Microalgae treatment of piggery wastewater

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Biological Engineering

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Abstract

The disposal of untreated or improperly treated wastewater into aquatic environments results in high nutrient loading into aquatic bodies, which may cause phytoplankton blooms and other environmental problems. Therefore, in order to discharge these effluents without causing any danger to human´s health or to natural environmental resources, it is necessary to find the most suitable technique for wastewater treatment. Recently, several studies have proposed the use of microalgae for wastewater treatment, highlighting their ability to uptake nutrients, such as phosphorus and nitrogen, and to produce a valuable biomass, which can be used for biofuels production.

The aim of this thesis was to investigate the efficiency of a microalgae consortium for treating a pre-treated piggery wastewater by assessing microalgae growth and nutrients removal efficiency, and also to investigate their ability to produce biogas. Microalgae proved to be able to grow on this high strength wastewater with high biomass yields and with high nutrients removal efficiency. The highest removal efficiency of $N\text{-}NH^{+}_{4}$ and $P\text{-}PO_{4}$ was 92 % and 82%, respectively. Moreover, the highest removal efficiency of COD was 64%, which was partly attributed to the synergetic relationship between microalgae and other microorganisms. Furthermore, the highest biogas production was 270.4 $mLCH_{4}.g^{-1}VS$.

For that reason, the hypothesis of using microalgae based treatment as the first step of biological treatment of piggery wastewater could be considered. In that case, algae biomass could be digested and the liquid phase of digestate could be recirculated upflow to be treated by algae/bacteria consortium along with the pre-treated piggery wastewater.

Keywords: Microalgae, Piggery wastewater, Biogas, Nutrients, Removal

Resumo

A descarga de águas residuais não ou inadequadamente tratadas em ambientes aquáticos resulta na acumulação de nutrientes, os quais podem causar diversos problemas para a saúde humana e para o meio ambiente. Por conseguinte, a fim de descarregar estes efluentes é necessário encontrar a técnica mais adequada para o tratamento de águas residuais. Recentemente, vários estudos têm proposto o uso de microalgas no tratamento de águas devido à sua capacidade de absorção de nutrientes, tais como fósforo e azoto, e de produção de uma biomassa valiosa, que pode ser utilizada para produzir biocombustíveis.

O objetivo deste trabalho foi investigar a eficiência de um consórcio de microalgas para o tratamento de um efluente de suinicultura pré-tratado por flotação e investigar a sua capacidade para produzir biogás. As microalgas utilizadas provaram ser capazes de crescer nestas águas residuais com rendimentos elevados de biomassa e eficiências elevadas de remoção de nutrientes. A maior eficiência de remoção de $N\text{-}NH^{+}_{4}$ e $P\text{-}PO_{4}$ foi de 92% e 82%, respetivamente. Além disso, a maior eficiência de remoção de COD foi de 64%, o que confirmou a relação de sinergia entre microalgas e outros microrganismos. A maior produção de biogás foi 270.4 $mLCH_{4}.g^{-1}VS$.

Assim, a hipótese de utilizar as microalgas como o primeiro passo de tratamento biológico de águas residuais de suinicultura pode ser considerado. Nesse caso, a biomassa de algas pode ser digerida e a fase líquida dos digestores pode ser recirculada e ser tratada pelo consórcio de microalgas/bactérias juntamente com o efluente de suinicultura pré-trado.

Palavras-chave: Microalgas, Efluente de suinicultura, Biogás, Nutrientes, Remoção

Abbreviations

AD	Anaerobic Digestion
AMT	Ammonium Transporter
AOB	Ammonium Oxidizing Bacteria
ATP	Adenosine Triphosphate
ATU	Allythiurea
BMP	Biochemical Methane Potential
BOD	Biochemical Oxygen Demand
COD	Chemical Oxygen Demand
DOP	Dissolved Organic Phosphorus
GHG	Green House Gas
HO	Heterotrophic organisms
HRT	Hydraulic Retention Time
NOB	Nitrite Oxidizing Bacteria
OD	Optical Density
OUR	Oxygen Uptake Rate
PAR	Photosynthetically Active Radiation
PBRs	Photobiorreactors
TSS	Total Suspended Solids
VFAs	Volatile Fatty acids
VS	Volatile Solids
WW	Wastewater
WWTP	Wastewater Treatment Plant

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1 Thesis Overview

The future of human population and Earth's capacity are highly unpredictable since they depend on both natural limitations and human behavior. Recent studies proposed that, although the growth rate of the world population appears to be decreasing, the total number of humans on Earth is estimated to rise by 50% during this century, reaching 11 billion of people (Ortiz-Ospina & Roser 2016) .This means that the world population will need to find more and more resources such as food, water, raw materials and energy in a world where they look already scarce and where our environmental impact is damaging the planet. The traditional "take, make, waste" of the linear economy approach to managing resources is no longer sustainable; therefore, the interest in optimized uses of natural resources and in their recovery from waste streams is growing and encouraging the shifting to a circular economy (Bradley 2015) (Figure 1.1).

Figure 1.1 Linear economy approach versus Circular economy approach (Bradley 2015)

One solution to the lack of resources and environmental pollution is the recovery of resources from waste streams. For example, during the last years many countries have developed techniques to recover resources from municipal solid wastes, which have allowed to reduce new resources withdraw from the environment and to limit the consumption of energy. Moreover, since good results have been achieved with solid waste streams, other fields are also being explored such as wastewater streams.

Currently, one of the most concerning environmental problems is the global water pollution. The disposal of untreated or improperly treated wastewater into aquatic environments causes serious harms such as health and environmental problems. Therefore, in order to discharge these effluents without causing any danger to human´s health or unacceptable damage to natural environmental resources, it is necessary to treat wastewaters with the aim of removing nutrients, pathogens and heavy metals to the acceptable limit concentrations prior to their discharge and reuse. Moreover, it should be highlighted that wastewater can be a resource of renewable energy, nutrients, such as nitrogen and phosphorus, and fresh water.

However, nowadays conventional wastewater treatment plants (WWTPs) are focused on the destruction of organic and inorganic pollutants, rather than on the recovery of these valuable resources.

Furthermore, most of these conventional wastewater treatment plants present high resources consumption (Figure 1.2), such as energy and chemicals, which are consumed by these systems to treat the wastewater to the required standards (Means 2004). For example, in the US it has been assessed that the energy used in WWTPs includes around one fifth of a municipality's total energy use by public utilities, and it is expected that it will keep rising by 20% in the next 15 years due to the increasing water consumption and to more stringent regulations (Mo & Zhang 2012). Likewise, more materials and chemicals are predictable to be used in the future for WWTPs construction and operation Therefore, in order to improve the sustainability of WWTPs, researchers are pushing towards the development of new technologies, which should recover resources from wastewater streams for secondary uses and revolutionize the wastewater treatment.

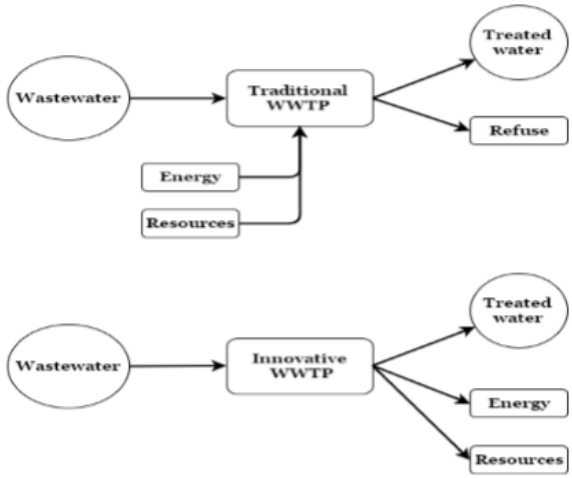

Figure 1.2 Main flows in a conventional WWTP and in an innovative WWTP

The aim of this thesis was to explore the efficiency of a microalgae consortium in the treatment of a pre-treated piggery wastewater due to their ability to uptake nutrients, such as phosphorus and nitrogen, as well as to investigate their suitability for biogas production. This thesis was focused on:

- The assessment of microalgae growth and stability on a high strength wastewater;
- The evaluation of microalgae/bacterial consortium capacity to remove nutrients (phosphorus and nitrogen) from the piggery wastewater as well as to remove chemical oxygen demand;
- The assessment of the used microalgae suitability to produce biogas by anaerobic digestion.

In this thesis, a literature review is presented in chapter 2. The materials and methods used in the experiments are then described in chapter 3, after which the results and discussion are presented in chapters 4 and 5, respectively. Finally, the main conclusions of this project are outlined in chapter 6 and in chapter 7 the future perspectives are presented.

2 Literature review

2.1 Importance of wastewater treatment

At present, it is universally recognized that fresh water and sustainable water management is one of the most important concerns of innumerous scientific, social or political groups. However, water resources seem to face severe quantitative and qualitative threats, since pollution and population growth, industrialization and rapid economic development inflict severe risks to the availability and quality of water resources in many worldwide areas (Abdel-Raouf et al. 2012).

The diagram represented in Figure 2.1 shows the impact of the expected population growth on water usage by 2025, taking into account the current rate of water use per person (Rekacewicz 2012).

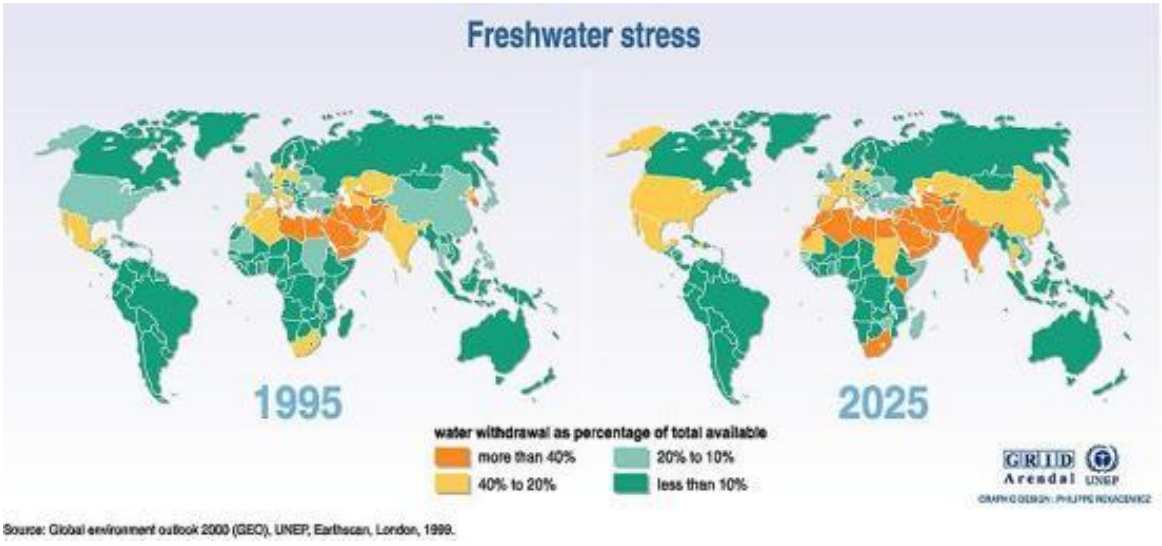

Figure 2.1 Worldwide freshwater sources availability in 2025 (Rekacewicz 2012)

As it is possible to observe in Figure 2.1, overall, the water withdrawal percentages are expected to rise substantially by 2025. For example, in the US, China and some European countries water scarcity will drastically increase. In addition to the reduction of fresh water resources, water quality degradation is rapidly joining the water scarcity as one of the biggest concerns of the world (Abdel-Raouf et al. 2012).

Currently, it is truism that human´s daily activities are the biggest cause of pollution and change of the environment. Such activities, although required for human development and future, lead to environmental degradation and to the release of dangerous and toxic materials causing the loss of quality of life.

One of the most severe environmental problems is water pollution and it is caused by different human activities such as industrial, domestic and agricultural. The major cause of water body pollution is the discharge of untreated or improperly treated wastewater that is associated with the disposal of pathogens, inorganic and organic compounds and macroscopic pollutants into water bodies (Pandey 2006). Therefore, in order to discharge agricultural, domestic and industrial effluents without causing danger to

human health or unacceptable damage to the natural environment resources, it is necessary to treat wastewaters with the aim of removing nutrients, pathogens and heavy metals to the acceptable limit concentrations prior to their discharge and reuse (Abdel-Raouf et al. 2012).

Unfortunately, an estimated 90 per cent of all wastewater in developing countries is discharged untreated directly into rivers, lakes or oceans (Ahlenius 2010). The ratio of treated and untreated wastewaters around the world is represented in Figure 2.2.

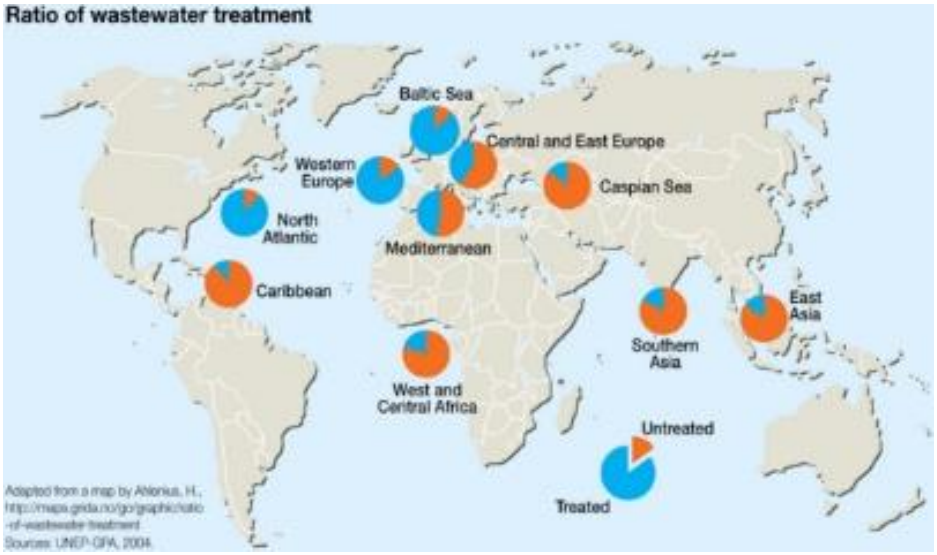

Figure 2.2 Ratio of treated and untreated wastewater reaching water bodies of 10 regions (Ahlenius 2010)

The disposal of untreated or improperly treated wastewater into aquatic environments causes serious harms such as health problems, especially due to the loading of pathogens that can cause diseases to humans and also environmental problems, like eutrophication of water bodies. Eutrophication has become more widespread since mid-20 century (Cai et al. 2013) and it is caused by the loading of high concentrations of nutrients, such as phosphorus and nitrogen, that affect the quality of water and can create conditions for undesirable phytoplankton (Manivanan 2008). Consequently, the uncontrolled bloom of algae in the water bodies limits the sunlight available and causes lack of oxygen in water, leading to the decline of the species in the aquatic environments (Manivanan 2008).

In fact, the nitrogen input in our environment has increased extremely during the last years. Nitrogen pollution causes acidification of soils and waters, loss of biodiversity in aquatic and terrestrial ecosystems and, as mentioned before, eutrophication of the water bodies. Ammonia nitrogen in surface waters is toxic for fish, while nitrate nitrogen in drinking water can cause health problems, among which the best known is methemoglobinemia, a disorder characterized by the presence of high levels of methemoglobin (MetHb) that results from the presence of iron in the ferric form instead of the usual ferrous form. Methemoglobinemia results in a decreased availability of oxygen to the tissues and can be caused by the reduction of nitrate to nitrite within the human organism, since MetHb is formed when nitrite oxidizes the ferrous iron in hemoglobin (Hb) to the ferric form (de Fernicola & de Azevedo 1981)(Fewtrell 2004). Another prejudicial effect of nitrogen is its contribution to ozone formation in the troposphere, causing a decreased forest and crops

production as well as human's health problems. Additionally, nitrogen in the form of ammonia can be released to the atmosphere contributing to the formation of acid rains.

Wastewater treatment methods are generally classified in three types: biological, chemical and physical. These methods can be applied individually or in combination, depending on the nature and type of pollution. Usually, physical and chemical methods are costly and chemical methods are normally associated with the increase of conductivity, pH and overall load of dissolved matter in the wastewater (Renuka et al. 2015). Therefore, biological treatment methods tend to be much more promising.

Agricultural wastewater, which is mainly obtained from livestock productions, is one of the biggest contributors to nitrogen discharge since it has high concentrations of ammonium (Lim et al. 2016). For years, the traditional method to treat piggery wastewater was using it as a fertilizer for lands, but the nitrogen in piggery wastewater cannot be completely uptaken by crops due to the unbalanced N/P ratio (Cai et al. 2013). The consequent accumulation of nitrogen in the soil can contaminate the receiving waters and cause all the problems previously described.

Therefore, it is important to find the most cost-effective and environmentally friendly treatment methods that require less inputs and simple infrastructures to treat piggery wastewater.

2.2 Conventional treatments of Agro-Industry wastewater

Livestock production started to raise during the end of 20^{th} century (Hjorth et al. 2010) and, nowadays, the increased demand for red meat in developing countries has led to the intensification of livestock production and consequently, to the development of large indoor animal houses, mainly pigs and poultry,(Bernet & Béline 2009), resulting in higher local emissions of odor and ammonia gas (Hjorth et al. 2010). In addition, the intensification of livestock production has led to the concentration of animals in limited areas with the aim of reducing production costs (Bernet & Béline 2009). In these areas, the local use of wastewater as organic fertilizer results in over-application of nutrients on soils, causing several environmental problems. For example, in Brittany (France) before the intensification of livestock production the average concentration of NO^{-}_{3} in a surface water used to be 5 $mg.L^{-1}$, but nowadays the concentration has reached the value of 35 $mg.L^{-1}$ (Bernet & Béline 2009).

For many years, the use of swine manure as organic fertilizer used to be one of the most economical and easiest methods of managing manure (Lim et al. 2016). However, as mentioned before, there are several environmental concerns related to over-application of animal manure, including a great risk of nutrient runoff, which will contaminate surface and ground water; eutrophication of surface waters; spread of pathogens; attraction of rodents, insects and other pests; and a potential higher energy used in the transport of manure to the cultivation crops (Iregbu et al. 2014) (Hjorth et al. 2010). Therefore, the use of manure as fertilizer should be limited and it is necessary to develop a cost-efficient piggery wastewater treatment, as an alternative to land application.

Animal wastewaters may have variable characteristics, depending not only on the different types of animals, but also for the same animals between countries and farms, depending on the water consumption, farm production and also, on the composition of the feed given to the animals (Boursier et al. 2005). Generally, piggery wastewater consists on a mix of urine, manure and flushing wastewater, which is characterized for having high concentrations of nitrogen, phosphorus, chemical oxygen demand (COD) and total suspended solids (TSS) (Girard et al. 2009). Many studies have been focused on the biological removal of carbon, nitrogen and phosphorus, however, few of these studies were carried out on wastewaters with high concentrations of these compounds, such as piggery wastewater.

Regarding the conventional treatment, firstly it is necessary to perform a preliminary treatment to remove large solid materials that can damage the downstream equipment and obstruct the flow. These large solids can be removed by passing the sewage through bars spaced at 20-60 mm (Abdel-Raouf et al. 2012).

After the removal of the coarse solids, sewage may be subjected to a primary treatment which consists in passing the sewage through sedimentation tanks with the aim of removing the settable solids by gravity (Abdel-Raouf et al. 2012). Moreover, it should be pointed out that a well design sedimentation tank can remove almost 40 % of the total COD (Abdel-Raouf et al. 2012).

Then, the secondary treatment process aims to reduce COD by reducing organic compounds and this is mediated by a mixed population of heterotrophic bacteria that use organic matter for energy and growth (Abdel-Raouf et al. 2012). Anaerobic digestion (AD) is one of the most frequently used methods for piggery wastewater due to its reliability, low cost and high efficiency (Wu et al. 2015) (Obaja et al. 2003) (Chynoweth et al., 1999). This process is defined as the decomposition of biodegradable material by microorganisms in the absence of oxygen and includes four phases. The first phase, named as hydrolysis, involves the decomposing of complex molecular organic compounds, such as proteins, fats and carbohydrates into smaller molecules by anaerobic bacteria using extra-cellular enzymes. Then, in the second phase, acid forming bacteria continue the degradation of the smaller compounds into carbon dioxide, organic acids, hydrogen sulfide and ammonia. After that, acetogenesis takes place, leading to the formation of CO_2, acetate and H_2 by acetogenic bacteria. Finally, methane forming bacteria produce biogas, chiefly made of methane and CO_2 (methanogenesis).

It is important to point out that anaerobic digestion may be operated in simple systems, such as anaerobic open ponds, or in closed systems, which are a very efficient way to decrease greenhouse gas (GHG) emissions into the atmosphere, allowing the production of renewable energy (methane) and avoiding the uncontrolled emission of GHG produced during animal manure management (Bernet & Béline 2009).

Generally, AD is operated in mesophilic conditions (35-40°C) (Chynoweth et al. 1999). As an alternative, it can be operated in thermophilic conditions, which benefits from having increased rates and higher sanitizing effects. However, thermophilic AD is less stable, due to volatile fatty acids (VFAs) accumulation, and more sensible to potential inhibitors, such as ammonia (Bernet & Béline 2009). Finally,

AD can be operated in psychrophilic conditions, at 20°C or less, but the rates are much lower than in mesophilic or thermophilic conditions (Masse et al. 1997).

Anaerobic digestion has been used for many years for the treatment of organic material in manure and the biogas produced, mainly composed of CH_4 (55-80%) and CO_2 (20-45%), can be used for energy production, as heat or for conversion into electricity (Bernet & Béline 2009). The liquid effluent that is obtained from the anaerobic digestion, named as digestate, has still high concentrations of nitrogen and thus needs to be further treated.

The removal of nitrogen is becoming one of the major steps in wastewater treatment plants since the discharge of its compounds in the water bodies causes important environmental problems, as previously described. The conventional digestate treatment is based on biological processes that include suspended and/or biofilm growth systems carrying out nitrification and denitrification (Obaja et al. 2003).

All the biological systems used to remove nitrogen compounds comprise an aerobic phase, where nitrification occurs, and an anoxic phase allowing denitrification.

The nitrification occurs in two steps, namely the conversion of ammonium to nitrite and the conversion of nitrite to nitrate. In the first step, chemo-autotrophic ammonium oxidizing bacteria, also known as AOB (ammonium oxidizing bacteria), convert ammonium to nitrite. Then, in a second step, another type of chemo-autotrophic bacteria, NOB (nitrite oxidizing bacteria) oxidize nitrite to nitrate.

The autotrophic bacteria responsible for nitrification, discovered in 1891 by Winogradsky (Tilley 2011), belong chiefly to the genera *Nitrosomonas* (ammonium oxidizer), *Nitrospira* (nitrite oxidizer) and *Nitrobacter* (nitrite oxidizer). These bacteria are chemoautotrophs and produce the energy they need to grow by oxidizing ammonia or nitrite. Thus, they require and consume oxygen and use CO_2 as carbon source for growing

The nitrification process is summarized in equations 2.1 and 2.2.

$$NH_4^+ + 3/2O_2 \rightarrow NO_2^- + 2H^+ + H_2O \qquad (2.1)$$

$$NO_2^- + 1/2O_2 \rightarrow NO_3^- \qquad (2.2)$$

The denitrification process occurs under anoxic conditions and has the aim to convert nitrate in nitrogen gas. In this phase, facultative aerobic organisms use the nitrate produced in the nitrification phase as electron acceptor instead of oxygen. These microorganisms are heterotrophic and require organic matter as a source of energy. Thus, it is generally necessary to add organic compounds, for example ethanol or methanol (Ra et al. 2000), unless a recirculation scheme is adopted, as it is the common case for municipal wastewater treatment plants, so that denitrification occurs before nitrification and can exploit the entering COD before it is oxidized. The denitrification phase is described by equation 2.3.

$$NO_3^- + 5e^- + 6H^+ \rightarrow 1/2N_2 + 3H_2O \qquad (2.3)$$

Most conventional wastewater treatment technologies are not economical for the treatment of agricultural wastewater, so it is compulsory to find alternative techniques. Microalgae based processes have been considered a promising biological treatment as microalgae remove nitrogen in one step together with various kinds of potentially toxic or hazardous pollutants from different types of wastewaters.

2.3 Microalgae general applications

Since the 1950s, researchers have focused on studying the use of microalgae to produce many important products in a wide range of processes, such as conventional industries, wastewater treatment and renewable energy production (Bux 2013). Microalgae biomass is mainly cultivated to obtain products with a commercial interest that are mostly used for animal and human nutrition, cosmetics and pharmaceuticals.

