0 ± 0.3) mm^2. These conditions corresponded to the same biomass concentration of 10^7 cells/mL in all shaking table cultures. As result,

the IBC did not influence flocs size since it seemed related to the biomass concentration in the culture. In case of air bubbles mixing (MC), flocs size resulted smaller (3.8 ± 0.1 mm^2 VS 14.0 ± 4.3 mm^2) but they presented more homogeneous sizes. Flocs size generally decrease after the exponential growth phase (day 7 for IBC of 10^6 cells/mL).

Table 1. – Dimensional analysis for settled flocs in case of shaking table (ST) and multi-cultivator (MC) samples corresponding to different initial biomass concentrations (IBC).

Flocs area (mm^2)		Time (d)			
		1	2	3	7
ST, ibc 10^6 cells/mL	*Max*	93.2 ± 1.9	156.1 ± 1.8	170.4 ± 1.9	72.2 ± 1.9
	Average	10.6 ± 4.2	14.7 ± 0.6	14.0 ± 4.3	7.9 ± 0.8
ST, ibc 10^5 cells/mL	*Max*		24.6 ± 1.9	88.3 ± 3.8	178.4 ± 1.9
	Average		5.0 ± 0.7	8.8 ± 2.0	8.5 ± 0.8
ST, ibc 10^4 cells/mL	*Max*			52.44 ± 2.8	166.3 ± 1.9
	Average			5.9 ± 1.1	7.7 ± 0.7
MC, ibc 10^6 cells/mL	*Max*			28.7 ± 4.8	13.9 ± 1.8
	Average			3.8 ± 0.1	2.1 ± 0.2

Biomass composition analysis contributed to explain the different flocs shape and dimensions observed for the two cultivation systems. The cultures of the two cultivation systems presented the same biomass composition at time zero (as reported in materials and methods, experimental setup). However, during the cultivation, biomass composition of the settled flocs resulted different for the two systems. Microscope pictures are reported in Figure 7 for the settled biomass collected at day 6 for shaking table (up) and multi-cultivator (down) cultures. In case of shaking table cultures, flocs were composed by the two species of cyanobacteria, which constituted the inoculum. More specifically, flocs structure showed an internal and dense nucleus constituted by *Pseudanabaena* filaments and external hairs formed by *Leptolyngbya* filaments. In case of air bubbles mixing, corresponding to multi-cultivator system, *Pseudanabaena* sp. resulted the predominant species causing little but dense and well shaped flocs structures. As main result, biomass mixing conditions resulted responsible of biomass speciation for the cultures. High

shear stress effects related to air bubbling limited *Leptolyngbya* filaments growth. Indeed, their conformation is longer and thinner compared to *Pseudananbaena sp*., so they are more fragile. On the contrary, *Pseudanabaena* filaments are shorter and wider; moreover they showed the capability to attach themselves in dense and well structured flocs. As consequence, in terms of biomass sedimentation, multicultivator flocs were heavy and easy to settle down. Shaking table flocs settling was slower because of their lower density, related to their hairy structures.