Since ancient times, microalgae have been widely used in many populations, mainly in Asiatic countries such as China, Japan and Korea. These organisms contain substances with very interesting antioxidant properties and are rich in proteins as well as in polyunsaturated fatty acids of the omega- 3 series, vitamins A, B, C, E, K and B12, potassium, magnesium and other minerals (Priyadarshani & Rath 2012). Being such a rich source of essential nutrients, they are considered a valuable dietary supplement for humans and animals (Bux 2013). Depending on the species, the extractable chemical compounds may be different and, currently, the production of microalgae for human consumption is widely increasing, and different species have been cultivated to obtain different natural compounds (Herrero et al. 2006).

During the 2000s, several cosmetic companies in Europe and the US start to launch cosmetics that use extracts from microalgae species, such as *Chlorella* and *Spirulina*, (Se-Kwon 2015) since they are able to produce important compounds that can be used as thickening agents, water binding agents and antioxidants (Priyadarshani & Rath 2012).

Regarding the pharmaceutical area, it has also been proven that microalgae are rich in biologically active compounds that present anticancer, antiviral, antifungal and antibacterial properties (Priyadarshani & Rath 2012). In addition, microalgae have the capacity to produce toxins that may have pharmaceutical applications (Se-Kwon 2015).

Summing up, depending on the different microalgae species, it is possible to extract diverse valuable compounds from microalgae biomass, such as fats, polyunsaturated fatty acids, sugars, oils, antioxidants, enzymes, esters, vitamins, pigments, carotenoids and chlorophyll (Bux 2013). Due to this diversity of high-value biological derivatives with many commercial applications, microalgae production is currently becoming much more promising in the area of nutrition and food additives, pharmaceuticals, cosmetics and aquaculture (as food for aquatic organisms) and, if grown on wastewater, they can also be used as a source of biofuels and for pollutant removal (Se-Kwon 2015). However, one major constraint to the use of microalgae is the difficulty in biomass harvesting due to their microscopic size, typically in the 2-40 μm range and also due to their low cell density, typically in the 0.3-5 g L^{-1} range (Brennan & Owende 2010).

2.4 Microalgae for wastewater treatment

In 1957, Oswald and Gotaas suggested for the first time the use of microalgae for wastewater treatment (Ruiz-Martinez et al. 2012) due to their photosynthetic capability, converting solar energy into valuable biomass, and to their capacity to incorporate nutrients like phosphorus and nitrogen. Since then, there have been several studies using microalgae systems to treat human sewage, livestock wastes, agro-industry wastes, industrial wastes and also other types of wastes such as piggery effluents and effluents resulting from food processing factories (Cai et al. 2013).

As an alternative to the conventional wastewater treatment methods, microalgae have proven to be efficient in removing nitrogen and phosphorus from wastewater and the algae based treatment process is named as phycoremediation (Renuka et al. 2015). The impact of microalgae wastewater treatment over conventional treatment must be evaluated in terms of energy and economic factors involved in the operation process. For example, the oxygen produced during the microalgae photosynthetic process can reduce the cost deriving from the need for artificial aeration and can limit the risk of pollutant volatilization. Moreover, wastewater normally sustain a mixed algae/bacteria consortium and the synergy between bacterial and algal biomass increases the process efficiency (Muñoz & Guieysse 2006).

Besides nutrient removal, phycoremediation involves the removal of BOD, of coliform bacteria, of toxic metals, like lead, cadmium, arsenic, mercury, bromine and scandium, of xenobiotic compounds and the sequestration of CO_2 (Renuka et al. 2015).

Microalgae enhance the removal of organic pollutants since they furnish O_2 to heterotrophic aerobic bacteria, which are responsible for mineralizing organic compounds, and in return, these bacteria release CO_2, which is used by microalgae for photosynthesis, thus establishing a symbiotic relation (Muñoz & Guieysse 2006). A limitation of this combined system is linked to the different speed of growth of microalgae and bacteria, which may reduce the benefits of oxygen production, at least in the starting phase, since the microalgae growth is slower, consequently it does not provide immediately the necessary oxygen for bacteria (Mezzanotte et al. 2015). In addition, microalgae can have a negative effect on the microbial community, as altering the pH and the temperature and they can also produce extracellular metabolites that may inhibit bacterial growth (Ruiz-Marin et al. 2010).

Another positive aspect due to the synergetic relation between microalgae and bacteria is the fact that wastewater may contain potentially hazardous and toxic substances for microalgae, such as polycyclic aromatic hydrocarbons, phenols or organic solvents, but their removal/conversion by bacteria allows microalgae to adapt to the growth substrate (Brennan & Owende 2010).

Regarding the removal of heavy metals, the most studied microbial biotreament is performed by using sulfate reducing bacteria, which remove the metals with the production of metal-sulfide precipitates. However, this treatment has some disadvantages including long residence times (weeks) and the need for continuous substrate supply (Perales-Vela et al. 2006). On the other hand, microalgae have proven to be efficient in removing heavy metals, such as zinc, cadmium, mercury, iron and nickel (Renuka et al. 2015)

with a specific metal uptake of 15 $mg.g^{-1}_{biomass}$ at 99% removal efficiency, showing that they can be competitive when compared with the other treatments (Muñoz & Guieysse 2006).

These metals are mainly removed by adsorption/diffusion or surface binding, which is facilitated by the properties of the algae cell walls (Rawat et al. 2016). The algal cell wall presents chemical affinity to metals as a result of the presence of several functional groups, such as carboxyl, carbonyl, amido, amino, sulfhydryl, hydroxyl, which gives negative charge to the cell surface, thus facilitating surface binding with positively charged metal ions by physical adsorption or chemical processes, including ion exchange, chelation with covalent bonds and precipitation (De Philippis et al. 2011). In fact, microalgae are able to release extracellular metabolites that have the capacity of chelating metal ions (Rawat et al. 2016) and the increase in pH due to photosynthesis may cause the precipitation of heavy metals (Mezzanotte et al. 2015).

The fast growing concern about the global warming, mainly caused by the increase of the CO_2 level in the atmosphere, made the United Nations to promote the protocol Kyoto (1997) with the aim of imposing the countries all over the world to reduce the greenhouse gas emissions, and more than 170 countries have ratified the protocol (Wang et al. 2008). Therefore, in the last decade several researches have been developed in order to find the most suitable techniques to reduce CO_2 in the atmosphere. Microalgae have proven to be promising in this field, since they can sequestrate CO_2 from the atmosphere or industrial exhausted gas, contributing to the reduction of GHG (Renuka et al., 2015) and also to the production of a valuable biomass that can be applied in the production of useful compounds such as biofuels and fertilizers (Pires et al. 2012).

Biological mitigation of CO_2 can be carried out by terrestrial plants and photosynthetic microorganisms. However, since microalgae have a simple structure, they have much higher growth rates and CO_2 fixation capacities when compared to conventional aquatic, agricultural and forestry plants, with an efficiency 50 times higher than the terrestrial plants (Li et al. 2008). On average, the production of 1kg of microalgae biomass involves the consumption of 1.8 kg of CO_2 (Chisti 2007).

Many studies on flue gas tolerance by microalgae concluded that some microalgae species can tolerate high concentrations of CO_2, but the levels of SO_X and NO_X can be a concern since they can inhibit microalgae growth (Lara-Gil et al. 2014). However, levels of SO_x and NO_x, up to 150 ppm can also be well tolerated by some microalgae species (Wang et al. 2008). The most suitable species in the fixation of CO_2 seem to belong to *Chlorella* and *Scenedesmus* genera (de Morais & Costa 2007).

Finally, the microalgae biomass produced during wastewater treatment can be used for the production of biofuels. The interest in the use of microalgae for renewable energy started growing in the 1970s during the first oil crisis (Spolaore et al. 2006) and in the last years the research in microalgae has become more extensive due to the depletion of fossil fuels and to the global increase of industrial activities, which are associated to a great need to balance the energy demand and the reduction of greenhouse gas emissions (Mata et al. 2010).

One of the main interests of using microalgae in the energy sector is due to the fact that they have a high lipid content and to the wide range of bioenergy products that can be obtained from culturing microalgae feed-stocks, including biomass for combustion to produce heat and electricity, fermentable sugars to

produce bioethanol, biobutanol or biogas, oil for conversion to biodiesel or even possibly algae biosynthesized biodiesel.

As an alternative to other biofuel sources, microalgae present many advantages such as their fast growth and short life cycle when compared to terrestrial plants (Sivakumar et al. 2016). For example, the average value of the maximum specific growth rate of microalgae species is nearly 1 day^{-1}, while for higher plants it is 0.1 day^{-1} (Chisti 2010). Additionally, in spite of being aquatic microorganisms, microalgae require less water than the terrestrial plant crops and since they can grow in harsher conditions and with less nutrients, they can be cultured in non-arable land, thus not competing for land with food crops (Sivakumar et al. 2016). Microalgae do not need freshwater for their growth, and consequently they can use different wastewaters as a growth medium, with no need for chemicals such as herbicides and pesticides (Brennan & Owende 2010).

Conventional wastewater treatment plants are mainly designed to remove organic compounds by anaerobic or aerobic biological processes, but the treated effluents still contain residual inorganic compounds such as nitrate, ammonium and phosphorus, as well as organic compounds quantified by COD and BOD_5. Thus, a tertiary treatment is often required before discharge (Rawat et al. 2016). Microalgae culturing can be a cost-effective option to remove nutrients from biologically treated effluents, acting as a tertiary treatment (Abdel-Raouf et al. 2012)(Cai et al. 2013). However, the potential to uptake nutrients varies among different species (Boelee et al. 2011) and also as a function of some environmental factors such as light and temperature (Cao & Orrù 2014).

Regarding piggery wastewater, many studies have been conducted in laboratory scale using microalgae as a tertiary treatment and assessing its potential to treat the liquid digestate obtained from anaerobic digestion (Uggetti et al. 2014)(Monlau et al. 2015) (Xu et al. 2015), which is the main conventional process used for removing organic compounds in these types of wastewater. The substances fed to the digester which cannot be converted into biogas are extracted as digestate, which consists of a mixture of undegraded organic and inorganic compounds, including heavy metals and nutrients. Particularly, the organic nitrogen and phosphorous present in the digester feed are found in the digestate mainly in mineralized form. AD offers two interesting products for the cultivation of microalgae, the off-gas that has a high content in CO_2 and the digestate, which contains high concentrations of nutrients, such as phosphorus and nitrogen. Therefore, it is interesting to consider the implementation of microalgae cultivation at anaerobic digestion plants of farm, agro-industry or sewage treatment plants.

The digestate is a suspension which typically contains 2-8% of dry solids and consequently, before being used for microalgae cultivation it must be subjected to a solid / liquid separation treatment in order to produce a clarified phase with reduced solids content and lower absorbance (Mezzanotte et al. 2015). The clarified digestate, possibly after dilution, is sent to microalgae cultivation. Finally, the produced algae can be separated from the culture liquid and fed back to the anaerobic digester, where they can in turn be converted into biogas.

In addition, some laboratory studies have been conducted with the aim of treating piggery wastewater as both secondary and tertiary treatment to remove COD, nitrogen and phosphorus nutrients (Godos et al.

2010). It is important to highlight that in this case the wastewater needs to be subjected to a previous treatment, such as flotation, in order to obtain the soluble fraction of carbon, phosphorus and nitrogen nutrients.

2.5 Production of Biogas using microalgae

Energy production is the principal sector that contributes to the release of GHG to the atmosphere, in particular CO_2, mainly due to fossil fuel combustion (Hook & Tang 2013). However, the associated harmful environmental, health and economic effects, as well as the depletion of fossil fuels, have led to the exploration of biofuel production from biomass (Arthur et al. 2011). Although biodiesel or biogas produced from terrestrial plants are considered as renewable energy sources, they are subjected to many critics because the cultivation of these crops require the deforestation of natural land, the use of fertile lands which could be used for food and also the use of high quantities of herbicides and pesticides (Ras et al. 2011). As mentioned in chapter 2.4, microalgae have proven to be efficient in producing biofuels, such as biodiesel and biogas, mainly due to their high lipid content and their high growth rate. However, the use of microalgae to produce biodiesel has shown some negative aspects such as the high need of fertilizers, as well as the high energy demand for lipid extraction and harvesting procedures (Collet et al. 2014).

Alternatively, AD is a spontaneous process mediated by microorganisms which can convert microalgae biomass into biogas or biomethane, a mixture mainly composed of CH_4 and CO_2 (Ras et al. 2011). The first authors to mention the anaerobic digestion of microalgae were Golueke et al. and they investigated the anaerobic digestion of *Chlorella vulgaris* and *Scenedesmus* species (Golueke et al. 1957).

The feasibility of converting microalgae biomass into biogas is closely linked to their anaerobic degradability, which can be determined by experimental tests, named as BMP tests (Biochemical Methane Potential) and in literature it is possible to find diverse results comprised between 153 and 600 L $CH_4.kg^{-1}$ VS (Ward et al. 2014). The methane yields obtained from anaerobic digestion of different microalgae species are presented in Figure 2.3.

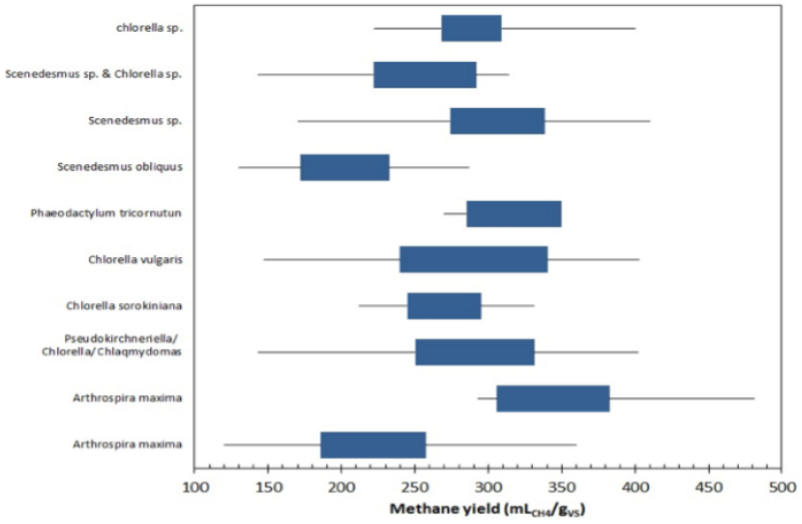

Figure 2.3 Methane yields ($mL_{CH4}.g^{-1}$ $_{VS}$) from different microalgae species (Mezzanotte et al. 2015)

An interesting characteristic of microalgae composition that favors their degradability over that of other plants is the absence of lignin as well as the low content of hemicellulose (Chen et al. 2013).Since these two compounds are slowly biodegradable, the anaerobic digestion of microalgae biomass is more advantageous because it presents higher conversion rates and efficiencies when compared with other plants (Vergara-Fernández et al. 2008).

However, one of the major constraints to take into account in this process is the presence of a cell wall and its composition. The complex cell wall structure of some microalgae species, mainly composed of cellulose, hemicellulose, pectin and glycoprotein, makes it highly resistant to bacterial attack, thus leading to low yields of biomethane production (Passos et al. 2013). Therefore, to overcome this constraint it is necessary to do a pretreatment, such as a heat treatment with temperatures between 70-100 °C, a thermo-chemical combined treatment, the application of ultrasounds or of freeze/thaw cycles (Ward et al. 2014).

It should be added that the production of biogas is also linked to the composition of microalgae biomass, particularly to the lipid content, since lipids have a higher theoretical methane potential than proteins and carbohydrates (Zamalloa et al. 2011). The lipid content allows the decision between biogas production and biodiesel production, taking into account which one is energetically more advantageous. It has been estimated that with a lipid content of less than 40%, the anaerobic digestion is more convenient (Sialve et al. 2009). However, the extraction of lipids for biodiesel production before AD of the residual biomass can be beneficial for anaerobic digestion since high lipid content can be inhibitory (Sialve et al. 2009)(Park & Li 2012)

Another important factor that influences anaerobic digestion is the ratio of carbon to nitrogen present in microalgae species (Sialve et al. 2009) and the data reported in the literature shows that this ratio (C/N) varies from 4.16 to 7.82 for microalgae species that have already been explored for anaerobic digestion (Ward et al. 2014). However, when the C/N ratio is lower than 20, there is an imbalance between carbon and nitrogen requirements for the anaerobic bacterial community, thus leading to the release of nitrogen in the form of ammonia, which can be inhibitory for the methanogenic bacteria and result in VFAs accumulation (Sialve et al. 2009).Therefore, one solution to overcome this problem is the co-digestion of microalgae with other waste streams or biomass with high C/N ratio, consequently leading to an optimal ratio (Santos-Ballardo et al. 2016). Many substrates have already been tested for co-digestion in laboratory scale, including glycerol, mixtures of oils and fats, residues from the paper industry and crop residues, such as corn (Santos-Ballardo et al. 2016).

Finally, it is important to refer the advantage in complementing anaerobic digestion with cultivation and production of microalgae due to the possibility of recycling nutrients. Regarding carbon, it is mainly released as a constituent of biogas and microalgae present the ability to purify the biogas since they can recover the CO_2 (Zamalloa et al. 2011). Similarly, the hydrolytic process that takes place inside the anaerobic digesters allows to dissolve the nutrients contained in the algae cells and these nutrients become present in the liquid phase of the obtained digestate (Ward et al. 2014). Therefore, this digestate can be fed back and used as a nutrient source for new algae growth, significantly reducing the need for fertilizers.

2.6 Microalgae cultivation

Microalgae cultivation at large scale can be done in closed systems, named as photobioreactors (PBRs), or in open systems, such as lakes and ponds.

Photobioreactors are flexible systems which can be optimized according to the physical and biological characteristics of the microalgae to be cultivated (Mata et al. 2010). These closed systems do not allow a direct exchange of gases or contaminants between the microalgae culture and the atmosphere and they are designed to optimize the diffusion of light, whether natural or artificial. Depending on their shape and design, PBRs present various advantages over open ponds since they offer an easier control of the parameters (pH, oxygen, temperature and nutrients), reduce CO_2 losses, prevent evaporation, allow to obtain higher microalgae densities and higher volumetric productivities and offer a safer environment, thus minimizing the risk of contamination (Mata et al. 2010). Despite these advantages, PBRs present some limitations, such as overheating, oxygen accumulation, bio-fouling, high building and operating costs , as well as difficulty in scaling up (Mata et al. 2010).

Among the various photobioreactor types, tubular photobiorreactor is one of the most suitable types for outdoor mass cultures. These photobioreactors are constructed with either glass or plastic tube and they appear either as straight tubes arranged flat on the ground or as long vertical rows (Abdel-Raouf et al. 2012). Tubular photobioreactors are very suitable for outdoor cultures since they have large illumination surface area, high productivities and are relatively cheap (Mata et al. 2010). However, one of the major constraints of this design is a poor mass transfer, which is a big problem in scaling up (Ugwu et al. 2008).

There are also flat-plate photobioreactors that are characterized by a large illumination surface area and consist of flat panels made of transparent material. Although these photobioreactors lead to a very low accumulation of dissolved oxygen when compared to the tubular photobioreactors, they also present some drawbacks, including difficulty in controlling temperature and the scale up requirement for many compartments and support material (Ugwu et al. 2008).

Over the years, the cultivation of microalgae in vertical column systems, such as air lifts and bubble columns, has also been developed since they are able to provide a good mixing, low shear stress, high mass transfer, high scalability potential and low energy consumption (Mata et al. 2010). The columns are positioned vertically, made of transparent material and aerated from the bottom. Compared to other types of photobioreactors, these vertical columns have a major disadvantage of having lower surface exposed to light (Ugwu et al. 2008).

The other systems for microalgae cultivation, known as open ponds, include natural waters (lakes, lagoons, ponds) and artificial ponds or containers and the most frequently used systems consist of shallow big ponds, tanks, circular ponds and raceway ponds. When compared to closed systems, open ponds are easier to construct and operate, they are cheaper and they are easier to clean after cultivation (Abdel-Raouf et al. 2012). However, open ponds present major limitations such as reduced light utilization by microalgae, evaporative losses, diffusion of CO_2 to the atmosphere, extreme dependence on weather conditions,

requirement of large areas of land and very low mass transfer rates, thus leading to low biomass productivities (Ugwu et al. 2008).

Finally, the cultivation of microalgae is completed with a system of microalgae collection from the dispersed phase in which they are grown and it consists in a separation of the solid phase (microalgae) from the liquid (the growth medium). As mentioned before, the harvesting of microalgae is a challenging phase of microalgae biomass production due to their small size and low concentration in the growth medium (Li et al. 2008). The choice of harvesting technique depends on the microalgae characteristics, such as size, density, and the value of the target products (Olaizola 2003). Generally, this process involves two phases: bulk harvesting, which consists on separating biomass from the bulk suspension by flotation, flocculation or gravity sedimentation, and thickening, which consists in concentrating the slurry by centrifugation, filtration and ultrasonic aggregation (Brennan & Owende 2010).

2.7 Microalgae classification and characterization

The term algae was first introduced by Linnaeus in 1753 to refer to a group of plants, which are known since ancient civilization. It refers to a wide group of unicellular and multicellular organisms present worldwide in different habitats, which can grow photoautotrophically, heterotrophically and mixotrophically (Perez-Garcia et al. 2011), depending on the different environmental conditions. In freshwater bodies and oceans, algae and cyanobacteria (photosynthetic prokaryote organisms, once considered microalgae too) constitute the phytoplankton and the primary producers, as they are the base of the aquatic food chain.

Microalgae are unicellular eukaryotic photosynthetic organisms, with an average size between 2 and 40 µm (Brennan & Owende 2010) that convert CO_2 and radiant energy of the sun into sugars for their energy and biosynthetic metabolism, and into oxygen. These microscopic algae typically found in freshwater or marine systems, can exist individually or in group and do not contain stems and roots as do higher plants. There is a vast biodiversity of microalgae and it is estimated an existence of 200 000-800 000 species but only around 50 000 species have been studied and described (Renuka et al. 2015). Thus, this vast biodiversity and the tendency of microalgae to adapt to extreme habitats encourages scientists to study and to identify promising species in order to use and develop more efficient microalgae based technologies for wastewater treatment (Roy et al., 2011).

Microalgae are categorized into diverse classes mainly considering their life cycle, pigmentation and cellular structure (Brennan & Owende 2010). Although the classification and division of algae and microalgae in groups is very controversial, the most known and studied algae are divided in Euglenophyta, Cryptophyta, Dinophyta, Chlorophyta, Heterokontophyta, Rhodophyta and Haptophyta. The following paragraphs represent a general description of the different classes, including the shape and the environments in which they can be found (Roy et al., 2011).

The Euglenophyta are unicellular ovoid or fusiform microalgae, with one or two flagella, mostly found in freshwater habitats, but also in brackish and marine environments. These microalgae have a grass green color and the common marine forms are 40-60 µm long, sometimes up to 500 µm, and around 10 µm wide. More than 800 species have been discovered until now.

The Cryptophyta consists mainly of photosynthetic nanoplanktonic flagellates and their color can be red, blue-green or gold, depending on their complement of photosynthetic pigments. These algae are commonly found in freshwater, brackish and marine environments and around 200 photosynthetic species have already been discovered. Regarding their shape and size, they are ovoid asymmetrical unicells, with an average size of 6-20 µm and with two flagella.

The Dinophyta, also known as dinoflagellates, are a diverse and complex group of unicellular flagellates, with a size comprised between 5 and 2000 µm and are constituted by two flagella. These organisms can be found in tropical, subtropical, temperate and polar oceans, also in freshwaters and at least 2000 species have been discovered, half of them being photosynthetic species. Dinoflagellates can easily form symbiotic relations with other eukaryotic microalgae and they can have diverse colors.

The Chlorophyta are photosynthetic eukaryotes which belong to the green algae lineage. These microalgae are mostly found in marine environments as planktonic individual cells and at least 17000 species have been discovered, being *Chlorella, Scenedesmus* and *Dunaliella* the most important species. Their morphology can be presented as small green flagellates, naked, coccoid or ovoid, with 10-40 µm in diameter.

Within Bacillariophyta group, there are diatoms, which are probably the best known of all the heterokont unicellular planktonic algae. Diatoms are unicellular or colonial organisms (2-200 µm) with no flagella and presenting the particularity of having a silica glass case. These organisms can be found in freshwater and marine environments and more than 100 000 living species have been defined.

The algae division Rodophyta, also known as red algae, includes primitive eukaryotes that belong to a very ancient lineage and it comprises a widely variety of pigmented unicells and macrophytes. Though only a few genera of unicells are known, more than 4000 macropythes species have already been screen and studied. The red algae are mainly found in marine environments, especially in hot and tropical waters but they can also be found in estuaries, freshwater and soils. Regarding their form and shape, they may appear as coccoid unicells or in colonies in a polysaccharide matrix, they don´t have flagella and their average size is 5-15 µm.

The Haptophyta division comprises unicellular species, mostly photosynthetic flagellates, which are mainly found in the nanoplankton of marine environments, especially in tropical waters. These microalgae have an average size of 5-10 µm, two flagella and they can have a variety of forms including coccoid, colonial, amoeboid and filamentous stages.

2.8 Microalgae metabolism

Generally, the organisms that exist in the world can be divided into two main categories, in autotrophic and heterotrophic organisms. Autotrophs are organisms that are able to synthetize all the complex organic compounds that they need, such as proteins, fats and carbohydrates, from nutrients and inorganic material, generally using energy from light (photosynthesis) or from inorganic chemical reactions (chemosynthesis) and CO_2 as carbon source. On the other hand, heterotrophs are the organisms that cannot fix carbon and that use organic compounds for growth.

As mentioned before, microalgae are considered as autotroph organisms and are the base of the aquatic food chain, since they can produce organic matter through photosynthesis. Photosynthesis is a fundamental process for the existence of life on Earth and it can be defined as a redox reaction, in which inorganic carbon and water are converted into oxygen and organic compounds, in the presence of light energy. Carbon is a constituent of all the organic compounds and is the main microalgae biomass element, contributing between 17% and 65% of dry weigh depending on the species (Markou et al. 2014). However, most of the species contain 50% of carbon (Richmond 2004).

Carbon is mostly taken up by photosynthetic organisms in the form of CO_2, however since microalgae live in aquatic environments, when CO_2 is dissolved in water, it reacts with water molecules, creating a weak acid buffer system, and in this system the form and the availability of inorganic dissolved carbon depends on diverse parameters, such as pH and temperature (Markou et al. 2014). Consequently, microalgae developed mechanisms that allow the uptake of different dissolved inorganic carbon, like $HC0_3^-$, for photosynthesis (Markou et al. 2014).

All the photosynthetic organisms comprise organic pigments to absorb the light energy, each one capable of absorbing light at different wavelengths. The most important pigment is chlorophyll which contains a heterocyclic ring (porphyrin ring). This ring has a magnesium atom at the center and is bonded to a hydrocarbon chain that makes the chlorophyll insoluble in water (Borowitzka et al. 2016). The main chlorophylls are chlorophyll a, absorbing mainly the blue-violet and red light, and chlorophyll b, which mainly absorbs blue and orange light (Borowitzka et al., 2016). Other two major organic pigments are carotenoids and phycobilins, which absorb light in the wavelength of blue-green and violet (Borowitzka et al., 2016).

Photosynthesis is divided in two stages, the light dependent reactions and the independent reactions (Chisti et al., 2014). The first process consists in a light-dependent series reaction that occurs in the grana and which requires the direct light energy in order to produce energy-carrier molecules that are further used in the second phase. So, in this phase the light is captured by chlorophyll to produce ATP and at the same time water is split, releasing oxygen, free electrons and hydrogen ions. Then, the free electrons react with $NADP^+$ and convert it in NADPH. In the second process, also called as dark reactions, ATP and NADPH molecules are used to convert CO_2 into carbohydrates (carbon fixation) through a reduction process that occurs in the stroma of chloroplasts.

The fixation of carbon dioxide occurs in the stroma of chloroplasts and it can be achieved through Calvin Cycle, also known as C_3 cycle, which is the most important process for carbon fixation that occurs in microalgae (Chisti et al., 2014). As described in Figure 2.4, the Calvin cycle is divided in four main steps and there is only one enzyme responsible for CO_2 fixation, ribulose 1,5-biphosphate carboxylase/oxygenase, also named as Rubisco. In the first phase, which is named as Carboxylation, CO_2 is incorporated in the five-carbon sugar ribulose bisphosphate (Ribulose-bis-P) to produce two molecules of phosphoglycerate (Glycerate-P) and this reaction is catalyzed by the enzyme Rubisco. Then, in the second step there is the reduction of Glycerate-P into 3-carbon sugars (Triose-P) by two sub steps, which are the phosphorylation of Glycerate-P to produce diphosphoglycerate (Glycerate-bis-P) in the presence of ATP and, the reduction of Glycerate-bis-P to phosphoglyceraldehyde (Glyceraldehyde-P) by NADPH.

Thereafter, there is the production of organic compounds, such as glucose, and the regeneration of ribulose-P for further CO_2 fixation in a complex series of reactions. So, three turns of the Calvin cycle are needed to produce one molecule of Glyceraldeyhde-P that can exit the cycle and go forward to produce glucose, and since it takes two molecules of Glyceradehyde-P to produce one molecule with six carbon atoms, it is necessary 6 CO_2, 18 ATP and 12 NADPH to form one molecule of glucose.

Besides the carboxylase activity, Rubisco enzyme displays also an oxygenase activity, thus, being able to uptake oxygen through a process known as photorespiration (Se-Kwon, 2015). During photorespiration, Rubisco enzyme oxygenates Ribulose 1,5- biphosphate, leading to a net loss in carbon dioxide fixation. Therefore, photorespiration is considered as a competitive process to carbon fixation and reduces the efficiency of photosynthesis. Photorespiration depends on the ratio of O_2/CO_2, where a high ratio favors this process whereas a low ratio favors carbon fixation (Chisti et al., 2014).

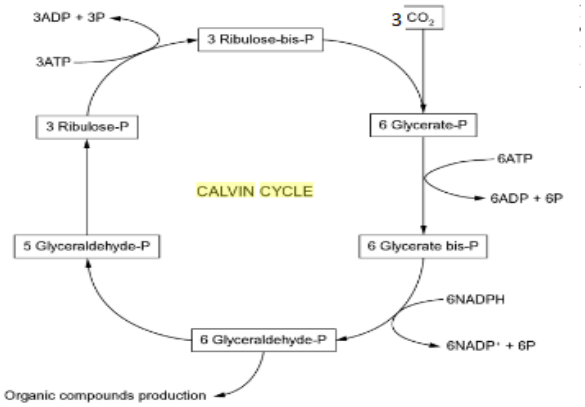

Figure 2.4 The dark process of carbon assimilation by photosynthetic microalgae (Chisti et al., 2014)

The main equation (2.4) that summarizes photosynthesis is represented bellow.

$$6C0_2 + 6H_2O \xrightarrow{\text{yields}} C_6H_{12}O_6 + 6O_2 \qquad (2.4)$$

While photosynthesis allows the accumulation of energy in the form of carbohydrates, which are the principal component for the life of all organisms, aerobic respiration is also a very important process, in which microalgae oxidize the organic matter into energy (ATP) and waste products. The aerobic respiration can be generally described in equation 2.5

$$C_6H_{12}O_6 + O_2 \xrightarrow{\text{yields}} H_2O + CO_2 \qquad (2.5)$$

In general, microalgae are considered as photoautotrophic organisms, however some studies have proven that some microalgae species can have heterotrophic as well as mixotrophic metabolism, and sometimes microalgae can alternatively implement the different kinds of metabolism, as a response to changing environmental conditions (Perez-Garcia et al., 2011). Some microalgae are able to grow in dark environments by assimilating the organic dissolved carbon as a source of energy and carbon (Perez-Garcia et al. 2011). One of the most interesting characteristics of heterotrophic microalgae cultivation is the

elimination of light requirements, allowing the use of innovative bioreactors without light constraints in their design (Bumbak et al., 2011). However, only few species have proven to grow under heterotrophic conditions (Lowrey et al., 2015) and this type of cultivation presents some disadvantages such as an addition of potential costs due to the need of meeting the organic carbon requirement which is most commonly delivered from glucose (Lowrey et al., 2015); diminishing of the pigmentation and of the production of high value phytochemicals of the microalgae cells due to dark reactions, thus causing economic problems in the cultivation (Mata et al., 2010) and production of carbon dioxide from the respiration of organic carbon during growth (Perez-Garcia et al., 2011).

Regarding mixotrophic conditions, it is defined as a mix of autotrophic and heterotrophic conditions, which means that microalgae are able to perform photosynthesis and consume CO_2 and organic matter as a carbon source (Lowrey et al., 2015). For example, (Heredia-Arroyo et al. 2011) reported that *Chlorella vulgaris* was able to grow under autotrophic, heterotrophic and mixotrophic conditions and that the mixotrophic conditions could produce more biomass concentration than the other conditions. However, mixotrophic microalgae, overall, are poorly studied and the available examples are quite limited to a few species. Hence, there should be more studies on mixotrophic microalgae since it takes advantage of both organic and inorganic resources to obtain maximum biomass production and, particularly interesting when integrating microalgae cultivation with wastewater treatment where wastewater is usually turbid and the sun light cannot penetrate well into the core of the wastewater body (Heredia-Arroyo et al. 2011).

However, certain species are characterized as obligate photoautotrophs and some species are able of having a photoautotrophic and heterotrophic metabolism simultaneously or sequentially (Perez-Garcia et al., 2011). The universal reason why some microalgae are obligate photoautotrophs is still unknown, however one of the proposed theories claims that it is a result of a deficiency in the cellular consumption of vital substrates, mainly sugars (Chen & Chen, 2006). For example *Phaeodactylum tricornutum* is a phototrophic microalga that became able to grow after the insertion of a gene which encodes a transporter for glucose (Morales-Sanchez et al., 2014).

Besides light and CO_2, microalgae need micronutrients and macronutrients for their growth. The micronutrients necessary for the growth of microalgae include potassium, metals, sulfur and calcium, while regarding macronutrients, microalgae need nitrogen and phosphorus compounds (Cao & Orrù, 2014). Nitrogen is a fundamental compound since it allows the production of amino acids and nucleic acids. In the case of phosphorus, it can be incorporated in a large variety of organic compounds, such as sugars phosphates, phospholipids and nucleotides.

2.9 Parameters that influence microalgae growth

As described in Figure 2.5, the growth cycle of microalgae in batch consists of 5 phases (Cao & Orrù, 2014):

1. Lag phase or Induction phase, during which microalgae adapt to the environment and to the new conditions thus, there is no growing of culture density;

2. Exponential phase, in which the culture density increases according to a logarithmic function with time;
3. Linear phase, in which cell division slows down when nutrients, light, CO_2 or other factors become limiting for growth;
4. Stationary phase, in which the limiting factors are balanced with growth rate, thus stabilizing the cell density;
5. Death phase, in which the death of microalgae prevails and this may be caused by several reasons, such as nutrients exhaustion, lack of oxygen, overheating, contamination or changes in the pH.

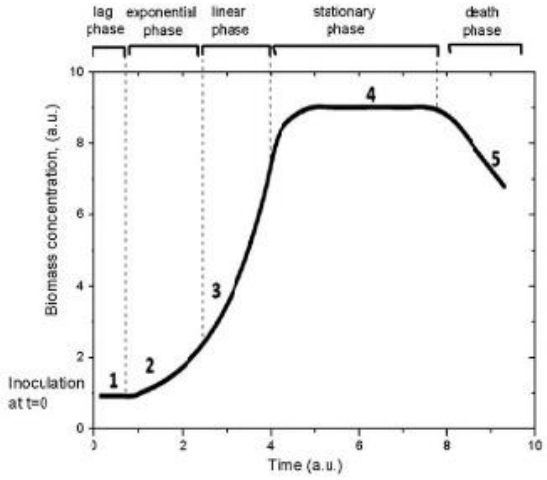

Figure 2.5 Schematic representation of microalgae growth in a batch culture (Cao & Orrù, 2014)

The factors that influence the growth of microalgae can be grouped in abiotic, biotic and operational factors. The first group consists in non-biological factors, such as temperature, pH, carbon dioxide, nutrients concentration, light, oxygen and the presence of toxic compounds. On the other hand, the second group includes all biological factors like the presence of bacteria, viruses and other pathogens, or the presence of other types of microalgae that compete for the same nutrients as the species with interest. The last group is related with all the parameters involved in the cultivation system , such as the hydraulic retention time (HRT) and the intensity of mixing (de la Noüe et al., 1992).

Regarding the abiotic factors, these can be easily controlled in laboratory. However, in large scale systems, whereas indoor or outdoor, it is more difficult to control these factors and, when possible, it is too costly (de la Noüe et al., 1992). The problem of abiotic factors is complicated due to big variations in the concentration of nutrients in effluents or in the environment.

Biotic factors are also not so easy to control. However, the purpose of using microalgae for wastewater treatment is not to produce biomass with a defined composition, but to remove efficiently unwanted compounds.

The solution to some of these problems can be solved by optimizing the operative parameters. It is extremely important to optimize the parameters that influence the growth of microalgae cultures in order to ensure a good productivity of the culture and an efficient treatment.

Some of the parameters mentioned before are described in detail bellow.

2.9.1 Nutrients

Among all factors that influence microalgae growth, nutrients availability is one of the most important factors to take into account during microalgae cultivation. Nutrient availability is crucial for cell division and for intracellular metabolic activities and once nutrients become limited, a steady decline takes place in microalgae growth cycle (Cao & Orrù, 2014).

As mentioned before, for their growth microalgae need micronutrients (Mg, Zn, Fe, K, Na, etc.) and macronutrients (C, P, N) as well as dissolved CO_2, transferred from the gas phase (Cao & Orrù 2014). Besides CO_2, whose importance has already been mentioned in the chapter 2.8, phosphorus and nitrogen nutrients are also fundamental keys for the growth of microalgae.

2.9.1.1 Carbon

As mentioned in chapter 2.8, carbon is the main microalgae biomass element and is mostly taken up by microalgae in the form of CO_2 (Markou et al., 2014). In natural conditions, microalgae uptake CO_2 from the ambient air, which contains around 360 ppmv of CO_2, however, most microalgae species have proven to tolerate and to uptake much higher concentrations, up to 150 000 ppmv (Brennan & Owende, 2010). CO_2 can be supplied from three different sources, such as atmosphere, exhaust gas from power plants and industries and soluble carbonates, like Na_2CO_3 and $NaHCO_3$ (Wang et al., 2008). The use of CO_2 from the atmosphere and from exhaust gas involves an environmental advantage since it contributes to the reduction of GHG emissions. However, the potential yield from the atmosphere is limited due to the low concentration of CO_2 (Brennan & Owende, 2010). On the other hand, CO_2 capture from power plant flue gas achieves better recovery as it contains higher concentrations, usually between 15-20% (Salih, 2011). This exhaust gas contains nitrogen oxides (NOx), sulfur oxides (SOx) and particulates (dust) causing some constraints related to their inhibitory effects on microalgae growth (Lara-Gil et al., 2014). However, some studies have proven that some microalgae species can tolerate NOx and SOx to some levels and use it as a source of nutrients (Chiu et al., 2011). For example, in an aqueous system NOx partially dissolves and oxidizes into nitrite, which could be used as nutrient for microalgae. Regarding sulfur dioxide, it can generate bisulfite, which can severely affect the pH of the culture or can generate sulfate that can be assimilated by microalgae (Lara-Gil et al., 2016). Finally, it is important to refer that these gases need to be cooled prior to the injection in the cultivation medium.

2.9.1.2 Nitrogen

After carbon, and not considering hydrogen and oxygen, nitrogen is quantitatively the most significant element, representing approximately 1 to 10 % of the dry matter of algae cells (Richmond 2004), and is a component of essential biochemical compounds, such as amino acids, nucleic acids and pigments like chlorophyll. It can be taken by microalgae in its inorganic form (nitrate, ammonium ion and nitrite) and converted in its organic form through assimilation but also, it can be taken in organic forms such as urea or amino acids (Flynn & Butler, 1986;Perez-Garcia et al., 2011).

Ammonium is the most favorite nitrogen source for microalgae since its uptake and assimilation process is simpler and requires less energy consumption than for other nitrogen sources (Markou et al.,

2014). Ammonium is transported through the membranes by a group of proteins that belong to the ammonium transporter family (AMT) (Perez-Garcia et al., 2011). The most important path for ammonium assimilation involves glutamine synthetase, which produces glutamine amino acid by the reaction between ammonium and glutamate and glutamate synthase, which produces two molecules of glutamate from glutamine and one molecule of α-ketoglutarate (De-Bashan et al., 2008).

One of the major constraints in using ammonium as a source of nitrogen is its toxicity at high concentrations which can be due to ammonium ion or free ammonia (Markou et al., 2014). Free ammonia is considered to be the most toxic form since it is uncharged and lipid soluble and can easily diffuse trough the membrane into the cells, which have low control on intracellular concentration, thus becoming toxic and damaging the photosynthetic system (Collos & Harrison, 2014). According to (Azov & Goldman, 1982) free ammonia presents a toxic effect for very low concentrations 2 mM. The relative concentration of ionized ammonia and free ammonia depends mainly on pH and temperature (Tosta & Mendonça, 2009).

Ammonia toxicity is a major concern to take into account when using wastewaters with high concentrations of nitrogen, as is the case of piggery wastewaters. One of the strategies to decrease toxicity effects is to control the pH of the medium, thus limiting the concentration free ammonia (Markou et al., 2014) and another strategy is to dilute the wastewater to avoid the inhibitory effect of ammonia (Olguín et al., 2003).

In addition, another important constraint to consider when using ammonium as a source of nutrient, is that it can be lost from cultivation medium though volatilization, mainly with high pH (Markou et al., 2014).

Regarding nitrate, it is one of the major sources of inorganic nitrogen to microalgae and its assimilation involves two reduction steps. First, nitrate is transported into the cell and a cytosolic nitrate reductase transforms it into nitrite, then it is transported to chloroplast, where nitrite reductase converts it into ammonium (Sanz-Luque et al., 2015). Finally, it can go through the glutamine synthetase pathway to produce glutamine amino acid. Although usually nitrate does not present toxic effects to microalgae, at high concentrations it can affect the microalgae growth since with the increase of nitrate, there is an increase of nitrate reductase activity, therefore resulting in an intracellular accumulation of nitrite and ammonium, which are toxic to the cells (Chen et al., 2009) .

Nitrite is regularly found in natural environments as an intermediate from nitrate reduction or from ammonia oxidation, however it can also be found intracellular as a result of nitrate reduction, which was previously described. Microalgae are able to up take nitrite from the medium, mainly through active transportation (Markou et al., 2014), but diffusion has been also reported for green microalgae. Nitrite is transported to chloroplast, where nitrite reductase converts it into ammonium. Then, as previously described, it can go through the glutamine synthetase pathway to produce glutamine amino acid. Although microalgae are able to uptake nitrite and use it as nitrogen source, at high concentrations it is a toxic compound and presents negative impacts in microalgae growth (Chen et al., 2012). Furthermore, the uptake of nitrite seems to be influenced by CO_2 concentration since under low CO_2 concentration the uptake of nitrite is reduced, while high CO_2 concentration affects positively the nitrite reductase activity, enhancing the nitrite assimilation (Markou et al., 2014).

Moreover, urea and some amino acids are taken up actively and are metabolized in the cells (Pérez Garcia, et al., 2011). Some green microalgae seem not only to be able to use organic nitrogen, but, when they use these molecules, their growth rate are similar or higher than when they use other inorganic forms of nitrogen. However, the possibility to use organic nitrogen compounds as nitrogen resource depends on the species.

It has to be highlighted that when different nitrogen sources are simultaneously present in the medium, microalgae firstly uptake the one with more affinity depleting it, and then they uptake the others.

2.9.1.3 Phosphorus

Phosphorus is another macronutrient that plays an important role in the metabolic processes of microalgae, forming many functional and structured compounds required for the growth and development of microalgae. Phosphorus is present in different organic molecules, such as nucleic acids, membrane phospholipids and ATP (Geider & Roche 2011) and its biomass content varies between 0.05% and 3.3% (Richmond, 2004)

Phosphorus is assimilated by microalgae mainly as orthophosphate, but other inorganic and organic forms can also be used for microalgae growth (Markou et al., 2014). Regarding dissolved organic phosphorus (DOP), it can either be actively up taken into the cells or it can suffer extracellular mineralization by phosphatase enzymes (Dyhrman & Ruttenberg, 2006). However, the uptake of DOP depends on its chemical composition. In fact, most of the DOP compounds cannot be directly assimilated by microalgae and therefore, these compounds need to be mineralized (Dyhrman & Ruttenberg, 2006). In the case of inorganic phosphorus forms other than orthophosphates, it is necessary to convert them into orthophosphates in order to have an efficient uptake by microalgae and this is also mediated by phosphatase enzymes (Markou et al., 2014). These enzymes can be intracellular, extracellular or attached to the cell wall of microalgae.

The uptake of phosphorus is affected by the cell condition and by environmental factors, such as pH, temperature, light and the concentration of the ions Na^+, Mg^{2+} and K^+ (Markou et al., 2014). The cellular production of phosphatase enzymes is increased when phosphorus availability decreases and when microalgae become phosphorus limited or starved (Dyhrman & Ruttenberg, 2006).

Another interesting fact is that under sufficient phosphorus supply, microalgae are able to accumulate intracellular phosphorus reserves as polyphosphate granules and this reserves can be used as phosphorus source when phosphate in the culture medium is depleted. This process is named as luxury uptake (Brown & Shilton, 2014).

Finally, another parameter to be controlled is the Redfield ratio, which is the atomic ratio between carbon, nitrogen and phosphorus found in phytoplankton. A stoichiometric formula for the most common elements in algae cells is defined as $C_{106}H_{181}O_{45}N_{16}P$ (Choi & Lee, 2015), thus the optimal Redfield ratio for algae cultivation is assumed to be 106:16:1 and this parameter allows to determine which nutrient is limiting (Geider & Roche, 2011). However, N:P ratio has an extra interest and it has shown to affect the growth, cellular elements and biochemical composition of the algae (Rasdi & Qin, 2014). According to (Choi & Lee,

2015) the Redfield ratio N:P (16:1) is not an optimal value for all the species but it represents an average of species-specific N:P ratio and the optimum value for biomass productivity and for nutrients removal is between 5 and 30.

2.9.2 Light

Light is one of the main factors that influences microalgae growth rate since it has a key role in photosynthetic reactions, thus the cultivation of microalgae requires light with appropriate wavelength, intensity and duration (Cao & Orrù, 2014). As it is widely known, solar energy is the main source of light for microalgae to perform photosynthesis and mainly the visible light since the pigments phycobilins, chlorophylls and carotenoids absorb light in the range 400-750 nm (Borowitzka et al., 2016). However, since only approximately 48 % of solar energy is photosynthetically active radiation (PAR) and since 10-20% of this energy is lost by surface reflection, only 12-14 % of the solar energy can be effectively converted into microalgae biomass (Park et al., 2011).

Light intensity is a major factor influencing microalgae growth. In fact, low intensity may lead to growth limitation of the culture and excessive intensity may cause photoinhibition (Parmar et al., 2011). As described in Figure 2.6, there is no growth of microalgae when the light intensity is too low, whereas the increase of intensity up to a certain point leads to the growth of the culture. However, over a defined threshold, the cells stop growing with increasing light intensity and this phenomenon is known as photoinhibition. This phenomenon generally causes reverse damage in the photosynthetic system due to exposition at excessive light, which results in a decrease of microalgae growth rate (Cao & Orrù, 2014).

The light intensity at which the cells stop growing is named as the light saturation level (Yusuf, 2007). Light saturation is characterized by a light saturation constant that corresponds to the intensity of light at which the growth rate is half of the maximum specific growth rate. In many cases, light saturation constants for microalgae are lower than the maximum solar irradiance at midday and for this reason, very often, the saturation level is exceeded and the efficiency of photosynthetic cells is not optimal. For example, as mentioned by Yusuf, (2007), the light saturation constant is 185 μE m^{-2} s^{-1} for *Phaeodactylum tricornutum* and 200 μE m^{-2} s^{-1} for *Porphyridium cruentum*, whereas the typical midday outdoor light intensity in equatorial regions is approximately 2000 μE m^{-2} s^{-1}.

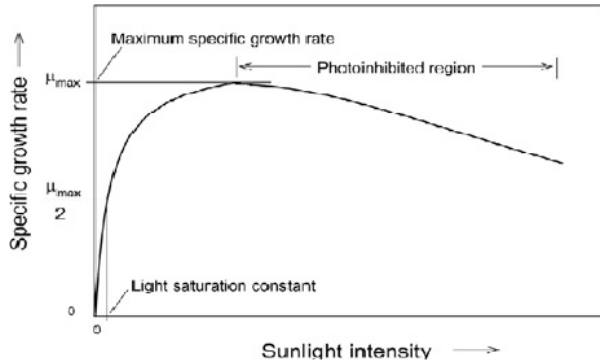

Figure 2.6 Effect of light in microalgae growth rate (Yusuf 2007)

Since the fluctuation of light intensities in the outdoor cultures can affect microalgae growth due to reduced light intensity during the rainy days or to excessive light intensity during summer days, the cultivation of microalgae with artificial light (LED or fluorescent lamps) is a good solution when the operation costs are justified (Zhao et al., 2013).

Finally, the duration of light is also an important factor to take into account during microalgae growth (Al-Qasmi et al., 2012). Microalgae need a light/dark system for an efficient photosynthesis since they need light for a photochemical phase to produce ATP and NADPH, and they need a dark phase to produce the crucial compounds for their growth. For this reason, if the light is artificial it is necessary to maintain a light/dark (LD) cycle similar to the natural conditions (maximum 16:8 LD and usually, 14:10 LD or 12:12) (Mezzanotte et al. 2015).