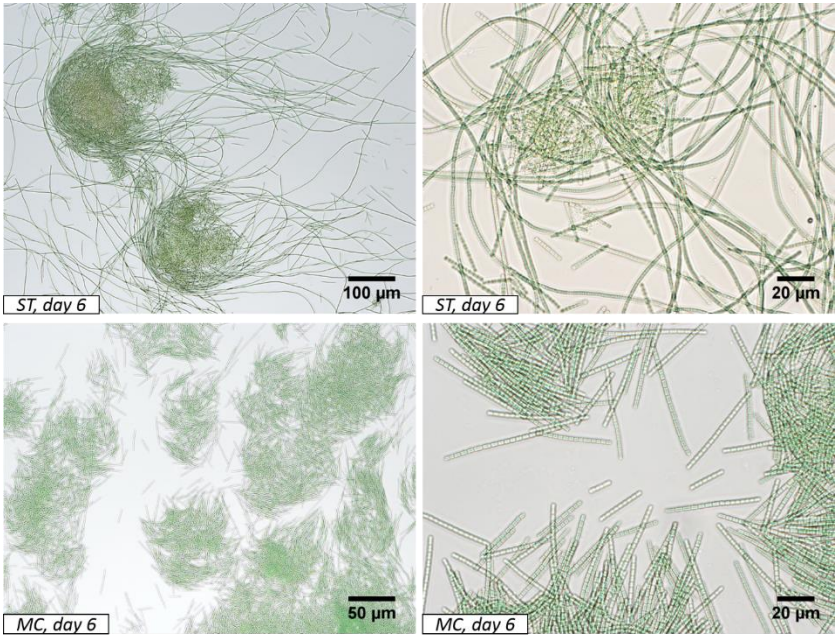

Figure 7. – Optical microscope pictures of settled biomass for multi-cultivator and shaking table samples with the initial biomass concentration of 10^4 cells/mL.

Biomass growth state influenced flocs features, as showed by stereomicroscope pictures (Supplementary materials, section D, Figure 1) captured at different time for shaking table cultures. Flocs forming occurred after one day thanks to filaments gliding and biomass mixing mode. Different green shades implied a higher biomass density at the flocs center site compared to the boundary. Moreover, floc formation seems due to both free filaments and free flocs bridging. At day 7, during the decline phase, biomass

color turned to yellow and flocs breaking occurred. Cyanobacteria death led to filaments disaggregation from the flocs, which showed low density and loose state.

Conclusions

Bioflocculation process observed for native wastewater filamentous cyanobacteria represents a real solution for low cost and environmental sustainable biomass harvesting. As a fact, the present work demonstrates that it is possible to recover the 70% of the cultivated biomass by natural flocculation. In a sequential batch system, the surnatant of the sedimentation process could be recovered as inoculum for new cultures. Cyanobacteria cultivation sustainability could be enhanced using wastewater as growth medium, even if cyanobacteria bioflocculation in wastewater need to be clarified. Finally, cyanobacteria flocs could represent a flocculation nucleus in microalgae cultivation promoting the combined biomass recovery.

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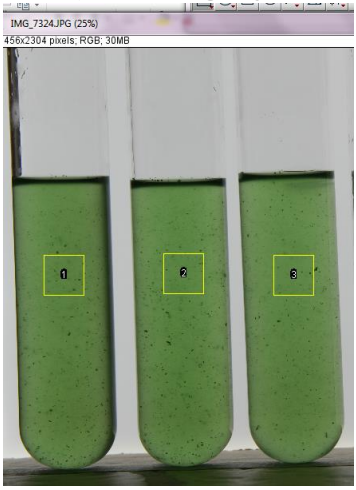
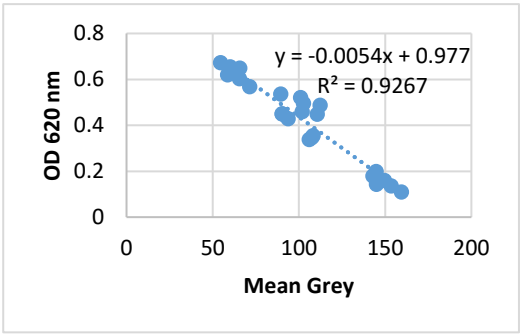
SUPPLEMENTARY MATERIALS

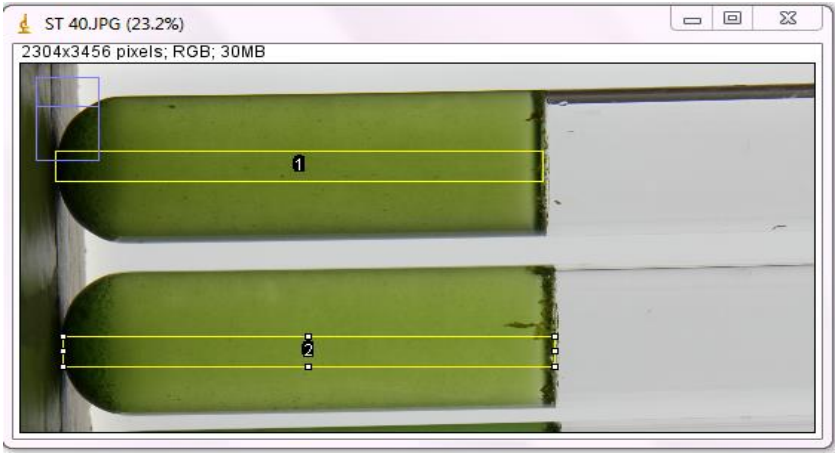
A) OD – grey values correlation

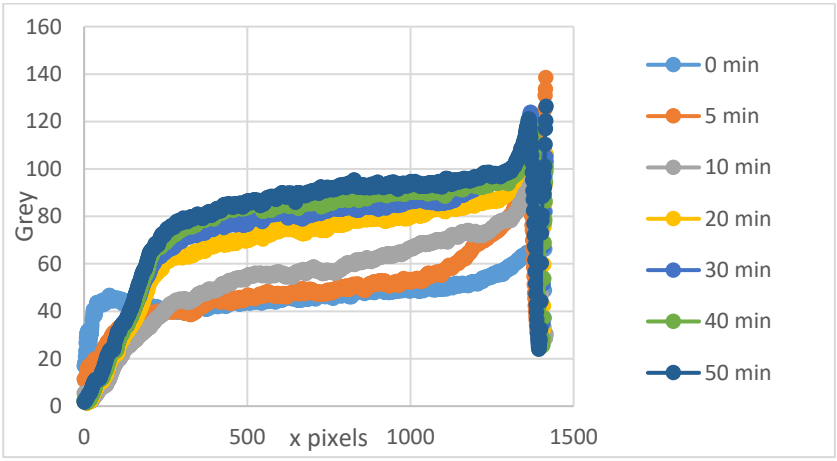




Yellow squares were drawn in the middle height of each tube; the relative pixels were transformed in grey values using the software Image J (commands: "transform, Image to Results"). As consequence, each square was converted in a matrix; the average value of the matrix was considered as "Mean Grey" showed in the graph above. The Mean Grey was correlated to the specific OD reading at 620 nm for the same sample. This method was applied at different cultivation days obtaining different points that allowed obtaining the direct correlation showed in the graph above.

B) Settling analysis





The software Image J was used to draw the yellow rectangles along the tubes for photos captured at fixed times. For each rectangle, a grey profile was obtained, showing the variation of the grey colour intensity along the tube. Grey profiles showed different trends in time, which were studied in order to simulate the biomass settling.

C) Settling modelling

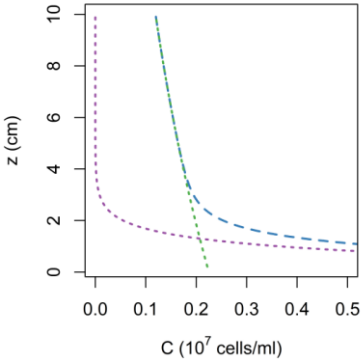


Figure 1. - Cells concentrations profiles predicted by the model for population 1 in green, population 2 in purple and for the global population in blue. Population 1 correspond to "suspended cells" that settles only slightly, while population 2 corresponds to "settling cells" that settle efficiently at the bottom of the tube.