2.9.3 pH

The monitoring of a medium´s pH during algae growth is an important tool to analyze the performance of the photosynthetic processes, since during photosynthesis microalgae uptake dissolved CO_2 from the medium and consequently, the pH increases. Although pH variation can be an indicator of the photosynthesis performance, it can also affect the kinetics of microalgae growth as it can modify the distribution of carbon dioxide species and carbon availability, it can change the availability of nutrients and trace metals, and at extreme pH levels it can cause direct physiological effects (Cao & Orrù, 2014)

The pH is closely connected to the concentrations of HCO_3^-, CO_2 and CO_3^{2-} of the carbonate system because as pH increases, carbonate concentration increases and the concentrations of carbon dioxide and bicarbonate decrease. The concentration of carbon dioxide in the medium is influenced by photosynthesis, respiration, water temperature and oxidative decomposition of organic matter (Zang et al., 2011). During the photosynthetic process, microalgae quickly uptake carbon dioxide, which causes a shift of the equilibrium, favoring the decomposition of HCO_3^- and thus, leading to a pH increase and a decrease of the partial pressure of CO_2 when the replacement process of CO_2 is slower that the utilization. The replacement of CO_2 includes the respiration, the fermentation, the influx of atmospheric CO_2 and dehydration and hydration reactions of dissolved CO_2 (Chen & Durbin, 1994). Therefore, the production of CO_2 by aquatic respiration processes may inhibit the decomposition HCO_3^- and consequently, result in the decrease of pH.

Many studies on the effect of pH in microalgae cultures have defined the optimum pH value as the parameter corresponding to the highest growth rate of microalgae, differing from species to species. As an example, regarding two of the most important species, the optimal pH is 6.5 for *Chlorella vulgaris* and 7 for *Scenedesmus obliquus* (Hodaifa et al., 2009). Generally, microalgae present a neutral or slightly alkaline optimum pH (7-9), though some species are able to grow in acidic conditions and this may be due to the fact that the photosynthetic system is inside chloroplasts, which are surrounded by cytoplasm. Since cytoplasm presents a neutral pH, when microalgae are subject to acidic environments, chloroplasts can be protected, subsequently protecting chlorophyll and the photosynthetic system (May, 1997).

As mentioned before, pH is the main parameter that influences the relative concentrations of ionized ammonia and free ammonia. Another important point is that the assimilation of different nitrogen sources

influences the pH of the culture medium since when ammonium is assimilated, pH may decrease due to the release of H^+ ions and when nitrate is assimilated there is the release of OH^- (Markou et al., 2014).

2.9.4 Temperature

Temperature is one of the main factors that affects microalgae growth since it influences the nutrient requirements, the nature of metabolism, the biomass concentration and the metabolic reaction rates of microalgae. Normally, at low temperatures microalgae growth rates decrease, whereas at high temperatures their metabolic rates accelerate (Muñoz & Guieysse, 2006).

In general, each microalgae species is characterized by having an optimum growth temperature, at which enzymes present the highest activity, thus providing a maximum growth rate and for most of the strains, this temperature ranges between 25 and 28°C (Richmond, 2004). Bellow optimal temperatures, a temperature increase causes an enhancement of enzymatic activities that are connected with the Calvin Cycle, thus leading to a more efficient photosynthesis and cell division (Ras et al. 2013). On the other hand, above the optimal temperature, microalgae growth rate tends to decrease, mainly due to heat stress, which can denature essential proteins/enzymes that are involved in the photosynthetic process (Ras et al., 2013).

Finally, if the temperature of the culture is not adequate to obtain the desired microalgae production, it is possible to control and regulate the temperature with an immersion heat system or exploiting the heat of the exhaust gas used to provide CO_2 to the system.

2.9.5 Dissolved Oxygen Concentration

As it is well known, oxygen is a product of photosynthesis. However, high concentrations of dissolved oxygen can limit microalgae growth. The main two mechanisms that negatively influence microalgae growth are the competitive effect of O_2 on Rubisco (photorespiration) and the photo-inhibition, which in the presence of high oxygen concentrations and high irradiance level causes cell damage of photosystem II with the production of reactive oxygen species (ROS) (Raso et al., 2011). Generally, with the aim of preventing inhibition and damage, the maximum tolerable dissolved oxygen level should not exceed around 400% of air saturation (Chisti, 2007).

Fortunately, high levels of dissolved O_2 are not a big problem in biodegradation processes, such as wastewater treatment, due to the continuous consumption of O_2 by bacteria (Muñoz & Guieysse, 2006).

2.9.6 Presence of other microorganisms

As mentioned before, the biotic factors also have an impact in microalgae growth since they are linked to the presence of other living organisms. So, these factors include the presence of pathogens (fungus, bacteria, virus), the competition between different microalgae species, since some microalgae have lower productivities than others, and the presence of predators, such as rotifers.

The presence of organisms which alter the normal functional characteristics of microalgae cultivation can be minimized by abruptly changing some parameters of the culture, such as pH, temperature and light radiation (Mata et al., 2010).

3 Materials and Methods

3.1 Wastewater Origin

In the described experiment, the wastewater used as growth substrate for microalgae is agro-industry type and was provided by Corte Grande WWTP in Italy (Figure 3.1). This plant is located at a piggery farm in Casaletto di Sopra (CR) and receives sewage from 20 000 pigs.

Figure 3.1 Satellite image of Corte Grande WWTP, in Italy

The plant comprises a first step of pig slurry purification by flotation to separate the liquid phase from the solid one. Then, the supernatant, called WW1, is sent to the company´s treatment plant while the sludge is sent to two anaerobic digesters that are operate in mesophilic conditions.

The first digester, which works in mesophilic conditions, is mainly fed with pig manure and corn and subsequently the product from anaerobic digestion is centrifuged to separate the solid phase from the liquid phase, called WW2. The liquid fraction, also referred as digestate, is sent to the company´s treatment plant and the solid phase is used as fertilizer in agriculture.

The second digester, which works in mesophilic conditions, in addition to pig slurry is also fed with manure, whey and corn and the product from digestion is separated using a system of screw presses. The liquid phase that is obtained is named as WW3.

In this project, the growth of microalgae was carried out on wastewater WW1, as an alternative to the conventional agro-industry wastewater treatments. As mentioned before, this wastewater is the liquid phase obtained from the flotation process.

With the purpose of allowing an optimal growth of microalgae, the substrate should not be too turbid to permit a good exposure to light, should have a N-NH_4^+ concentration not inhibitory for the growth (Markou et al. 2014) and also a ratio of N:P around 10:1 (Xin et al., 2010). Therefore, the effluent WW1 arising from

the flotation process was characterized in order to evaluate the need to perform a pre-treatment before adding it to the microalgae culture.

The characterization of the wastewater consisted in analyzing some parameters such as Chemical Oxygen Demand (COD), nitrogen and phosphorus nutrients concentration, pH, conductivity, turbidity, optical density (OD) and Total Suspended Solids (TSS). Table 3.1 reports the average composition of the wastewater WW1 and the standard deviation.

Table 3.1 Average parameters of wastewater WW1 and standard deviation

Parameters	Average	Standard deviation
Conductivity ($mS.cm^{-1}$)	2.0	0.63
pH	7.7	0.26
OD (680 nm)	0.7	0.02
$N\text{-}NH^{+}_{4}$ ($mg.L^{-1}$)	396.0	41.78
$N\text{-}NO^{-}_{2}$ ($mg.L^{-1}$)	0.030	0.026
$N\text{-}NO^{-}_{3}$ ($mg.L^{-1}$)	3.29	0.74
$P\text{-}PO_{4}$ ($mg.L^{-1}$)	21.96	2.488
COD ($mg.L^{-1}$)	2277	387.20
Turbidity	732	35.81
TSS ($g.L^{-1}$)	0.76	0.03

3.2 Microalgae Culture

The microalgae inoculum used to study the growth on the described wastewater is a mixed culture of *Chlorella spp.* and *Scenedesmus spp*. (Figure 3.2) acquired from previous experiments on digestates.

Figure 3.2 Chlorella spp. (left) (Škaloud 2007) and Scenedesmus spp (right) (Morgan 2005).

Chlorella spp. is a genus of single-celled green algae that belongs to the phylum Chlorophyta and can be found both in aquatic habitats and in terrestrial habitats. They are characterized for being nonmotile, globular, unicellular green algae with an average diameter of 4 to 10 µm and they do not have flagella

(Prescott 1964). Under an optical microscope it is possible to recognize them by their green color and spherical shape. *Chlorella* present a higher concentration of chlorophyll and photosynthetic capacity when compared with higher plants. There are 20-30 different species of *Chlorella* which are classified according to the shape of the cells and characteristic of chlorophyll (Costa & de Morais 2014). They were one of the first microalgae to be culture and they have been one of the most used microalgae for laboratory experiments since they have the ability to quickly absorb and assimilate carbon dioxide and phosphorus and nitrogen nutrients. Also, when compared to other algae species, they are more durable and less susceptible to contamination from the wastewater.

Scenedesmus spp. is also a kind of green algae, no motile, specifically of the Chlorophyta. It is often associated in colonies of four or eight cells within a wall (John et al. 2002). This species is used in the treatment of wastewater due to its high-speed growth, and especially for its ability to remove ammonia and phosphorus rapidly.

3.2.1 Experimental plan

Firstly, two vials (1 and 2) were prepared with the centrifugation of 300 mL of algae suspension from previous studies during 10 minutes at 300 rpm, using an ALC 4222 centrifuge.

The supernatant was removed and 0.12 g of microalgae were placed in each new vial. Then, the vials were filled with 150 mL of wastewater WW1. However, the desired concentration of ammonium nitrogen was around 200 $mg.L^{-1}$ and since the concentration of WW1 was 400 $mg.L^{-1}$, it was necessary to dilute it with tap water before adding it into the vials.

After 11 days, two new vials (A and B) were similarly prepared in order to have a replica of the experiment. The vials were filled with 150 mL of wastewater WW1 and microalgae were also obtained from previous studies.

The cultures were grown at room temperature (20-25°C) with continuous aeration and artificial light. The four test vials were distributed in Plexiglas supports (Figure 3.3) and subjected to 12 h/12h of light /dark cycles, which was provided by 6 fluorescent lamps (FLORA model from OSRAM) with 18 W each. The mixing and diffusion of CO_2 was provided by air that was bubbled at the bottom of each vial through a fine bubble diffuser. A good mixing of the suspension was crucial to prevent sedimentation of the algae, to improve gas exchange between the culture and the air, and also to guarantee that all microalgae were equally exposed to nutrients and to light. Moreover, a 2 $g.L^{-1}$ solution of Na_2HPO_4 was added to the vials to ensure that the N/P ratio was around 10. The four glass vials were covered with cotton to avoid evaporation.

All the glass test vials were working in semi batch conditions with an average HRT of 9 days during the first 29, 35, 30 and 30 days, for vials 1, 2, A and B, respectively, and an HRT of 5 days between the 32nd and the 91st day for vial 1; of 6 days between the 39th and the 91st days for vial 2; of 6 days between the 32nd and the 88th days for vial A and of 7 days between the 32nd and the 88th days for vial B. The calculation of HRT was based on the volume of each vial and the slope of the graph of accumulative volume addition (Annex I).

The average test conditions and standard deviations of each vial are represented in Table 3.2.

Table 3.2 Average temperature (°C), pH, conductivity ($mS.cm^{-1}$) and its standard deviation for vial 1, 2, A and B

	Temperature (˚C)		**pH**		**Conductivity($mS.cm^{-1}$)**	
	Average	Standard Deviation	Average	Standard Deviation	Average	Standard Deviation
1	23.4	1.94	9.43	0.871	1.72	0.215
2	23.7	2.26	9.54	0.995	1.71	0.221
A	24.2	2.56	9.32	1.26	1.67	0.198
B	24.1	2.55	9.46	1.35	1.66	0.208

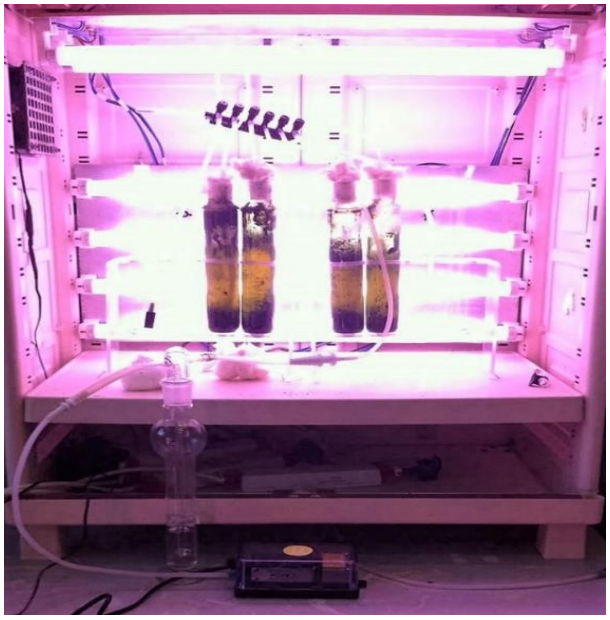

Figure 3.3 System used for the growth of microalgae cultures

3.3 Analytical Methods

The effect of algae growth was controlled and followed by performing chemical analyses on nutrient concentration and COD in the medium. These chemical analyses were carried out using the test kits (Hach Lange, Figure 3.4). The measured parameters were:

- N-NH_4^+ (LCK303);
- P-PO_4^{3-} (LCK348);
- N-NO_2^- (LCK342);
- N-NO_3^{2-} (LCK339);
- COD (LCK514).

In order to perform the chemical analyses on the microalgae wastewater culture, 5 mL were taken from each culture suspension and filtered using a porous membrane filter with 0.45 microns as a first step.

Then, before each analysis and depending on the range of concentrations read for each compound by the spectrophotometer, a dilution with distilled wastewater was done when necessary. The reading of the concentrations was done using the spectrophotometer VIS Hach DR3900 (Figure 3.4).

Figure 3.4 Kit Hach Lange and Spectrophotometer DR3900

It is also important to refer that for phosphorus and COD analyses a digestion step was required and performed using the Hach Lange LT200. In the case of phosphorus, the digestion was performed at 100 °C during 1 hour and for COD it was performed at 148°C during 2 hours.

Conductivity, pH and temperature were measured using a Tecnovetro XS PC510 pH-meter without filtering the samples.

3.4 Algae growth measurement

The algae growth was assessed by four parameters:

- optical density
- turbidity
- total suspended solids
- counting of algae cells

It is essential to refer that these four parameters were measured in the raw culture suspension without filtration.

3.4.1 Optical density

The optical density of the culture is a rapid and nondestructive spectrophotometric measurement of light scattered by the suspension and is an indirect measurement of biomass in cultures of microorganisms (Griffiths et al., 2011). The standard procedure involved a first blank reading with the spectrophotometer (VIS Hach DR3900) by filling the proper cuvette (10 mm) with distilled water. After that, the optical density of the samples was measured in the range of 340-900 nm.

In the determination of the optical density, when the values obtained were higher than 1, it was necessary to do a dilution of the sample with distilled water since the values read by the spectrophotometer are more precise when lower than 1.

Regarding the more accurate wavelength to use, there is little consensus about which value to use in microalgae suspensions. However, the use of a wavelength between 650-680 nm has been reported to correspond to the maximum absorbance value since it corresponds to the maximum absorbance of chlorophyll (Griffiths et al., 2011). Figure 3.5 reports an example of a spectrum at different wavelengths and it is possible to observe that the maximum absorbance in the visible range corresponds to 680 nm.

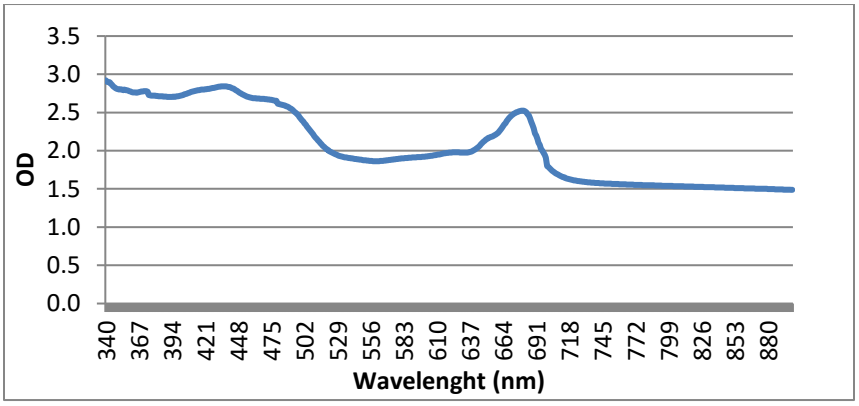

Figure 3.5 Example of a curve obtained from the measurement of absorbance at different wavelengths

3.4.2 Turbidty

Turbidity is an optical determination of liquid clarity and is an expression of the amount of light that is scattered by the suspension when a light is shined through the sample. Turbidity is an important water quality parameter that detects the presence of suspend solids like algae.

The turbidity measurement was carried out by spectrophotometer (840 nm) and, as for the optical density, the standard procedure started with a blank reading. For this parameter, a 5 cm cuvette was used and usually, since the range of turbidity values read by the spectrophotometer was between 40-400 FAU, it was necessary to dilute the samples with distilled water.

3.4.3 Total suspended solids

The parameter total suspended solids is also important for the assessment of algae growth and corresponds to a laboratory gravimetric procedure where the solids from the culture are filtered through a 47 cm glass fiber filter (1.2 µm porosity) (Sartorius stedim biotech).

The first step for the determination of TSS consisted in placing the filters in the oven (MPM Instruments, type M80-VF) for 3 hours at 105 °C. Then, the filters were placed in a dryer and two hours later, they were weighted in a balance (Acculab) .Then, 10 mL samples from each vial were filtered with the aid of a vacuum pump (KNF NEUBERGER, D-79112). Finally, the filters were dried and weighed using the procedure described previously.

The TSS concentration is expressed in g.L^{-1} and can be calculated using equation 3.1. In this equation, $w_{filter,f}$ is the weight of the filter after filtration (in g), $w_{filter,i}$ is the weight of the filter before filtration (in g), and V is the filtered volume (in mL).

$$TSS = \frac{(w_{filter,f}) - (w_{filter,i})}{V \times 10^{-3}} \quad (3.1)$$

3.4.4 Microalgae counting

In addition to the described parameters, counting of algae is also an essential method for analyzing their growth and it also allows to check the type of algae present in the suspension and observe other organisms such as rotifers and nematodes.

The counting procedure started with sample preparation, which was done by mixing well the suspension and then, diluting it to an appropriate concentration because if the concentration was too high, the algae would overlap and become difficult to count. On the other hand, if the concentration was too low, this would lead to count too few algae per square and, consequently, to get poorly representative results. Later, the dilution used was considered in the determination of the final concentration.

For the counting, a microscope Optika B-350 with a magnification of 40 x and a hemocytometer (Burker chamber) were used. The hemocytometer is a rectangular glass slide (7.5 cm x 3.5 cm), 4 mm thick, with two gridded areas of 3x3mm, 0.1 mm deep, each divided into 9 squares of 1mm, each with 16 sub-squares of 0.2 x 0.2 mm, and the surface of 1/25 mm².

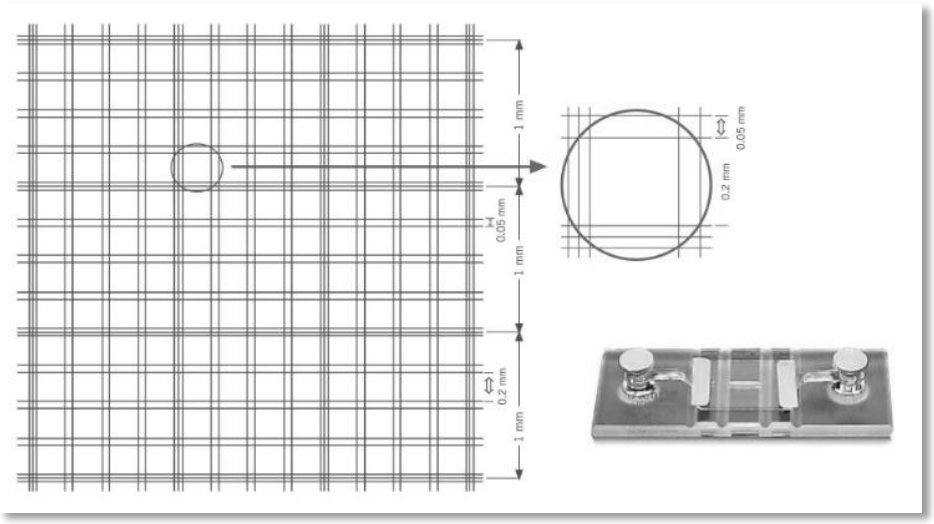

Figure 3.6 One of the two gridded areas (3x 3mm) and a hemocytometer (right bottom) (Cellis 2006)

Before the counting, it was important to ensure that the hemocytometer was clean, dry and free of dust. Then, 0.1 mL of the prepared sample was injected with a pipette on the hemocytometer where the liquid flowed by capillarity. Later, the hemocytometer was fixed in the microscope and, first, the presence of predators, such as rotifers and nematodes, was determined using a magnification of 4x.

After that, the counting of algae was done with a magnification of 40 x. During the first days of the experiment the counting was only performed on 9 little squares (0.04 m^2), randomly chosen. However, after 40 days for vial 1 and 2 and 29 days for vial A and B, the count was extended to 36 little squares in the bottom part of the hemocytometer and 36 in the top one, each square with 0.04 mm^2, in order to obtain more reliable data. In each big square, 3 lines were randomly chosen, each with 12 squares.

Another important rule involved in the counting was that the algae that were on the line of a grid that touch the bottom and the right lines of a square should not be counted. On the contrary, cells on the top and left side should be counted.

Finally, the concentration of algae, expressed in cell.mL^{-1}, was determined using the equation 3.2, where the volume of the square is expressed in mL. Since the considered squares have 0.04 mm^2 surface, the volume of the square is 0.000004 mL.

$$Cell_{concentration} = \left(\frac{number\ of\ cells\ counted}{number\ of\ squares}\right)\left(\frac{dilution}{volume\ of\ the\ square}\right) \qquad (3.2)$$

3.5 Experimental procedure

The analyses of all the previously described parameters were performed 3 times per week. Each day, before starting the analyses, distilled water was added to the four vials in order to compensate the evaporated water. Then, 5 mL of suspension from each vial were filtered on 47 glass fiber filter (Sartorius stedim biotech) and the concentration of $N\text{-}NH_4^+$;$P\text{-}PO_4^{3-}$;$N\text{-}NO_2^-$;$N\text{-}NO_3^-$ and COD (in mg.L^{-1}) were determined on the filtered samples.

The turbidity was determined using 1 mL of suspension, without filtering, and diluting it with distilled water. In addition, optical density, pH, conductivity and temperature were also measured.

If the concentration of ammonium nitrogen was lower than 100 mg.L^{-1}, a mass balance was done to determine the volume of wastewater which had to be replaced in the vials in order to ensure that the concentration of ammonium nitrogen inside each vial was maintained around 200 mg.L^{-1}.

The calculations used to determine the volume of wastewater necessary to add (V_{add}) are represented in equation 3.3 and 3.4.

First, the mass of ammonium nitrogen which should be added (m_{add}) was calculated by equation 3.3, where$[N-NH_4]_{obj}$ is the objective concentration of ammonium nitrogen inside the vial; V_{vial} is the volume of each vial in L (0.150 L for each vial); $[N-NH_4]_{meas}$ is the measured concentration of ammonium nitrogen in mg.L^{-1} ; $V_{vial,d}$ is the volume of the vial in the day of analyses.

Then, knowing the concentration of ammonium nitrogen in the wastewater ($[NH_4]_{WW1}$) that is expressed in mg.L^{-1}, it was possible to determine the V_{add}.

$$m_{add} = [N-NH_4]_{obj} \times V_{vial} - [N-NH_4]_{meas} \times V_{vial,d} \qquad (3.3)$$

$$V\ add = \frac{m_{add}}{[N - NH_4]_{WW1}} \quad (3.4)$$

Every time the addition of wastewater was necessary, all the analyses were repeated later and the TSS ($mg.L^{-1}$) were measured following the procedure described in chapter 3.4.3.

After the addition of wastewater, it was important to check if N/P was lower than 10. Otherwise, a 2 $g.L^{-1}$ solution of Na_2HPO_4 was added to the vials to keep the ratio lower or equal to 10.

Algae counts were only performed before addition, following the procedure described in chapter 3.4.4.

3.6 Respirometric tests

As it was already discussed, microalgae are able to remove inorganic nitrogen from wastewaters. However, part of ammonium nitrogen is removed by nitrifying bacteria, which require the oxygen produced by microalgae during photosynthesis and therefore, establishing a symbiotic relation with microalgae (Muñoz & Guieysse, 2006). Normally, the nitrification process mediated by bacteria involves two steps, ammonium oxidation into nitrite by a group of bacteria and nitrite oxidation into nitrate by other bacteria group (Tilley, 2011).

Respirometry is the measurement of the respiration rate of organisms and is defined as the amount of oxygen per unit of volume and time that is consumed by the microorganisms. Respirometric tests allow to plot oxygen concentration against time and the rate of oxygen consumption can be obtained by calculating the slope in that time (Nollet & Gelder, 2014).