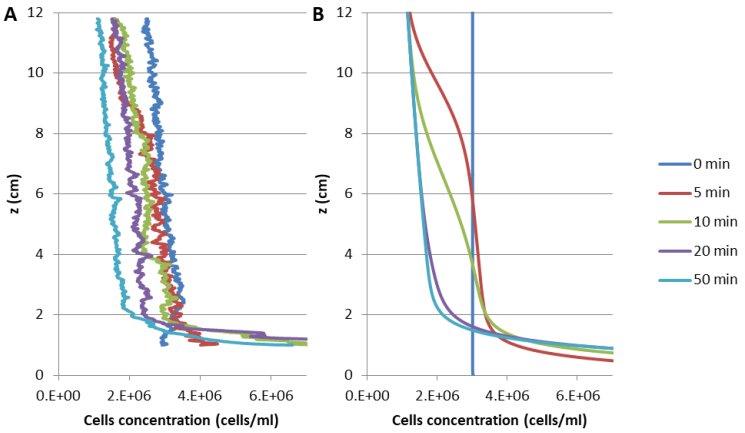


*Figure 2. - Measured (**A**) and modelled (**B**) sedimentation profiles at different sedimentation times for MC IBC 10^5 cells/ml day 8 experiment.*

Table 1. - D/v ratios and relative proportions of populations 1 and 2 estimated from the modelling of experiments with MC and ST systems for various Initial Biomass Concentrations (IBC).

ST, IBC 10^4 (cells/l)				
	day 6	day 7	day 8	day 9
D1/v1 (cm)	24	24	22	32
f1	98%	91%	83%	77%
D2/v2 (cm)	0.22	0.78	0.95	0.76
f2	2%	9%	17%	23%

ST, IBC 10^5 (cells/l)				
	day 3	day 6	day 7	day 8
D1/v1 (cm)	31	54	28	21
f1	99%	91%	73%	62%
D2/v2 (cm)	0.69	0.91	0.64	0.71
f2	1%	9%	27%	38%

MC, IBC 10^4 (cells/l)				
	day 6	day 7	day 8	day 9
D1/v1 (cm)	58	77	48	28
f1	100 %	99%	87%	68%
D2/v2 (cm)	2.95	0.93	0.78	0.51
f2	0%	1%	13%	32%

MC, IBC 10^5 (cells/l)				
	day 3	day 6	day 7	day 8
D1/v1 (cm)	20	32	26	21
f1	99%	95%	72%	51%
D2/v2 (cm)	0.18	0.73	0.48	0.40
f2	1%	5%	28%	49%

ST, IBC 10^6 (cells/l)				
	day 3	day 6	day 7	day 8
D1/v1 (cm)	35	27	31	30
f1	92%	80%	75%	78%
D2/v2 (cm)	2.58	0.74	0.62	0.73
f2	8%	20%	25%	22%

MC, IBC 10^6 (cells/l)				
	day 3	day 6	day 7	day 8
D1/v1 (cm)	29	26	19	16
f1	96%	72%	43%	60%
D2/v2 (cm)	0.55	0.44	0.37	0.53
f2	4%	28%	57%	40%

Table 2. - Modelled settling velocities for population 2 in experiments with f2 > 20%

	v_2 (m/s)					
	ST			MC		
Day	IBC 10^4 cells/mL	IBC 10^5 cells/mL	IBC 10^6 cells/mL	IBC 10^4 cells/mL	IBC 10^5 cells/mL	IBC 10^6 cells/mL
6			5.0E-05			5.0E-05
7		8.0E-05	2.4E-04		1.1E-04	3.7E-04
8		2.1E-04	1.6E-04		1.1E-04	1.5E-04
9	7.0E-05			6.0E-05		

D) Biomass flocculation

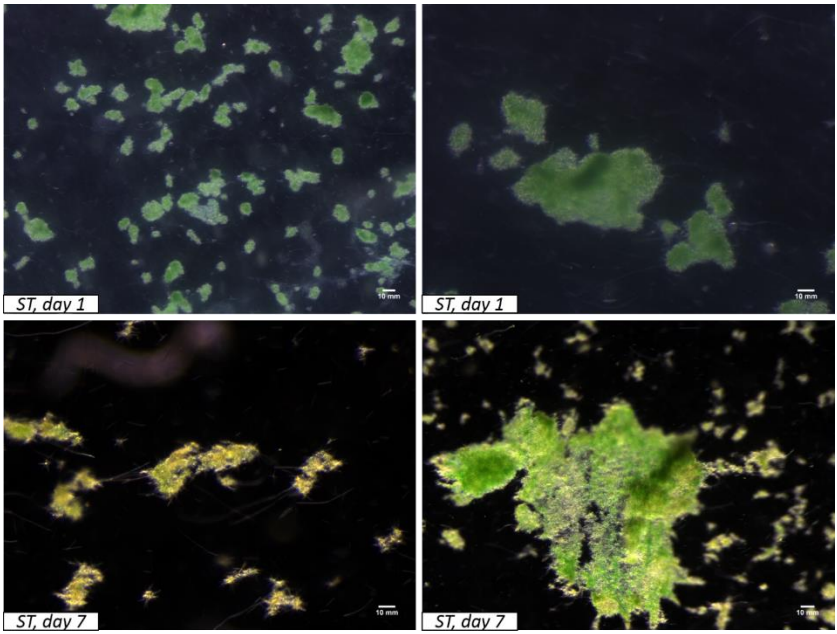


Figure 1. – Stereomicroscope flocs pictures: flocs bridging (up), flocs breaking (down) for shaking stable cultures (ST) for the initial biomass concentration of 10^6 cells/mL.

Chapter 7

Conclusions

The use of microalgae for wastewater treatment processes offers several advantages, such as CO_2 emission reductions, energy saving and nutrients recovery. Furthermore, microalgal biomass as a feedstock for a sustainable production of biofuels is preferable to terrestrial plant biomass. Currently, the most relevant approaches use sterilized wastewater for microalgal cultivation. Nevertheless, only a few studies have been carried out at pilot scale and in outdoor conditions. Microalgae harvesting, actually, remains the principal challenge for a large-scale use of microalgal cultivation. In this context, bioflocculation is the most promising process as it is cost-effective and eco-friendly.

In this thesis, the principal aspects related to microalgal cultivation in wastewater and its further valorisation, principally as biofuel feedstock, were analysed. For this purpose, microalgae cultivation was conducted in both closed and open systems. The effects of light intensity and nutrients supply were investigated in order to improve lipids accumulation in closed cultivation systems. Low nutrients supply and high light intensity promoted lipids accumulation in the produced microalgal biomass, reaching a maximum concentration of 29% lipids/dry weight. In case of open cultivation systems, lipids accumulation was enhanced by testing the combined culture of microalgae with yeast. Indeed, yeast lipids fraction could reach a 70% of

dry weight; moreover, yeast and microalgae could create a symbiotic system capable to improve the growth of both species. In case of urban wastewater, yeast growth was limited by the low concentration of readily fermentative organic substrates; anyway, the presence of yeast, during the initial microalgal lag phase, improved the microalgal production. Lipids concentration was evaluated during the whole cultivation time and two peaks were found: the first (7% lipids/dry weight) corresponding to yeast growth and the second and highest peak (15%) measured at the end of the cultivation time, when conditions of nitrogen starvation occurred for the microalgal biomass. Lipids concentration evaluated for the open culture resulted lower than that measured for the indoor culture. Despite this result, outdoor cultures use the solar radiations for light supply, which is cost-free and eco-sustainable; moreover, the biomass volumes produced in open systems are much higher than those obtained indoor.