The aim of performing respirometric analyses was to confirm the presence of other microorganisms besides microalgae and to measure the uptake of oxygen by three different organisms which could be present in the culture suspension: AOB; NOB and heterotrophic organisms (HO).

In order to analyze separately the different activities present in this mixed culture containing microalgae, selective inhibitors were used taking into account that the inhibition should be instantaneous and complete for the target population and not affect other populations. Therefore, allythiourea (ATU) (Ginestet et al., 1998) was used to inhibit AOB and potassium chlorate (Hynes & Knowles, 1983) for inhibiting NOB , without affecting any other populations.

The respirometric test was performed using a suspension obtained by taking 20 mL of each vial (1, 2, A and B) and mixing it in just one vial. Then, it was added wastewater WW1 until reaching 100 mL of volume and the vial was aerated and exposed to light in the same conditions described in 3.2.1 chapter. In addition, the vial was fed following the normal procedure described in chapter 3.5, during one week, with the aim of adapting and growing more microalgae.

Sodium nitrite and ammonium chloride were added with the aim of ensuring that ammonia and nitrite nitrogen concentrations were not limiting during the experiment. Besides, a solution of sodium acetate was also prepared to be used as a substrate for heterotrophic organisms. Thus, in order to prepare these solutions it was necessary to do calculations taking into account the need for 10 $mg.L^{-1}$ of $N\text{-}NO^{-}_2$,10 $mg.L^{-}$

[1] $N\text{-}NH^{+}_{4}$,10 $mg.L^{-1}$ of ATU (Ginestet et al., 1998), 834.5 $mg.L^{-1}$ of ClO_3 (Hynes & Knowles, 1983) and 20 $mg.L^{-1}$ of acetate, in 250 mL of suspension. The concentrations of the prepared solutions are represented in Table 3.3 .

Table 3.3 Concentration of the prepared solutions used during respirometric analyses

Reagents	Brand	Concentration ($g.L^{-1}$)
Allythiourea (ATU)	Sigma-Aldrich (assay 98%)	2.5
Potassium chlorate ($KClO_3$)	Sigma-Aldrich (assay ≥99%)	61.3
Sodium Acetate ($C_2H_3NaO_2$)	Merck (assay ≥99%)	5,0
Ammonium chloride (NH_4Cl)	Merck (assay ≥99.8%)	9.5
Sodium nitrite ($NaNO_2$)	Riedel-de Haen (assay ≥99%)	12.3

Before starting the respirometric analyses, it was necessary to analyze some parameters in the mixed culture, such as $N\text{-}NH_4^+$ and $N\text{-}NO_2^-$, to check if it was necessary to add ammonium chloride and sodium nitrite solutions, as well as the absorbance to check if it was not too high, thus not affecting the penetration of light in the system.

An Erlenmeyer flask was prepared with 90 mL of the mixed culture and the suspension was diluted with distilled water until reaching 250 mL volume. Then, the culture suspension was exposed to the same light conditions of the original vials and mixing was provided by a magnetic stirrer (VELP Scientifica). Before starting the experiment, the culture suspension was maintained in these new conditions for a few minutes in order to allow microalgae to adapt.

The measurement of dissolved oxygen concentration during time was performed by a DO probe (Hach HQ40d) every 30 seconds and, since the probe could only measure 500 points, each phase of the experiment was set to last about 20 minutes.

In the first 10 minutes, the lights were on and no reagent was added in order to determine the gross oxygen production rate of microalgae. Then, 1 mL of $NaNO_2$ and 1 mL of NH_4Cl solutions were added to ensure that the concentration of ammonia and nitrite were not limiting.

After that, the culture suspension was covered with aluminum paper with the aim of stopping the light penetration and consequently, microalgae photosynthesis and oxygen production. So, the oxygen uptake rate (OUR) by algae respiration NOBs, AOBs and HOs was measured.

Then, 1 mL of ATU solution was added to inhibit the ammonium oxidizers, thus allowing the measurement of OUR by algae respiration, NOBs and HOs. Later, 5 mL of KClO were added to inhibit the NOBs, therefore allowing the measurement of OUR by algae respiration and HOs.

Thereafter, the culture was exposed to light once more so that it would be possible to analyze the oxygen production rate and to check if the addition of the previous compounds had caused any inhibition on microalgae. The Erlenmeyer flask was then covered again with aluminum paper in order to measure the

OUR by algae respiration and HOs. Finally, 1 mL of sodium acetate was added as a substrate for the heterotrophic organisms.

During the entire procedure, the concentration of dissolved oxygen was controlled so that there was not risk of oxygen limitation. For this reason, if the concentration of dissolved oxygen was under 5 $mg.L^{-1}$, it was necessary to start aeration in the suspension until oxygen concentration reached 8 $mg.L^{-1}$ The phases in which aeration was needed had to be slightly longer to compensate for the time measurements that could not be carried out during aeration.

3.7 BMP tests

For biochemical methane potential (BMP) tests, 130 mL from vials 1 and 2 and 105 mL from vials A and B were collected and mixed and two sub-samples, called C and D, were then sent to Laboratory A.Rozzi, in the Cremona site of Politecnico di Milano.

The biomethane potential tests were carried out in batch conditions with the aim of measuring the maximum amount of biomethane produced per g of volatile solids (VS) content in the organic substrate used for the anaerobic digestion, which in this case was microalgae biomass. The microalgae biomass sample was introduced in glass bottles with an inoculum of anaerobic bacteria (substrate to inoculum rate 0.530 and 0.549 gVS substrate/gVS inoculum for sub-samples C and D, respectively) and put in a thermostatic bath in the mesophilic temperature range (33-35°C). Each bottle was continuously mixed with a mechanical stirrer and the biomethane production was monitored over time. Digestion time was 35 days. Figure 3.7 represents the equipment used for BMP tests.

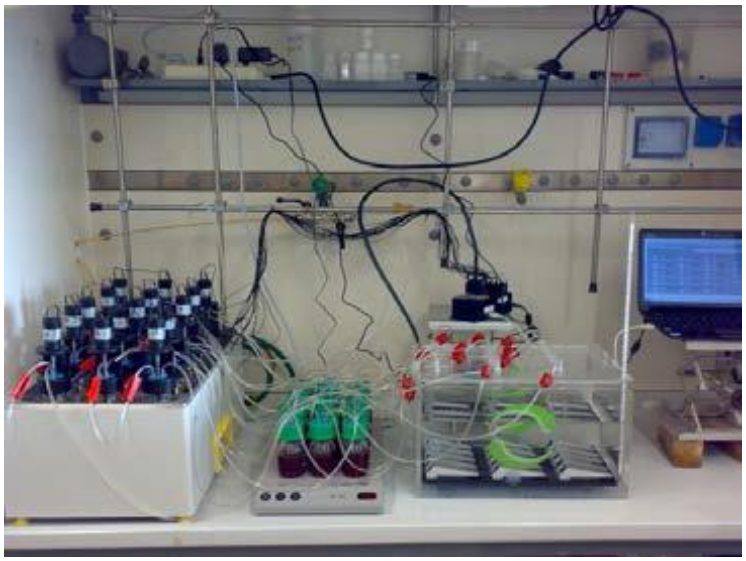

Figure 3.7 Example of BMP tests performed in the laboratory A.Rozzi, in the Cremona site of Politecnico di Milano

4 Results

4.1 Algae growth parameters

4.1.1 Correlation between different parameters

Turbidity, algae count (in 72 little squares of the hemocytometer) and TSS were found to be consistent with OD measurements, as described in Figure 4.1, Figure 4.3 and Figure 4.4, respectively. On the other hand, the counting during the first days of experiment, which corresponded to the count of 9 squares, was not consistent with OD (Figure 4.2).

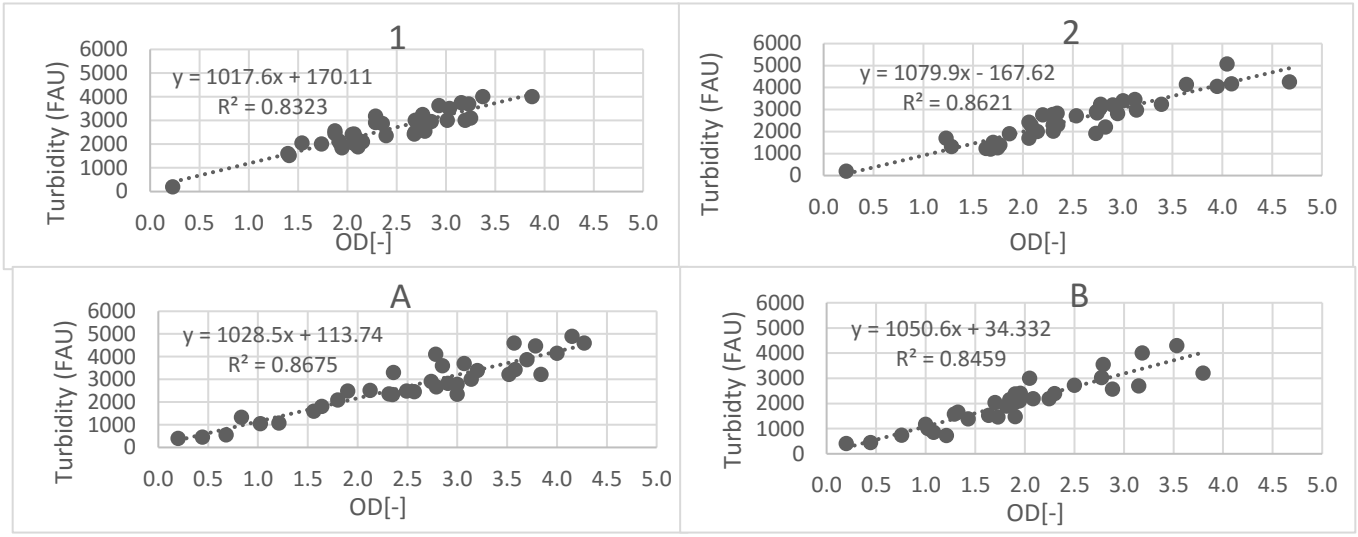

Figure 4.1 Relationship between optical density [-] and turbidity (FAU) for vials 1, 2, A and B

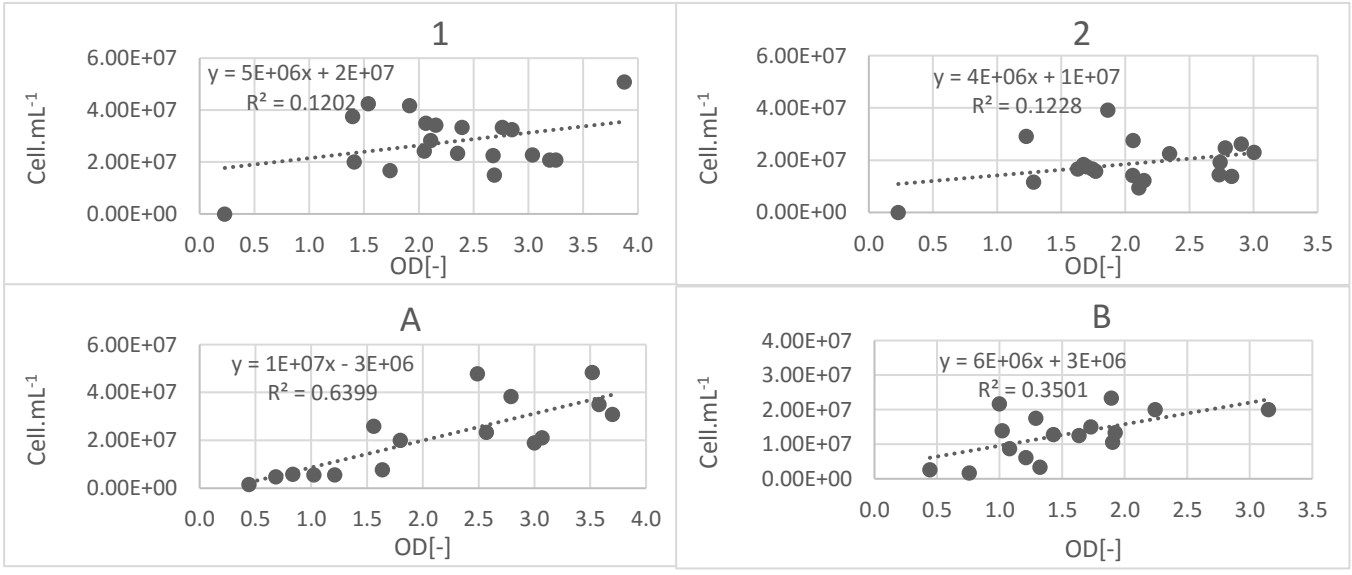

Figure 4.2 Relationship between optical density [-] and microalgae count (Cell.mL^{-1}) of 9 little squares of the hemocytometer for vials 1, 2, A and B

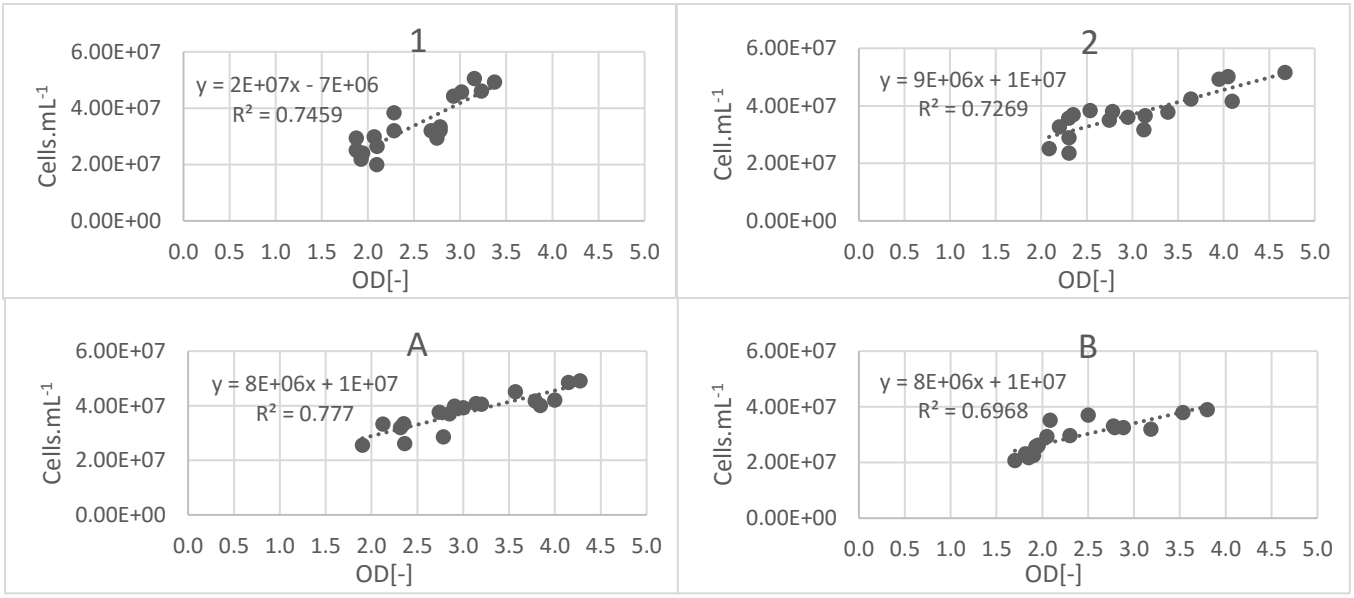

Figure 4.3 Relationship between OD [-] and microalgae count (cell.mL^{-1}) of 72 squares of the hemocytometer for vials 1, 2, A and B

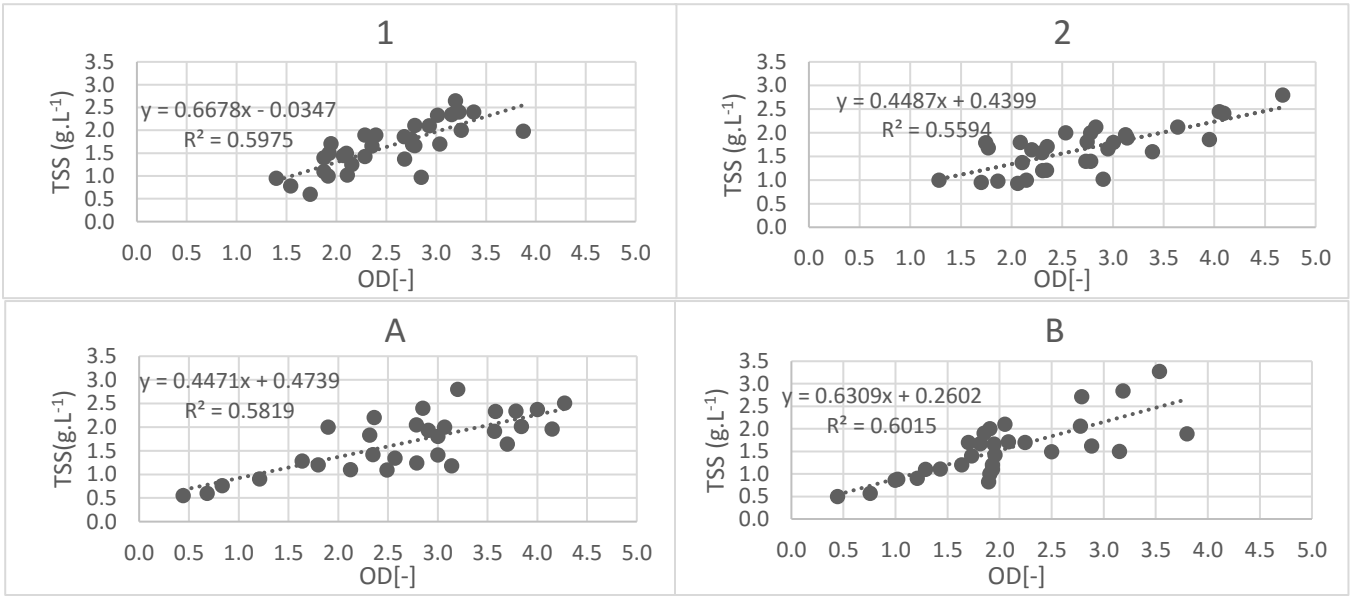

Figure 4.4 Relationship between optical density [-] and total suspended solids (g.L^{-1}) for vials 1, 2, A and B

As observed in Figure 4.1, the relationship between OD and turbidity presented the highest correlation with R^2 values comprised between 0.8323 and 0.8675. Regarding the relationship between OD and microalgae count, in the first 40 days of experiment for vials 1 and 2 and 29 days for vials A and B, the counting procedure was only performed on 9 squares of the hemocytometer and the correlation between these two parameters was very low, except for vial A, as shown in Figure 4.2. Therefore, in the following days the number of squares counted was increased to 72 and the correlation of these two parameters increased with R^2 values comprised between 0.6968 and 0.7770. Finally, it is possible to observe that for

all the four vials, OD and TSS measurements were found to be consistent, however, these two parameters presented lower R^2 values (comprised between 0.5594 and 0.6015) than turbidity measurements and microalgae counting of 72 squares (0.04 m^2).

4.1.2 Variation of TSS with time

The parameter total suspended solids was chosen to represent the biomass concentration in the vials during the experiment, since it is one of the main parameters used in literature to describe microalgae biomass variation during time.

Although in this study the cultures were replicas of each other, it was possible to observe that their performance was different and, therefore, Figure 4.5 represents the variation of total suspended solids, in $g.L^{-1}$, separated for each vial.

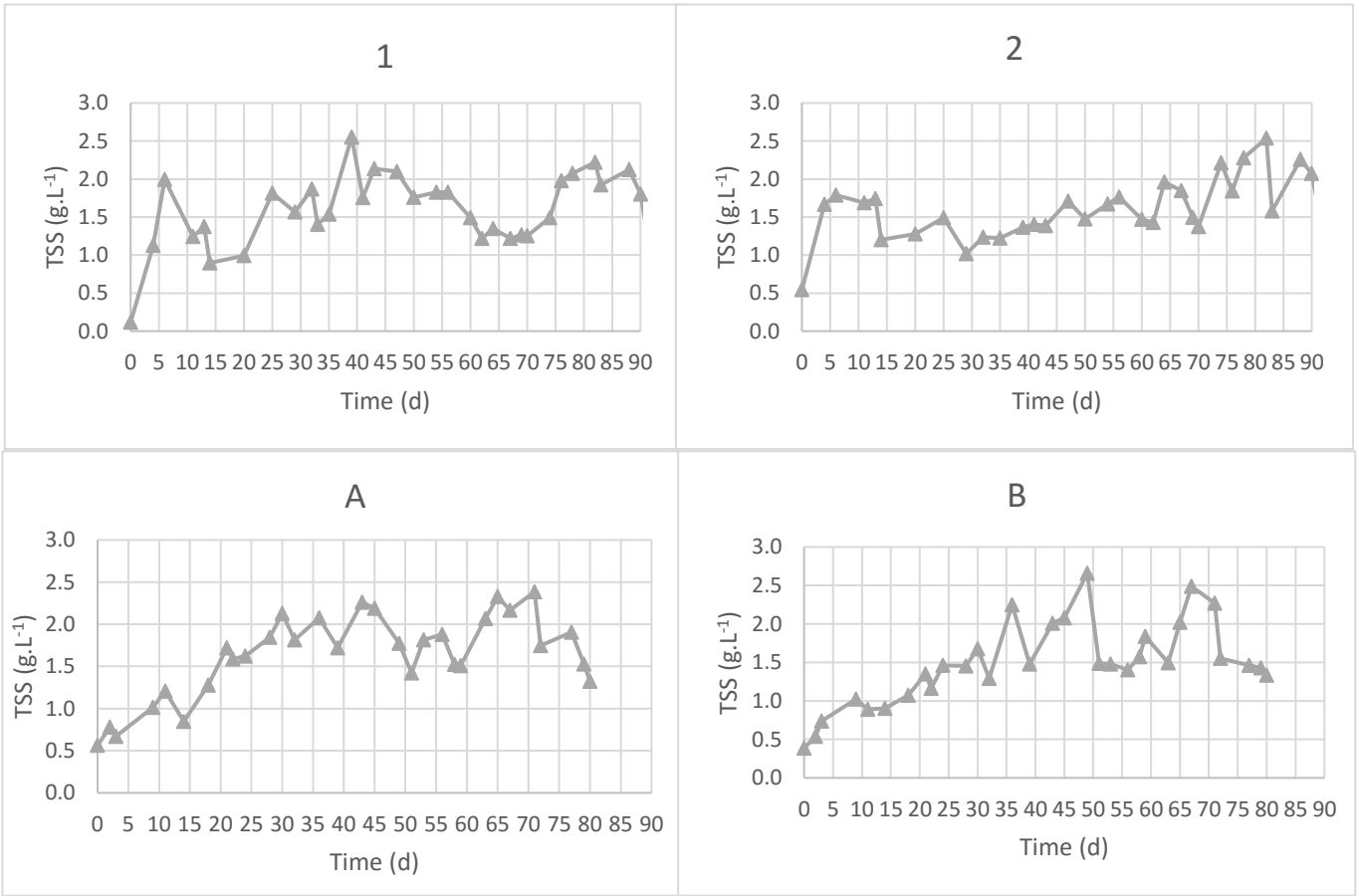

Figure 4.5 Variation of total suspended solids, expressed in $g.L^{-1}$, for the four vials

Generally, in all the vials the mixed culture of *Chlorella spp.* and *Scenedesmus spp.* was able to grow on high organic and high nutrient concentration substrate (piggery wastewater) as well as to adapt and recover after some decreases in the biomass.

Regarding the culture in vial 1, it is possible to observe that in the first 6 days the biomass growth was fast, while then it tended to oscillate around the same values. However, there was a drastic decrease

after the 56th day and biomass concentration only started to recover after the 70th day. The maximum value of TSS was 2.55 $g.L^{-1}$.

For the culture in vial 2, the fast-growing phase lasted 4 days. After that, the total suspended solids tended to stabilize with less variations than in vial 1. The maximum value of TSS was 2.54 $g.L^{-1}$.

In the case of vial A, the growth was more gradual and reached a steady state only after 30 days. However, there was a high decrease after 45th day followed by recovery only after the 59th day. Likewise, there was a decrease of TSS in the last days of the experiment. The maximum value of TSS was 2.39 $g.L^{-1}$.

Similarly to vial A, the culture in vial B grew gradually in the first days, had a decrease in density after the 49th day and a recovery after the 63rd day. In addition, there was an evident drop during the last days. The maximum value of TSS was 2.66 $g.L^{-1}$.

4.1.3 Microalgae productivity

The determination of microalgae productivity involved a standardized method which consisted on using cumulated values of total suspended solids as an approximation to a continuous system. The calculation was based on equation 4.1

$$Biomass_{PR} = \frac{\left(m_{TSS,cum}\right)_{t_i} - \left(m_{TSS,cum}\right)_{t_{i-1}}}{\Delta t \times V_{vial}} \quad (4.1)$$

where, $Biomass_{PR}$ is the microalgae productivity, expressed in $g.L^{-1}.day^{-1}$; $m_{TSS,cum}$ is the cumulate TSS, expressed in g; Δt is the time elapsed between time t_i and time t_{i-1}; V_{vial} is the volume of the vial, expressed in L.