The thesis also focused on the capability of the open microalgae culture to capture carbon dioxide (CO_2). This system is actually considered the most economically viable solution for microalgae cultivation. Only a fraction of the total CO_2 added to the open pond can solubilize in the culture, depending on different physical-chemical characteristics of both water and gas, according to the two-film theory. Moreover, solubilized CO_2 cannot be completely absorbed by microalgae, indeed, high CO_2 concentration in the water culture could even inhibit microalgae growth. Low gas flowrate (0.2 L/min) enhanced both CO_2 water solubility and bio-available CO_2 fixation during daylight, thus resulting the most efficient condition for the microalgae cultivation pond fed with untreated urban wastewater. On the other side, in case of high gas flowrate (1 L/min), a high CO_2 concentration would be found in the liquid, but a high concentration, CO_2 would escape to the atmosphere at the same time, even if microalgal production would be increased.

Finally, the thesis examined the bioflocculation process as microalgae harvesting method. Native wastewater filamentous cyanobacteria were capable to flocculate spontaneously, without chemicals addition and at pH around neutrality value. These conditions resulted in harvesting a not contaminated biomass, separated from liquid by gravity settling. Cyanobacteria interactions with microalgae led to their bioflocculation, which improved, as the harvesting conditions were enhanced when a combined cyanobacteria-microalgae cultivation was tested. Furthermore, the

combined cyanobacteria-microalgae cultivation led to produce a more valuable biomass thanks to its higher lipids content. Filamentous cyanobacteria bioflocculation was further analysed in this thesis using two different mixing systems (air bubbles and shaking table) and setting different initial biomass concentrations. Air bubbles mixing resulted in being the most favourable system in order to improve cyanobacteria flocculation and subsequent settling; moreover, higher initial biomass concentrations enhanced their bioflocculation tendency. The biomass growth phase also resulted in being an important parameter to optimize flocculation, which is well-performed at the end of the exponential growth phase. The different mixing systems, finally, influenced cyanobacteria speciation, which affects structure of formed flocs.

Curriculum vitae

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Birthplace: Caserta, Italy

Education and training

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University of Molise, Bioscience and Territory department.

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University of Naples Federico II, Department of Civil, Architectural and Environmental Engineering

Master Degree in Environment and Territory Engineering (LM-35), vote 110/110 cum laude

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1/11/2009 – 31/10/2012

University of Naples Federico II, Department of Civil, Architectural and Environmental Engineering

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11/09/2004 – 13/07/2009

State scientific high school G. Galilei, Piedimonte Matese (Caserta), vote 100/100

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List of publications

Articles in peer-reviewed journals

- Vulnerability Assessment of Drinking Water Treatment Plants. A. Panico, F. Iasimone, G. Fabbrocino, F. Pirozzi. Chemical Engineering Transactions, vol. 43, 2015.

- Experimental study for carbon dioxide emissions reduction in wastewater treatments plants applying microalgal cultivations. F. Iasimone, V. De Felice, A. Panico, F. Pirozzi. Journal of CO2 utilization 22 (2017) 1-8.

- Combined yeasts and microalgae cultivation in pilot scale raceway pond for urban wastewater treatment and potential biodiesel production. F.Iasimone, G. Zuccaro, V. D'Oriano, G. Franci, M. Galdiero, D. Pirozzi, V. De Felice, F. Pirozzi. Water Science & Technology, in press, 2017

Conference contributions

Workshop "Chimica, Ambiente e Territorio", poster "Le microalghe dalle acque reflue: una coltivazione ad impatto zero". Caserta, 30 settembre 2015.

X International Symposium on Sanitary and Environmental Engineering, oral presentation "Carbon dioxide sequestration and wastewater treatment high rate algal pond for low-cost biofuel production". Rome 19-23 giugno 2016.

XXI IUPAC chemrawn conference "Solid Urban Waste Management", presentazione poster "Microalgal wastewater treatment for biofuel production". Rome, 6 – 8 aprile 2016

FITEMI (Forum Italiano sulle Tecnologie Microalgali), poster "Understanding the bioflocculation potential of wastewater native filamentous cyanobacteria", oral presentation "Coltivazioni ecosostenibili di microalghe: dai fotoreattori ai sistemi aperti". Palermo 6 –7 aprile 2017.

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