The average microalgae productivity, as well as the standard deviation, are represented in Table 4.1 for each vial.

Table 4.1 Representation of average daily productivity, expressed in $g.L^{-1}.day^{-1}$, and standard deviation for each vial

	Average productivity ($g.L^{-1}.day^{-1}$)	**Standard deviation**
1	0.314	0.109
2	0.263	0.128
A	0.400	0.139
B	0.352	0.126

4.1.4 Microalgae predominance

During these experiments, a mixed culture of *Chlorella spp.* and *Scenedesmus spp.* was used. Figure 4.6 represents the total algae count (Cells.mL^{-1}) as well as the total *Chlorella spp.* and *Scenedemus spp.,* expressed in cell.mL^{-1}, for each vial.

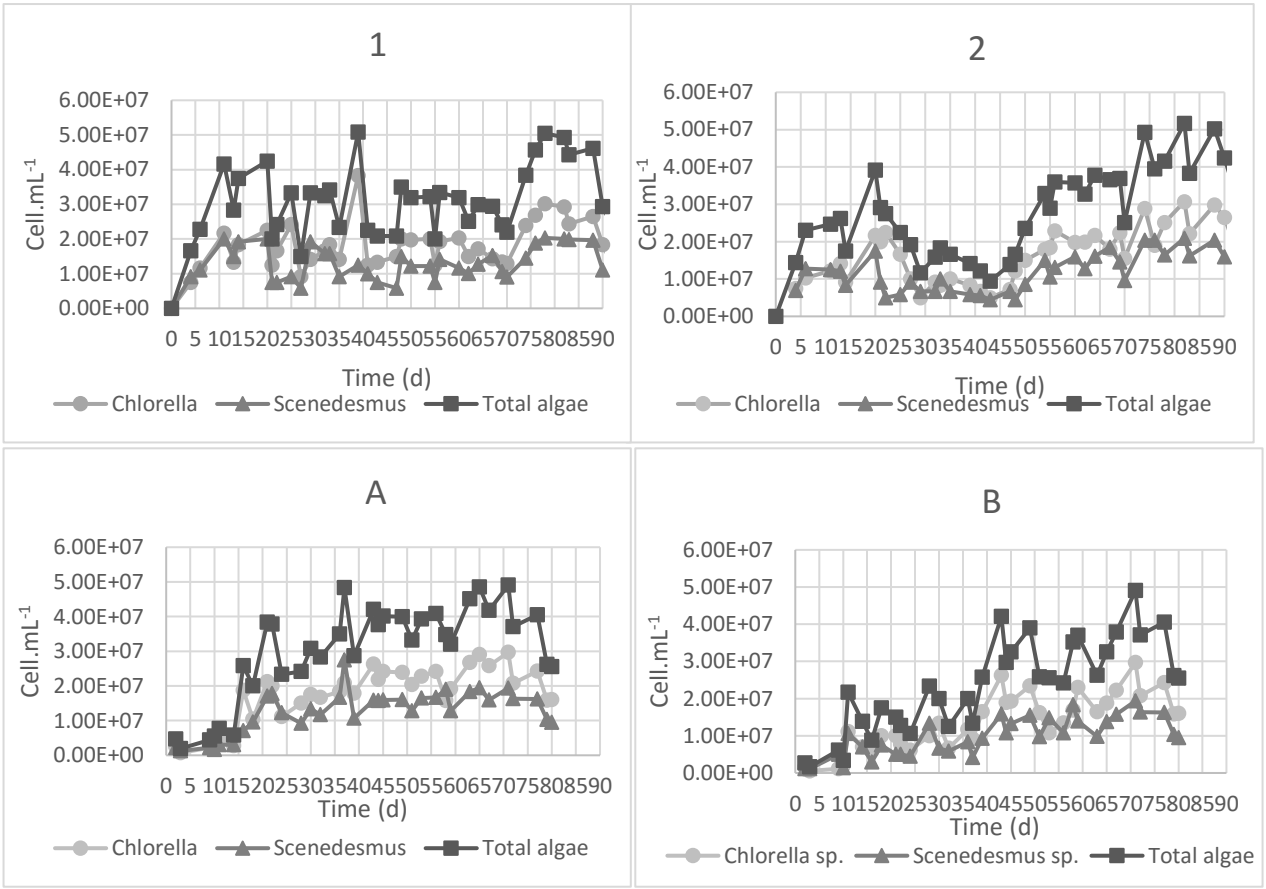

Figure 4.6 Representation of total algae as well as total Chlorella spp. and Scenedesmus spp., expressed in cell.L^{-1}, for each vial

As observed in Figure 4.6, generally, it is possible to observe that in the first days the presence of *Chlorella spp.* and *Scenedesmus spp* was relatively similar, however, after a few days *Chlorella spp.* was often the most dominant species.

4.1.5 Variation of TSS with pH

Figure 4.7 represents the variation of total suspended solids with time as well as the variation of pH with time. The average pH for vial 1 was 9.43±0.871, for vial 2 was 9.54±0.995, for vial A was 9.32±1.26 and for vial B was 9.46±1.35.

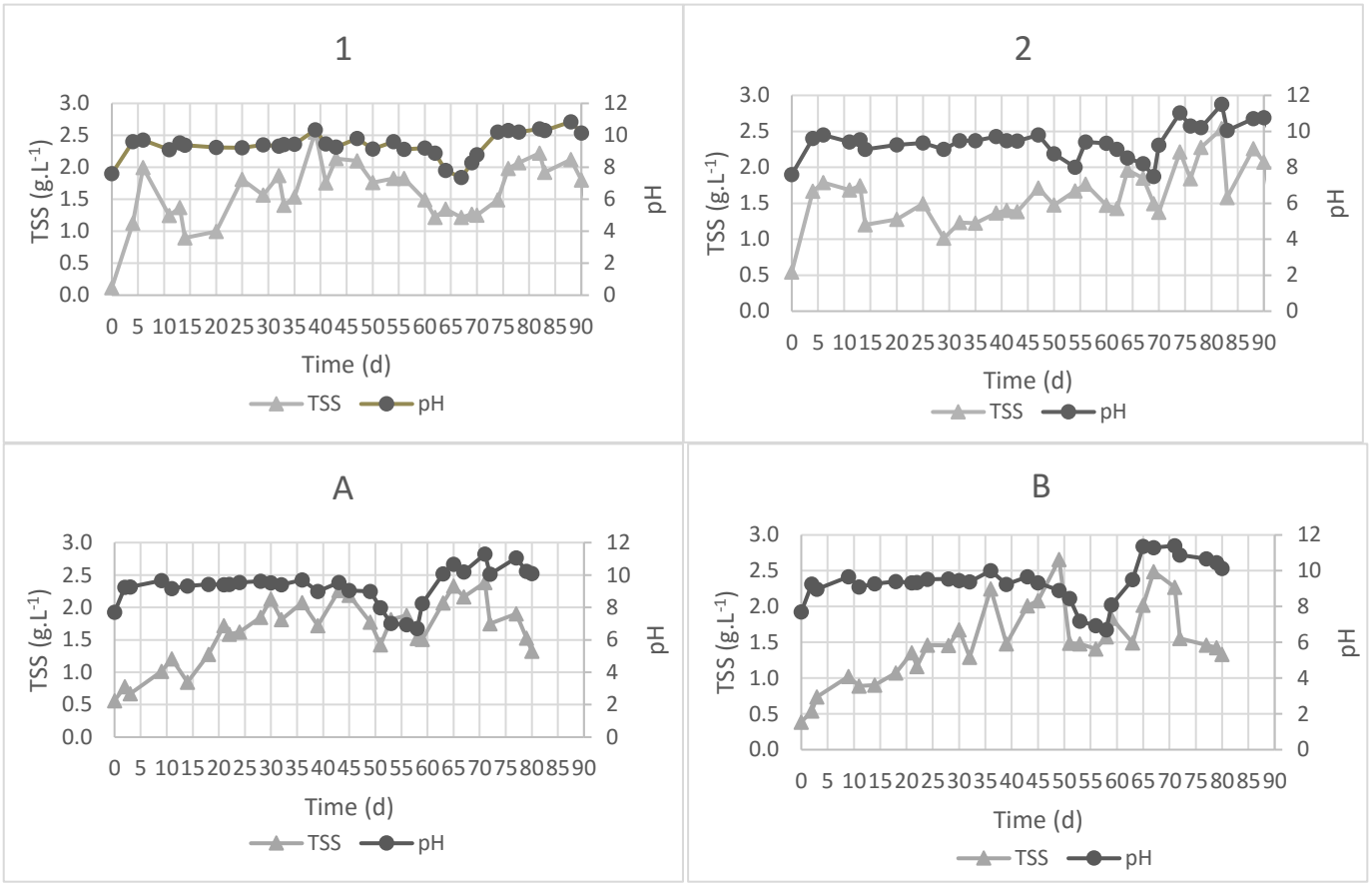

Figure 4.7 Representation of the variation of total suspended solids, expressed in $g.L^{-1}$, with pH

For all the vials, it is possible to observe a similar trend between pH and total suspended solids since generally, when TSS increased, pH also increased. Hence, the highest pH values corresponded to the highest total suspended solids concentration.

Likewise, when TSS decreased pH slightly decreased. However, it is important to refer that for all the four vials there were days in which the pH drastically decreased. For example, in the case of vial 1, pH abruptly decreased between the 62nd and 70th days, reaching a value of 7.36 and in the case of vial 2, it occurred between the 50th and the 54th days as well as between the 64th and the 69th, reaching a value of 7.49. Regarding vial A and B, pH drastically decreased between the 49th and the 59th days, reaching a value of 6.70 and 6.71, respectively.

4.1.6 Possible inhibitory effects

Among the different inorganic nitrogen compounds, it has been reported that unionized ammonia and nitrites are the most toxic forms for microalgae and may have inhibitory effects. Therefore, in Figure 4.8 and Figure 4.9, it is represented the variation of unionized ammonia and nitrite concentration with the variation of total suspended solids, respectively.

Concentration of unionized ammonia was calculated as a function of pH, temperature and total ammonia concentration by equation 4.2 (Tosta & Mendonça 2009):

$$\frac{Free\ NH_3}{Total\ NH_3} = \left(\frac{1}{1 + 10^{\left[0{,}09018 + \left(\frac{2729{,}92}{T}\right) - pH\right]}}\right) \qquad (4.2)$$

where free NH_3 and total NH_3 are expressed in $mg.L^{-1}$ and T is the temperature expressed in Kelvin.

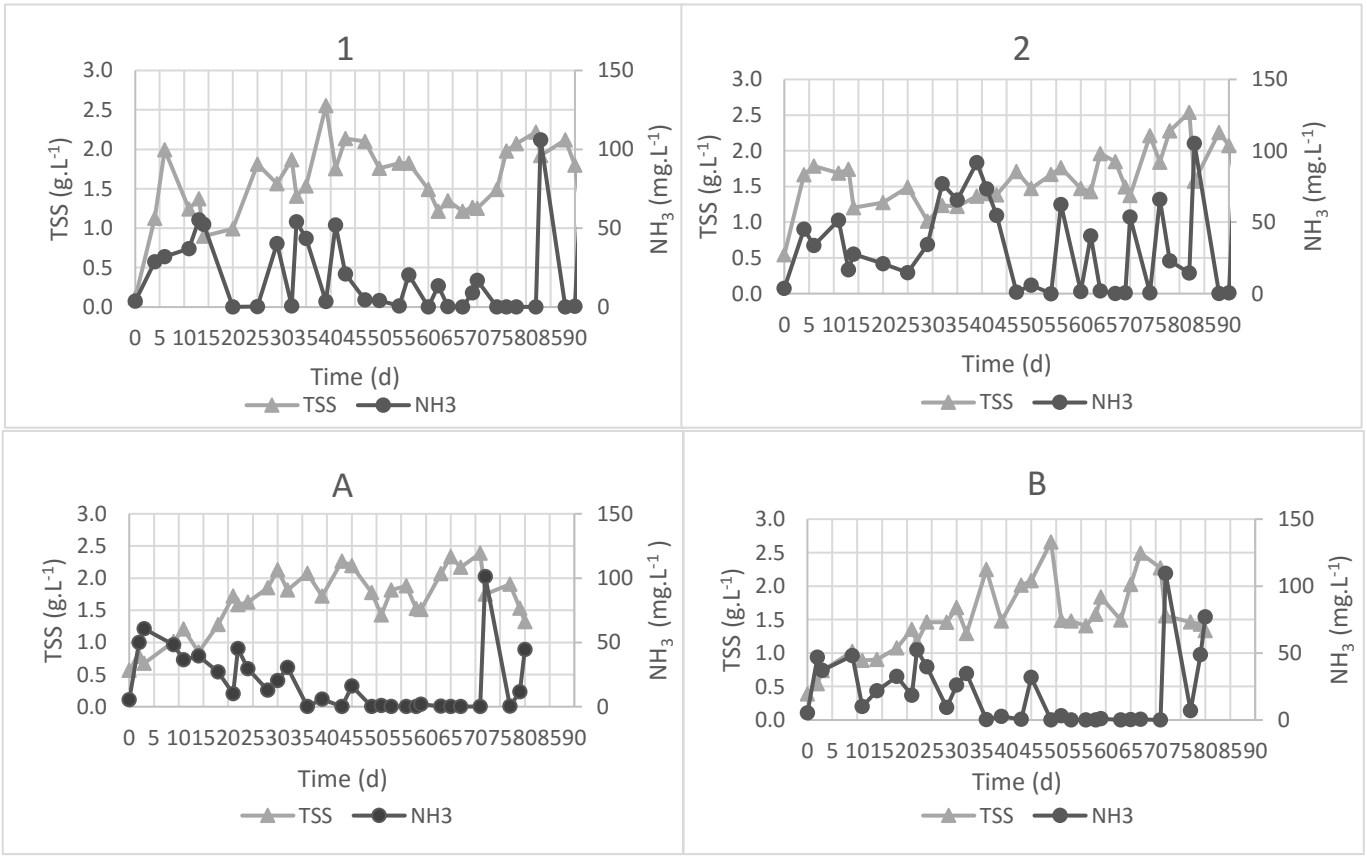

Figure 4.8 Variation of TSS, in $g.L^{-1}$, and unionized ammonia concentration, in $mg.L^{-1}$, during the experiments

As described in Figure 4.8, unionized ammonia concentration was variable during the experiments and for all the four vials it is possible to observe that the increase of unionized ammonia concentration corresponded to a decrease of TSS, and vice-versa. In addition, the highest unionized ammonia

concentration was reached in the last days of the experience, being 126.1, 105.3, 101.2 and 118.9 mg.L^{-1} for vials 1, 2, A and B, respectively. Hence, in these days there was a drop in the TSS values in all the vials.

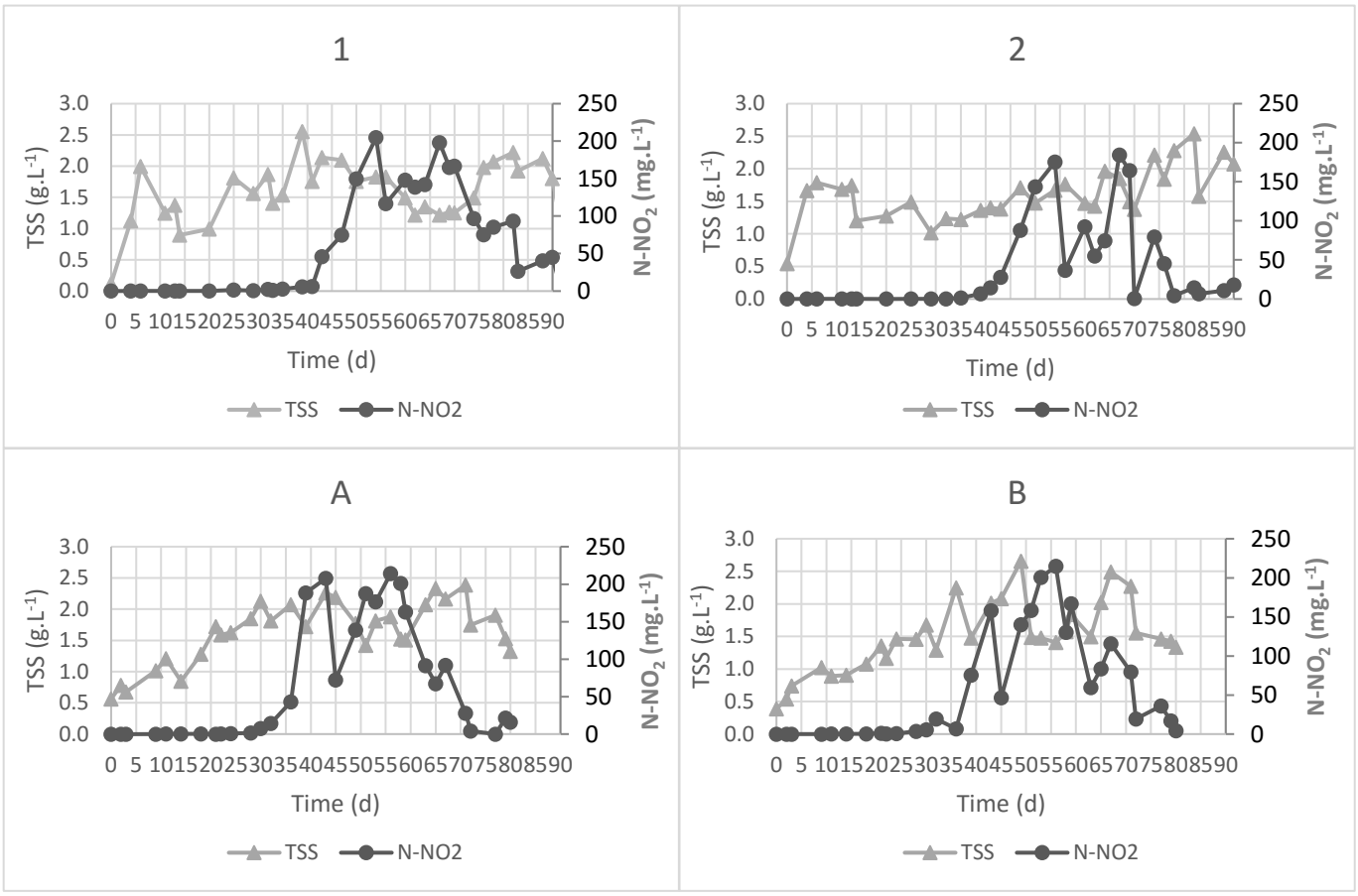

Figure 4.9 Variation of TSS, in g.L^{-1}, and nitrite concentration, in mg.L^{-1}, during the experiments

As described in Figure 4.9, nitrite concentration was nearly zero for all the four vials in the first days. However, after some days, there was formation of nitrite, reaching concentrations of 204.8, 184.1, 200.9, and 214.7 mg.L^{-1} in vials 1, 2, A and B, respectively. Furthermore, nitrite concentration progressively decreased in the last days.

4.2 Treatment efficiency

4.2.1 Nitrogen removal

Figure 4.10, Figure 4.11 and Figure 4.12 represent the concentration of N-$NH^{+}_{4,}$ N-NO^{-}_{2} and N-NO_3 in the effluent, meaning the concentration before the addition of wastewater, expressed in mg.L^{-1} during the whole experiment for the four vials, respectively.

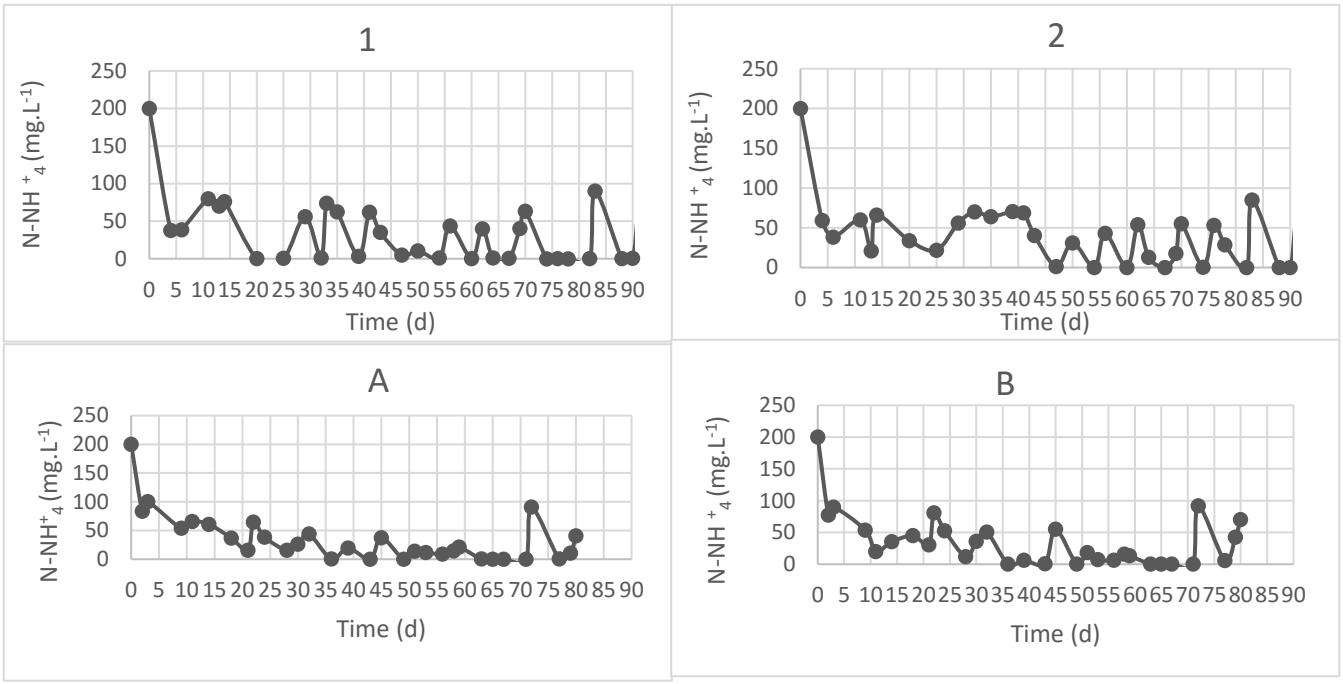

Figure 4.10 Time course of $N\text{-}NH_4^+$ concentration in the effluent, expressed in $mg.L^{-1}$

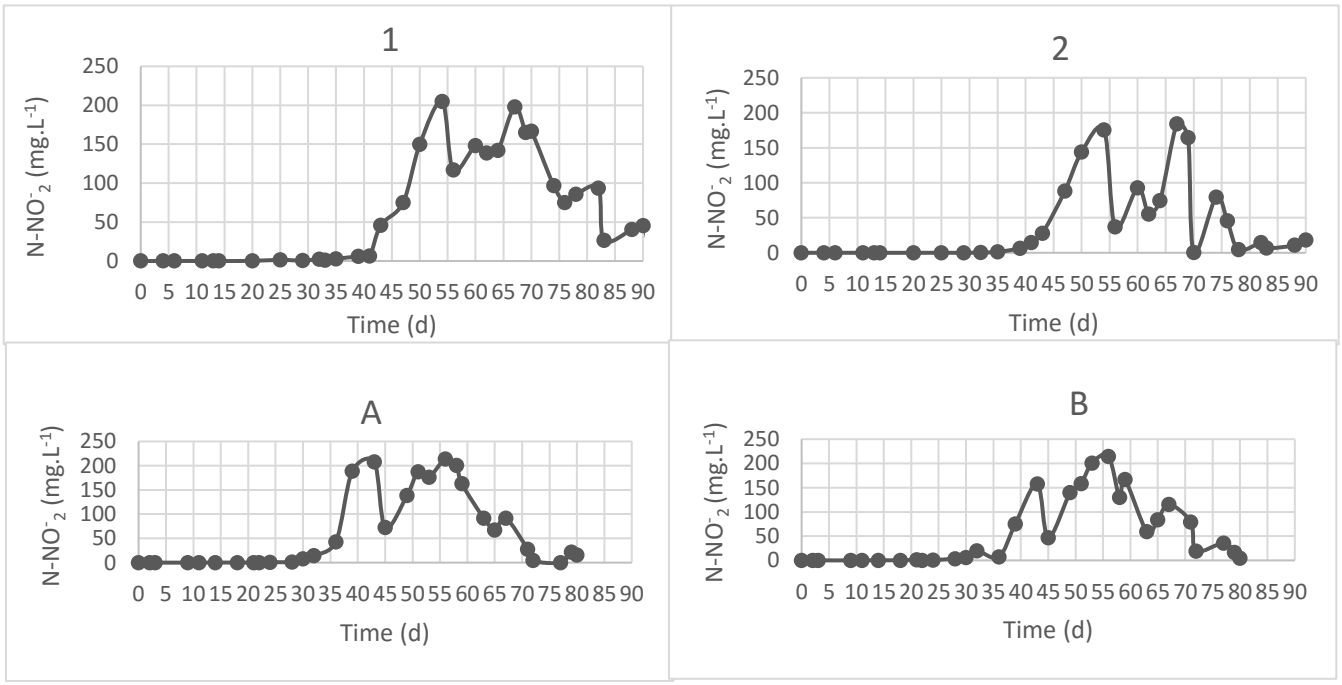

Figure 4.11 Time course of $N\text{-}NO^-_2$ concentration in the effluent, expressed in $mg.L^{-1}$

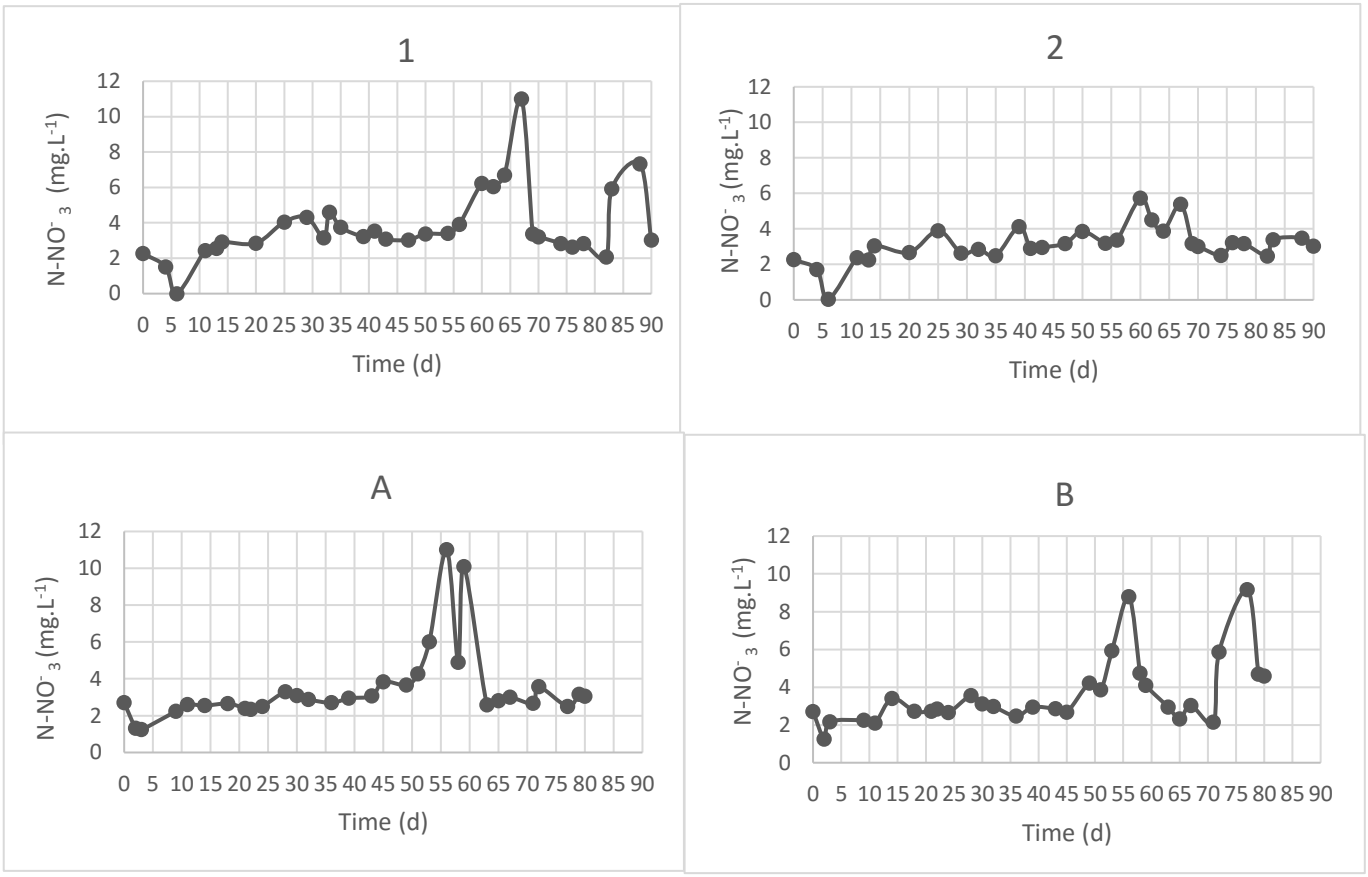

Figure 4.12 Time course of N-NO^-_3 concentration in the effluent, expressed in $mg.L^{-1}$

The average N-NH^+_4 removal by the microalgae-bacterial based system was calculated taking into account the average starting concentrations of the ammonium nitrogen and the average concentration of ammonium nitrogen in the effluent. Table 4.2 represents the average removal efficiencies of N-NH^+_4.

Table 4.2 Removal efficiencies of N-NH_4^+ from piggery wastewater by microalgae-bacterial based system

	Removal (%)			
Vials	**1**	**2**	**A**	**B**
N-NH^+_4	88	85	92	90

Regarding the removal of ammonium nitrogen, different mechanisms may be involved such as biomass uptake, ammonia stripping and nitrification. Therefore, the determination of the average contributions of the different mechanisms involved a standardized method which consisted in using cumulated values as an approximation to a continuous system.

Firstly, for this calculation it was necessary to calculate the total nitrogen in the effluent (N_{out}) as well as the total nitrogen in the influent (N_{in}), basing on equation 4.3 and equation 4.4, respectively.

$$\left(m_{N_{out,cum}}\right)_{ti} = \left(m_{N_{sol},cum}\right)_{ti} + \left(m_{N_{biomass},cum}\right)_{ti} + \left(m_{N_{out,cum}}\right)_{ti-1} \quad (4.3)$$

where $\left(m_{N_{out,cum}}\right)_{ti}$ corresponds to the cumulate total nitrogen in the effluent in time t_i, expressed in mg; $\left(m_{N_{sol},cum}\right)_{ti}$is the total cumulate dissolved nitrogen in time t_i, which corresponds to the amount of $N\text{-}NH^{+}_{4}$ remained in effluent plus the amount of $N\text{-}NO^{-}_{2}$ and $N\text{-}NO^{-}_{3}$, expressed in mg; $\left(m_{N_{biomass},cum}\right)_{ti}$ is the cumulate value of nitrogen incorporated in microalgae biomass in time t_i, expressed in mg, considering that nitrogen concentration in algae cells is generally estimated as 10% of the microalgae dry weight, which has been approximately considered as TSS. Finally, $\left(m_{N_{out,cum}}\right)_{ti-1}$ is the cumulate value of total nitrogen in the time t_{i-1}.

$$\left(m_{N_{in,cum}}\right)_{ti} = V_{add,t} \times [N - NH_4]_{W1} + \left(m_{N_{in,cum}}\right)_{ti-1} \quad (4.4)$$

where $\left(m_{N_{in,cum}}\right)_{t}$is the cumulate value of total nitrogen in the influent in time t_i, expressed in mg; V_{add} is the wastewater volume added in time t_i to the vials, expressed in L; $[N - NH_4]_{W1,t}$ is the $N\text{-}NH_4^{+}$ concentration of the piggery wastewater. It is important to refer that in the previous equation as an approximation it was only considered the nitrogen in the form of ammonium, since the concentrations of $N\text{-}NO^{-}_{2}$ and $N\text{-}NO^{-}_{3}$ in the wastewater were negligible.

Moreover, the determination of the nitrogen apportioning existing as $N\text{-}NH^{+}_{4}$, existing as N-NOx, existing as N in biomass and stripped as NH_3 was based on equations 4.5, 4.6, 4.7 and 4.8, respectively.

$$\%N - NH_4 = \frac{\left(m_{N-NH_4}\right)_{out}}{(m_N)_{in}} \quad (4.5)$$

$$\%N - NO_X = \frac{\left(m_{N-NO_X}\right)_{out}}{(m_N)_{in}} \quad (4.6)$$

$$\%N_{biomass} = \frac{\left(m_{N_{biomass}}\right)_{out}}{(m_N)_{in}} \quad (4.7)$$

$$\%N_{stripped} = 1 - \frac{(m_N)_{out}}{(m_N)_{in}} \quad (4.8)$$

Table 4.3 summarizes the obtained values from the previous equations for all the four vials, allowing to get a nitrogen mass balance.

Table 4.3 Nitrogen apportioning, expressed in %, for each vial

	Nitrogen apportioning for the different vials			
	1	**2**	**A**	**B**
$N\text{-}NH^{+}_{4}$	5	5	3	4
$N\text{-}NO_x$	25	19	29	26
N biomass	44	45	47	45
N stripped	26	31	21	25

4.2.2 Phosphorus removal

Figure 4.13 represents the concentration of $P\text{-}PO_4$ in the effluent, expressed in $mg.L^{-1}$, during time for the four vials.

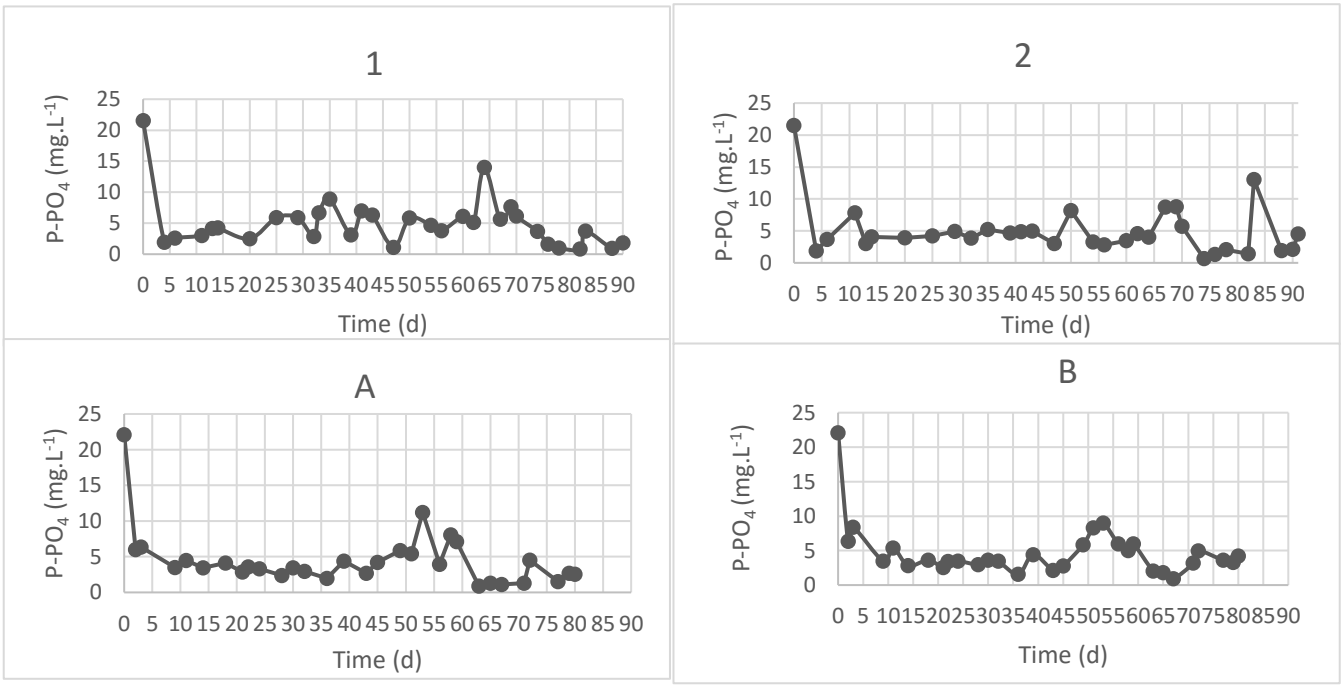

Figure 4.13 Time course of $P\text{-}PO_4$ concentration in the effluent, expressed in $mg.L^{-1}$

The average $P\text{-}PO_4$ percentage removal by the microalgae-bacterial based system was determined taking into account the starting concentration of $P\text{-}PO_4$ as well as the concentration of $P\text{-}PO_4$ added to ensure the adequate N/P ratio and the average concentration of $P\text{-}PO_4$ in the effluent. Table 4.4 represents the $P\text{-}PO_4$ average percentage removal for each vial.

Table 4.4 Removal efficiencies of P-PO_4 from piggery wastewater by microalgae-bacterial based system

	Removal (%)			
Vials	**1**	**2**	**A**	**B**
P-PO4	78	79	82	81

4.2.3 COD removal

Figure 4.14, represents the COD in the effluent, expressed in $mgO_2.L^{-1}$, for the four vials.

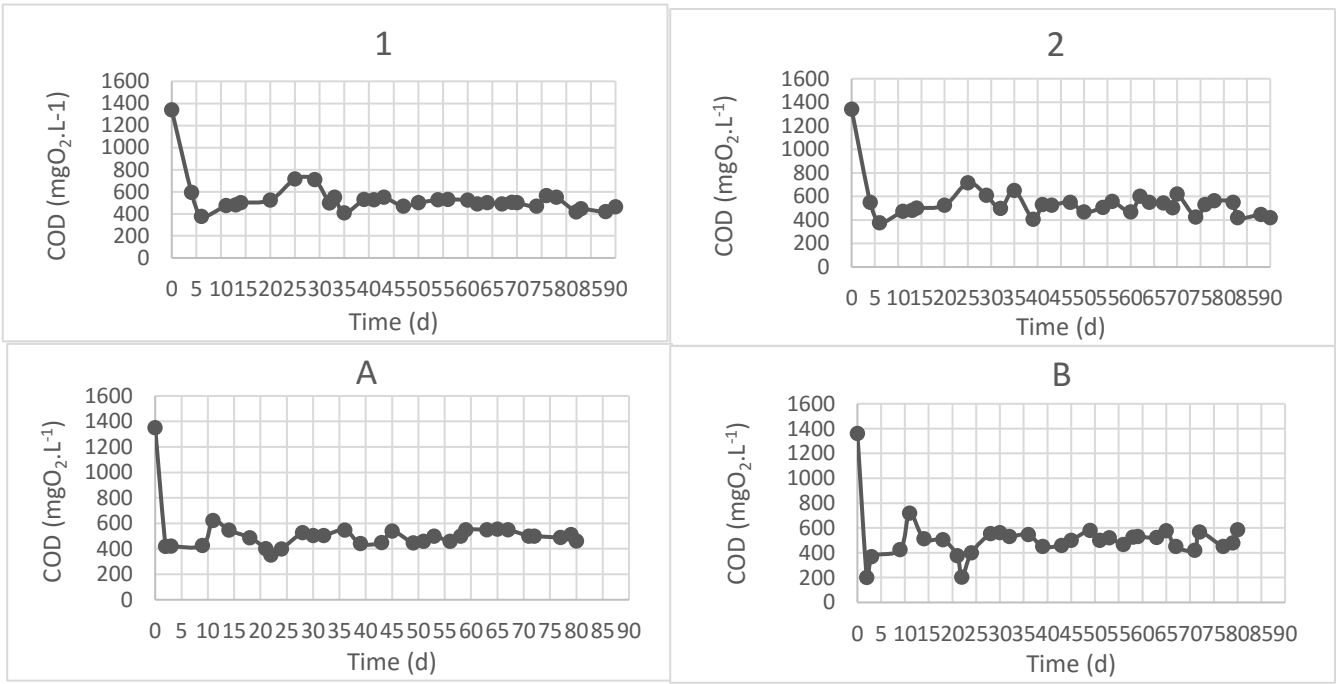

Figure 4.14 Time course of COD concentration in the effluent, expressed in $mgO_2.L^{-1}$

The average COD percentage removal by the microalgae-bacterial based system was determined by using the starting COD values and the average COD values in the effluent. The obtained removal efficiencies are represented in Table 4.5.

Table 4.5 Removal efficiencies of COD from piggery wastewater by microalgae-bacterial based system

	Removal (%)			
Vials	**1**	**2**	**A**	**B**
COD	64	62	63	63

4.3 Respirometry

In order to assess the presence of other microorganisms in microalgae culture, such as ammonium oxidizing bacteria, nitrite oxidizing bacteria and heterotrophic organisms, respirometric analyses were performed by using specific inhibitors and measuring the dissolved oxygen during time. Figure 4.15 represents the concentration of dissolved oxygen during time as well as the oxygen uptake rate.

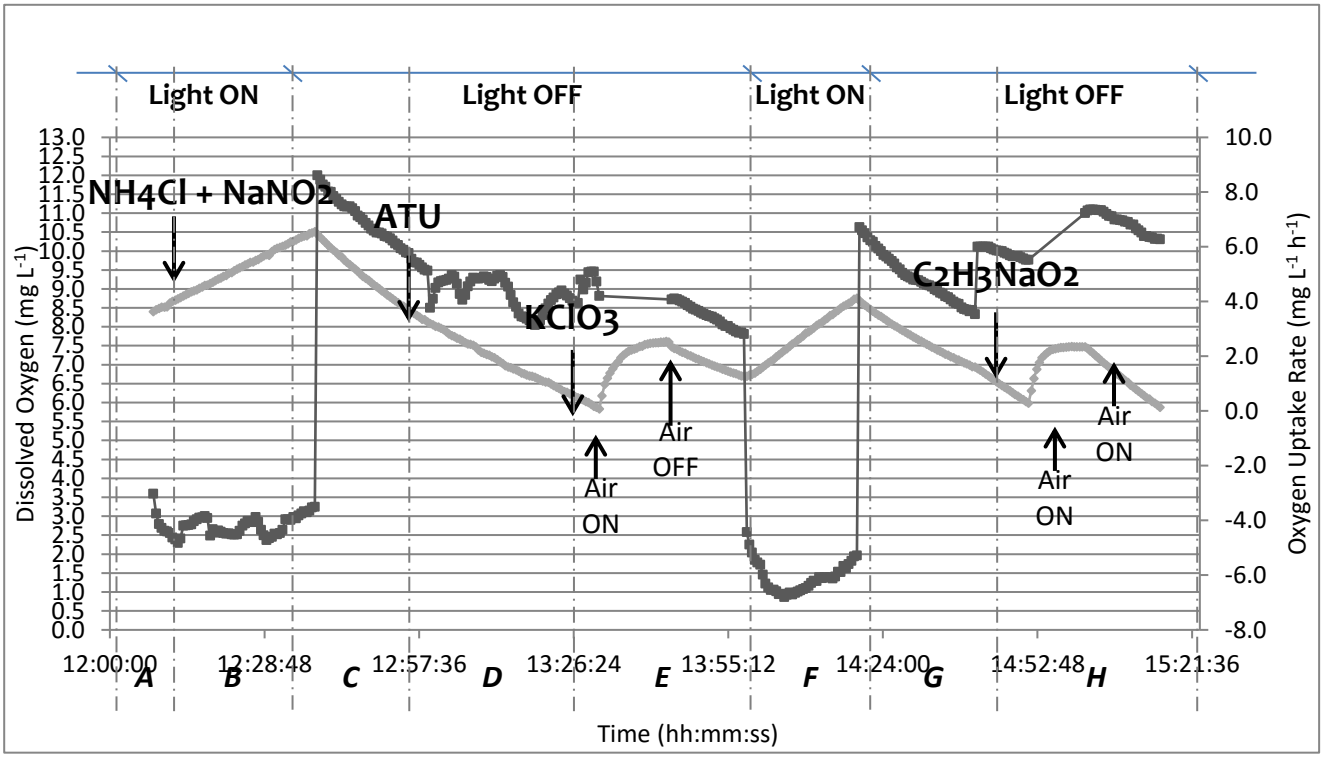

Figure 4.15 Representation of dissolved oxygen concentration, expressed in $mg.L^{-1}$, during time as well as oxygen uptake rate ($mg.L^{-1}.h^{-1}$); Dissolved Oxygen (—●—) Oxygen Uptake Rate (—■—)

As described in Figure 4.15, it is possible to observe 8 different phases during the respirometric analyses and all the phases were linear with R^2 values higher than 0.9. Regarding phase A, it corresponded to a period of light on, in which microalgae culture was adapting to the new conditions without chemical compound addition, whereas phase B corresponded to the period after addition of ammonium chloride and sodium nitrite, which were used as substrates for microalgae. Then, phase C corresponded to the first 20 minutes of a light off period and phase D corresponded to the period after the addition of allylthiourea, which was an inhibitor of ammonium oxidizing bacteria. With regard to phase E, it corresponded to the period after the addition of potassium chlorate, which was an inhibitor of nitrite oxidizing bacteria and during this phase, it should be highlighted that it was necessary to provide aeration to the system since the oxygen level was too low. Phase F corresponded to a new period of light on and phase G to the first 20 minutes of a new cycle of light off. Finally, phase H corresponded to the period after addition of sodium acetate, which functioned as substrate for the heterotroph organisms.

Furthermore, the representation of the dissolved oxygen concentration had a very high linearity (Table 4.6) and the OUR values were obtained by calculating the slope of the concentration variation of dissolved oxygen during time and the average OUR, expressed in $mg.L^{-1}.h^{-1}$, for each phase as well as the standard deviation are represented in Table 4.6. It should be emphasized that the values represented in the table below correspond to the opposite signal of the calculated slope in order to have positive OUR values when there was consumption of oxygen.

Table 4.6 Representation of R square and the average oxygen uptake rate, expressed in $mg.L^{-1}.h^{-1}$, for the different phases as well as the standard deviation

	R^2	Average OUR ($mg.L^{-1}.h^{-1}$)	Standard deviation
A	0.994	-4.17	0.402
B	0.999	-4.33	0.342
C	0.994	6.62	0.985
D	0.996	4.28	0.556
E	0.997	3.68	0.673
F	0.999	-6.11	0.556
G	0.994	4.78	0.902
H	0.999	6.37	0.626

4.4 BMP

Figure 4.16 represents the cumulative biomethane produced during time.

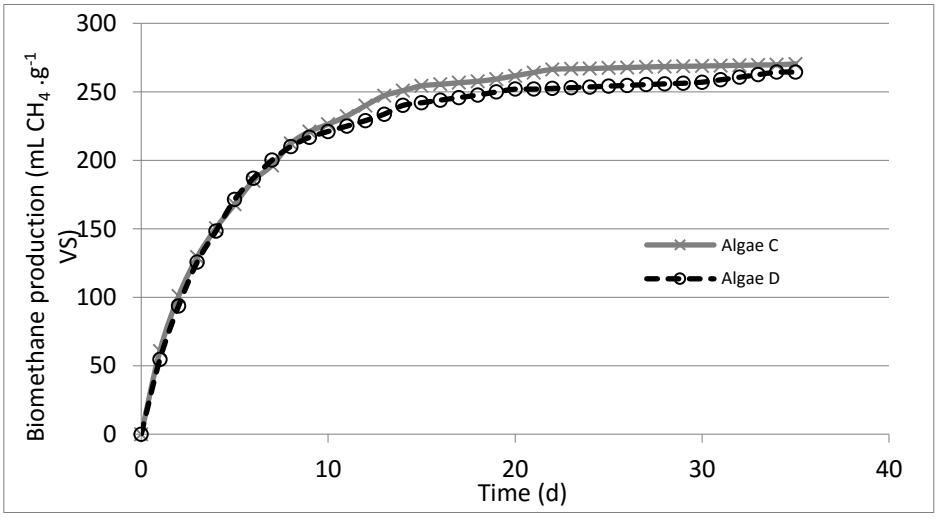

Figure 4.16 Cumulative biomethane production curves in $mLCH_4.g^{-1}$ VS, over time.

The slope of the tangent line to the cumulative curves at any point shows the biomethane production rate at that specific time. As observed in figure 4.16, methane production presents an inverse exponential

curve that is characterized by three main zones. During the initial phase there was a rapid biomethane production, which slows down during the intermediate phase and tend to zero at the end of the final phase.

Furthermore, both algae cultures had a similar curve and the maximum methane production was 270.4 and 264.3 $mLCH_4.g^{-1}$ VS for algae C and D, respectively.

5 Discussion

During the present study, microalgae growth on piggery wastewater was monitored by measuring optical density at 680 nm, turbidity, total suspended solids concentration and by counting microalgae on an optical microscope. In general for all the vials, turbidity, TSS and algal counts were found to be consistent with OD measurements. As expected, the highest correlation found was between OD and turbidity measurements.

Concerning the relationship between OD measurements and microalgae counting, during the first days of experiments a very low correlation was observed between these two parameters (Figure 4.2), which may be due to the fact that during these days the counting was performed only on 9 little squares randomly chosen, which were not representative of the culture suspension. Therefore, in the following days the counting was performed on 72 little squares and, as expected, the correlation between OD measurements and microalgae counting results increased drastically. However, there was still a small variability between these two parameters, which can have many explanations but it was most probably attributable to human error, to the small sample size analyzed and to the occasional formation of microalgae agglomerates.

Furthermore, with regard to OD measurements and TSS concentration (Figure 4.4), the correlation was lower than expected, which may be explained by homogeneity variability of the cultures, different microalgae size and weigh distribution, different particle shapes distribution and occasionally formation of microalgae aggregates (Geographer & Irvine 2002).

Total suspended solids were chosen to show the trend of biomass concentration in the vials since it has been one of the main important parameters used in literature reports to describe microalgae biomass variation during experiments, as well as since it is one of the most direct ways to estimate biomass productivities. Although the four vials were replicas of each other, their behavior along the experiment was different (Figure 4.5). In the case of vial 1 and 2, there was a fast biomass growth in the first days and then it tended to stabilize around the same values. This is likely to depend on the penetration of light which was limited by the high algae density and, thus, limited any further density increase. Regarding vial A and B, it was possible to observe a slower increase in the first days, which may be due to the fact that they were prepared 11 days later than sample 1 and 2 and also, to the starvation period (6 days) that they were subjected after the 3rd day of cultivation. Moreover, there were some days in which TSS drastically decreased, in particular for vial 1, A and B, and this may be caused by different reasons which are further discussed. However, for all the vials, microalgae have shown the capacity to survive to these possible reasons and to recover the value of equilibrium, which may be an essential aspect in wastewater treatment, since the input conditions can easily vary and therefore, microalgae should be capable to adapt and to recover from adverse conditions. The average biomass productivity was ranged between 0.263±0.128 and 0.400±0.139 $g.d^{-1}.L^{-1}$, which was similar than that in the research by Zhu et al. (2013), who achieved 0.296 $g.d^{-1}.L^{-1}$ biomass growing *Chlorella* sp. in a tubular photobioreactor using piggery wastewater. Moreover, the biomass productivity obtained in the present study was also similar to the productivity reported by (Xu et al., 2015) , who achieved 0.311 $g.d^{-1}.L^{-1}$ growing *Scenedemus* spp. on a piggery digestate.

During the experiments, the cultured species were *Chlorella spp.* and *Scenedesmus spp*. which are within the top 10 more resistant microalgae based on their capability to grow in organic polluted environments (Godos et al., 2010). As presented in Figure 4.6, in the first days the presence of *Chlorella spp*. and *Scenedesmus spp.* was relatively similar, however, after a few days *Chlorella spp*. adapted better and was often the most dominant specie due to its resistance, higher growth rate and its preference on environments with high organic matter (Costa et al., 2006), whereas *Scenedesmus spp.* prefers environments with medium organic loads (Godos et al., 2010). Likewise, a recent study has reported that a strain of *Chlorella* has shown better performance in terms of biomass productivity and growth rate in a batch system when compared to a strain of *Scenedesmus* (Gao et al., 2016).

As described in Figure 4.7, there was a similar trend between TSS and pH, which was expected since when algae density was higher, there was more photosynthetic activity and, consequently, more consumption of CO_2 and HCO^-_3,, leading to an increase in pH (Zang et al., 2011). According to (Park et al., 2011), algae photosynthesis often leads to pH values higher than 11 and this is in agreement with the highest pH values achieved in these cultures, 10.85, 11.5, 11.3 and 11.89 for vials 1, 2, A and B, respectively.

Likewise, when algae density was lower, there was lower photosynthetic activity, which lead to a decrease in pH. It is important to emphasize that, generally, pH did not have drastic variations, which indicates that the substrate was well buffered and that the bubble aeration was enough to replace the CO_2 depleted during photosynthesis. However, for all the four vials there were a few days where pH drastically decreased and this might be explained by high accumulation of nitrites during these days (Figure 4.11), since the oxidation of ammonia into nitrite by the bacterial community leads to the release of H^+ ions.

The equilibrium ammonium/ammonia is dependent on pH and temperature of the culture medium. Hence, as pH increases, there is a shift on the equilibrium towards the formation of unionized ammonia, which can significantly inhibit algal growth, since it is uncharged and lipid soluble and easily diffuses across membranes, damaging the photosynthetic system (Collos & Harrison 2014). As observed in figure 4.8, there were some days in which the increase of unionized ammonia corresponded to a decrease on TSS and this may be associated with its potential inhibitory effect. Furthermore, special attention should be taken in the last days of the experiment in which there was decrease on TSS that may have been caused by the drastically increase of unionized ammonia concentration, reaching much higher values than reported inhibitory concentrations of 36 $mg.L^{-1}$ and 51 $mg.L^{-1}$ (Park et al. 2011).

Although it has been reported that high concentrations of nitrite may inhibit microalgae growth (Markou et al., 2014), in these experiment it seems that there was a poor relation between nitrite and TSS (Figure 4.9). However, regarding vial 1 after the 56^{th} day, there was a drop in TSS, which corresponded to the period of high nitrite concentration in the medium. Additionally, TSS started to increase again after the 70^{th} day, which also corresponded to the day when nitrite concentration started to decrease. In addition, regarding vial A and B, there was a drop of TSS after the 45^{th} and the 49^{th} days, respectively, which also corresponded to the period when nitrite concentration was high. Therefore, one possible explanation of this drastic drop may be the toxic effect of high nitrite concentrations.

Bioremediation of piggery wastewater using a mixed culture of *Chlorella spp*. and *Scenedesmus spp.* was studied by assessing the removal of nutrients such as ammonium nitrogen and phosphorus, as well as the removal of COD. The average ammonium nitrogen removal was ranged between 85-92% for all the four vials (Table 4.2) and concerning this nutrient, there were three main removal mechanisms (biomass uptake, ammonia stripping and nitrification) considered.

Nitrogen biomass uptake was determined by considering the total biomass produced as well as the total nitrogen contained in the biomass. For all the vials, nitrogen uptake was the main mechanism reaching values of 44, 45, 47 and 45% for vials 1, 2, A and B, respectively, which are higher than 21% nitrogen uptake obtained from previous studies on piggery wastewater using a mixed culture with a strain of *Chlorella*, *Scenenedesmus* and a strain of *Chlamydomonas* (Molinuevo-Salces et al., 2015). However, a study on urban wastewater with the same microalgae species reported a nitrogen uptake ranged between 85 and 96 % (González-Fernández et al. 2016). Furthermore, it should be remembered that ideally the use of microalgae for bioremediation should not only be focused on the removal of nutrients from wastewater but also on their recovery when using algae biomass as nutrient source, for instance, for agriculture, or on the use of algae biomass for biofuel production.

Regarding ammonia stripping, it was observed a significant contribution for ammonia removal, which was expected, since this mechanism is promoted at alkaline pH levels, higher than 9 (Chu et al. 2015). The obtained values were 26, 31, 21 and 25% for vials 1, 2, A and B, respectively. This means that in full scale applications, pH should be controlled by means of CO_2 addition to avoid such phenomenon.

Lastly, nitrification is the biological process of NH^+_4 oxidation into NO^-_2 and then NO^-_2 oxidation into NO^-_3. As observed in Figure 4.11, nitrification did not occur in the first days of experiment, since nitrite concentration was nearly zero. However, after some days of experiment, nitrite concentration started to increase, which has been reported as a bacterial contamination of the cultivation (Molinuevo-Salces et al., 2015). The obtained values for nitrification were 25, 19, 29 and 26% for vials 1, 2, A and B, respectively. As reported by (Risgaard-Petersen et al. 2004), a mixed consortium of microalgae, AOB and NOB can compete for CO_2 and for N itself. One point which has not yet been fully understood is the reason why nitrite was not oxidized to nitrate. However, it is also possible that part of the produced nitrite was actually oxidized to nitrate and that it was fastly uptaken by algae so that its concentration was usually constant. Moreover, the causes and effects of NO^-_2 accumulation should still be studied: in principle algae should be able to use N-NO^-_2, but as previously mentioned, high concentrations could be inhibitory.

In the case of phosphorus, phosphate can be removed by biomass uptake, since microalgae use it for metabolic activities, or by precipitation due to the increase in pH during photosynthesis (Dahmani et al., 2016). Regarding the removal of phosphates by precipitation, it has been reported that it occurs at pH values between 9 and 11 (Godos et al. 2009). Generally, the pH of each vial was higher than 9, except for a few days as already discussed, therefore, the removal of phosphorus may have been due to both mechanisms. If precipitation took place, the availability of phosphorus for algae was strongly reduced and this would mean that algae grew and reached high densities with N/P ratios in the substrate well far from the reported optimum values. The average phosphorus removal efficiency ranged between 78-82% for the

four vials. The effective phosphorus removal efficiency has been reported in other studies. For example, as previous mentioned, Molinuevo-Salces et al. (2015) cultivated *Chlorella*, *Scenenedesmus* and *Chlamydomonas* on piggery wastewater and obtained a removal efficiency of 82%, which is similar to the ones obtained in these experiments. Moreover, another study using a cultivation of *Chlorella* spp. with anaerobic digested dairy manure reported 76-83% phosphorus removal (Wang et al. 2010).

Chemical oxygen demand represents roughly the overall organic load (dissolved and suspended matter) of the wastewater. The average removal efficiencies in this study ranged between 62 and 64 %, which are closer to the minimum 66% and lower than the maximum 80% obtained from other studies on piggery wastewater (Zhu et al., 2013). The obtained removal efficiencies were partly due to the synergetic relationship between microalgae and bacteria, allowing bacteria to use the oxygen released by microalgae to decompose the organic matter, and partly to the mixotrophic metabolism of algae, which is especially well known for *Chlorella* spp..

Moreover, in order to prove the presence of other microorganisms in the microalgae culture, respirometric analyses were performed in a mixed culture of all the vials, by using inhibitor compounds and by measuring the concentration of dissolved oxygen during time. First of all, it should be considered that the studies already reported in the literature about the inhibitory effects of the compounds used during respirometry were for single cultures. However, during the current analyses the inhibitory effect by adding the chemical compounds to a mixed culture were also observed. As described in Figure 4.15, there were 8 phases during respirometric analyses and as expected, it was possible to observe that during phases A, B and F, which corresponded to the periods of light on, microalgae were able to produce oxygen due to photosynthesis reactions and thus, there was an increase in the concentration of dissolved oxygen in the system. With regard to phases A and B, if the test had been performed on a pure algae culture, it should be expected a higher increase in the slope after the addition of substrate. However, in the studied system there were also bacteria, which also consumed the substrate and consumed oxygen, hence, there was more and less an equilibrium. The global oxygen production was 4.33±0.342 $mg.L^{-1}h^{-1}$. Regarding phase F, the obtained oxygen production rate was 6.11±0.556 $mg.L^{-1}h^{-1}$, which allows to conclude that microalgae were not affected by the inhibitory compounds and, since ammonium oxidizers and nitrite oxidizers were inhibited, there was less competition with microalgae for the substrate, thus, a higher oxygen production than phase B was attained.

On the other hand, during phases C, D, E, G and H, which corresponded to the periods of light off, there was consumption of oxygen in the system and a decrease in the concentration of dissolved oxygen was observed, as expected, with OUR values equal to 6.62 ±0.985,4.28±0.556,3.68±0.673, 4.78±0.902 and 6.37±0.626 $mg.L^{-1}h^{-1}$, respectively. With regard to phases C and D, it was possible to observe that after the addition of allylthiourea there was a decrease in the oxygen consumption, which was expected since allylthiourea had an inhibitory effect on ammonium oxidizing bacteria and therefore, there were less microorganisms consuming oxygen. Furthermore, the average OUR obtained during phase E was lower than the previous phases, as predicted since there was an addition of potassium chlorate that inhibited nitrite oxidizers and therefore, the oxygen consumption of the system was lower. Regarding phases G and

H, as expected, there was an increase in the OUR after the addition of sodium acetate since it activated heterotrophic organisms by working as a substrate and therefore, there was more consumption of oxygen.

Finally, another target of this study was to evaluate the suitability of the mixed microalgae culture used for the piggery wastewater treatment as a substrate for biogas production by anaerobic digestion. Literature (Yen & Brune, 2007) reports that the methane production by a mixed culture of *Chlorella vulgaris* and *Scenedesmus sp*. is characterized by an inverse exponential curve, which is in agreement with the results obtained (Figure 4.16) in the present study, where it was possible to observe a rapid methane production increase during the first days, an intermediate phase in which biomethane production rate decreased and a plateau phase after the 10th day of degradability. This inverse exponential curve can be explained by the fact that the BMP tests were performed in batch conditions and, as time went by, the amount of substrate decreased as well as the biomethane production rate.

In general, BMP values for microalgae found in the literature are diverse and usually ranged between 153 and 600 $mLCH_4.g^{-1}$ VS (Ward et al. 2014). Hence, it should be pointed out that methane production is strongly dependent on the species and it has been observed (Mussgnug et al. 2010) that microalgae without cell wall as *Dunaliella salina* or with protein-based membranes, such as *Chlamydomonas reinhardtii*, *Arthrospira platensis* and *Euglena gracilis*, present a higher BMP values, between 481 and 587 $mLCH_4.g^{-1}$VS, than microalgae species with a cell wall containing cellulose or hemicellulose as *Scenedesmus obliquus* and *Chlorella kessleri*, which present BMP values comprised between 287-335 $mLCH_4.g^{-1}$ VS. Therefore, it is possible to conclude that the cell wall degradability is strongly correlated with the quantity of methane produced and sometimes pre-treatments should be considered to degrade the cell wall prior to digestion. With regard to this experiment, microalgae were found to have an interesting methane production potential and similar biomethane values were obtained for algae C and D, showing the good repeatability of the test. The obtained data between 264.3 $mLCH_4.g^{-1}$ VS and 270.4 $mLCH_4.g^{-1}$ VS was comprised in the usual range reported in the literature. Also, it was similar to the previous mentioned values for *Scenedesmus obliquus* and *Chlorella kessleri*. Furthermore, the methane conversion during a study with a mixed culture of *Chlorella spp.* and *Scenedesmus spp*. reached 143 $mLCH_4.g^{-1}$ VS (Yen & Brune, 2007), which is lower than the results of the present study. In addition, the anaerobic digestion of piggery manure from Corte Grand plant reached a BMP value of 150 $mLCH_4.\ g^{-1}$ VS (Marazzi et al. 2015), which is also much lower than the BMP values obtained during this study. Therefore, the obtained values confirm that microalgae biomass cultivated in nutrient-rich streams, such as piggery wastewater, is a feasible option for biogas production.

6 Conclusions

Agricultural wastewater is one of the most relevant contributors to environmental pollution due to its high concentration of nutrients, such as nitrogen and phosphorus, as well as its high organic load. For years, the traditional method to treat piggery wastewater was using it as land fertilizer, but the nitrogen in piggery wastewater cannot be completely uptaken by crops due to the unbalanced N/P ratio and consequently, the accumulation of nutrients in the soil can contaminate the receiving waters. Therefore, it is important to investigate and to find the most efficient and environmentally friendly treatment method for piggery wastewater.

The present study was devoted to evaluate the capacity of a mixed culture of *Chlorella spp*. and *Scenedesmus spp*. to grow on a piggery wastewater (pre-treated with a flotation process) as well as their bioremediation capacity to remove nutrients, such as nitrogen and phosphorus, and the culture suitability for energy production.

The results from this study proved that these green microalgae were able to efficiently grow on the high organic load medium, which is in agreement with previous studies that reported that *Scenedesmus spp.* and *Chlorella spp.* are within the top 10 more resistance species. Moreover, these species also seemed to adapt and recover from some drastic biomass decreases, which is an important aspect to take into account in a WWTP since the input conditions can easily vary.

Furthermore, the microalgae based system proved its ability to remove phosphorus, ammonium nitrogen as well as COD and the obtained removal efficiencies were in agreement with the literature data. Regarding the removal of $N\text{-}NH_4^+$, the average removal efficiencies were ranged between 85-92% and different mechanisms were responsible for the removal of nitrogen (nitrification, biomass uptake and ammonia stripping), being the biomass uptake the most important mechanism. In the case of phosphorus, the average removal efficiency was ranged between 78-82 % and the considered removal mechanisms were biomass uptake and phosphorus precipitation due to high pH levels. With regard to COD, the average removal efficiency was ranged between 62-64% and these results enhance the synergetic relation between microalgae and bacteria, since the removal of COD was partly attributed to the microorganisms that made use of the oxygen produced by microalgae during photosynthesis to decompose the organic matter present in the wastewater and also, partly to the mixotrophic metabolism of microalgae.

Besides the removal of COD, the presence of other microorganisms was also proved by respirometric analyses, which consisted in using inhibitors of ammonium oxidizers and nitrite oxidizers and measuring the dissolved oxygen during time. Moreover, the used inhibitors proved to have the desired effect, since after the addition of these compounds, it was observed a decrease in the oxygen consumption.

Finally, the other aim of this work was to investigate the feasibility of anaerobic digestion for biogas production from the mixed culture of *Chlorella spp*. and *Scenedesmus spp*. used in the piggery wastewater treatment. The rapid economic growth has led to high-energy consumption around the world and

consequently to the increase of environmental pollution. Biogas is an alternative energy source that can overcome these concerns and microalgae are one interesting substrate for biomethane production due to their high growth rate, high biomass yields and to their capacity to grow on wastewaters. The obtained biomethane production was ranged between 264.3 and 270.4 $mLCH_4.g^{-1}$ VS, which is in agreement with the literature data and is much higher than the BMP values obtained from the anaerobic digestion of piggery manure from Corte Grande plant. Hence, the obtained values confirmed that microalgae biomass cultivated in nutrients-rich streams, such as piggery wastewater, is a feasible option for biogas production.

Considering an appropriate process evaluation and design, the hypothesis of using algal-based treatment as the first step of biological treatment of piggery wastewater could be considered. In that case, algae biomass could be digested and the liquid phase of digestate could be recirculated upflow to be treated by algae/bacteria consortium along with the pre-treated piggery wastewater.

7 Future Perspectives

Based on the work done and the results obtained, the following recommendations can be suggested:

- Studying the effects of N/P ratio on the mixed culture to investigate if it affects microalgae growth and the nutrient removal efficiency;
- Investigating with detail mixotrophic microalgae since they are poorly studied and they can take advantage of both organic and inorganic substrates to obtain higher biomass production, which is very interesting in the integration of microalgae cultivation with wastewater treatment processes where the wastewater is usually turbid and the light cannot penetrate into its core body. Moreover, studying these mixotrophic conditions to improve the removal of COD;
- Improving the nitrogen biomass uptake mechanism, for example, by a tight control of the pH values, which will decrease the ammonia volatilization;
- Investigating the nitrification process in order to understand the reasons for its discontinuity and for the eventual accumulation of nitrites and its influence on algae growth. In fact, competition for CO_2 could take place and a certain lag is likely to be needed by algae population grown on ammonium nitrogen to shift towards the uptake of nitrate nitrogen.
- Investigating possible pre-treatments for microalgae before BMP tests.

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ANNEXES I

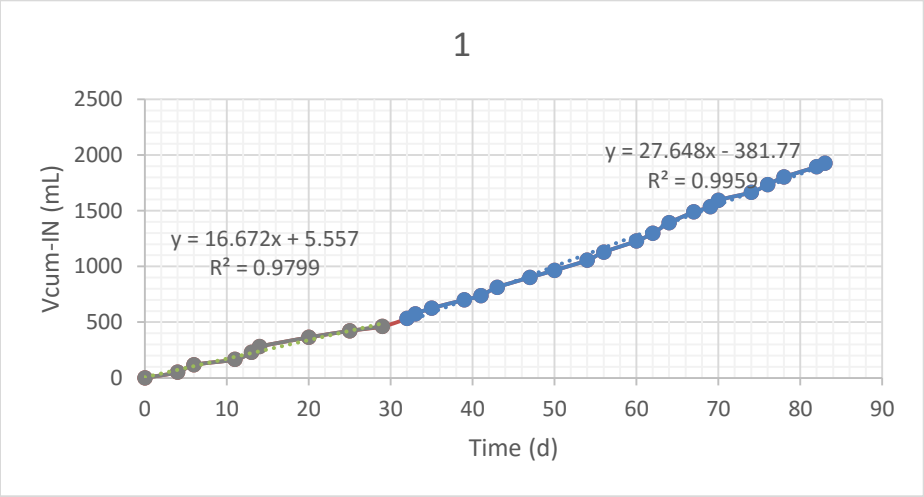

Figure a. Cumulate volume addition for vial 1, expressed in mL

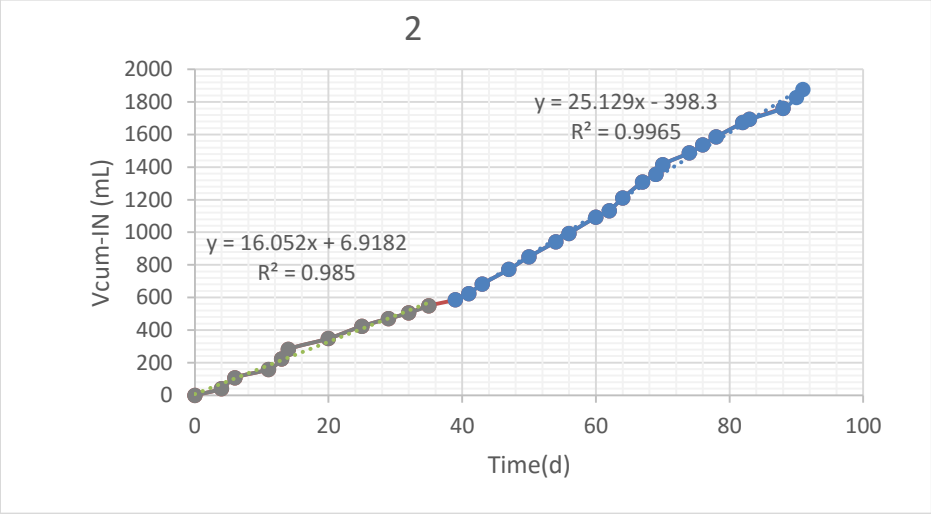

Figure b. Cumulate volume addition for vial 2, expressed in mL

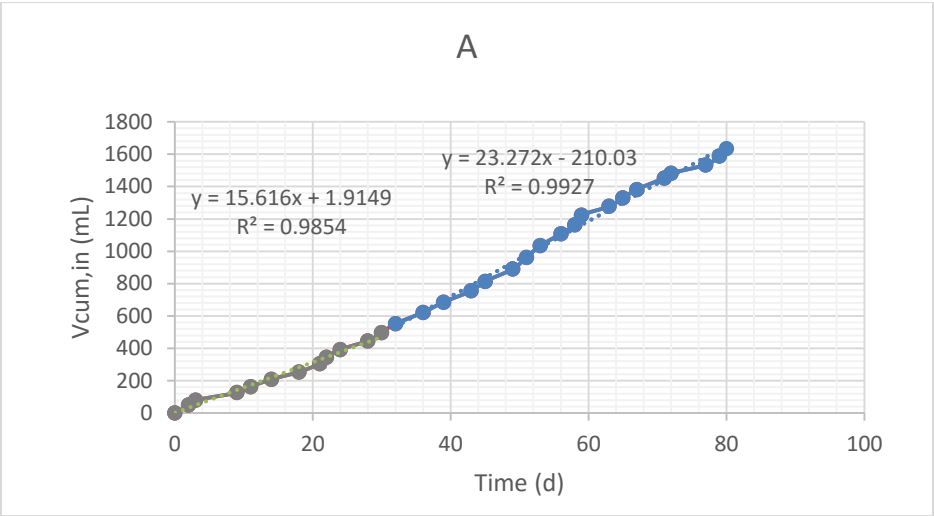

Figure c. Cumulate volume addition for vial A, expressed in mL

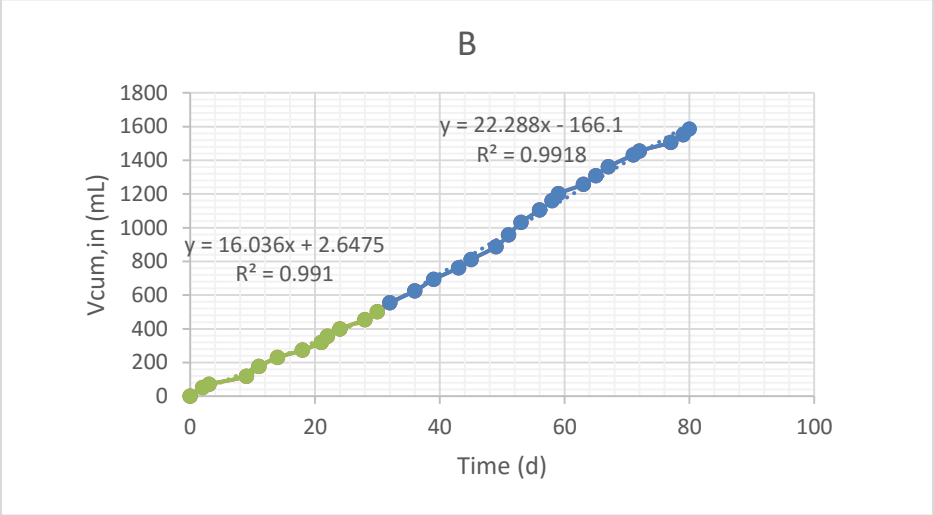

Figure d. Cumulate volume addition for vial B, expressed in mL