UNIVERSIDADE DA BEIRA INTERIOR

Ciências

Bioremediation of liquid effluents using synergy between *Chlorella vulgaris* and bacteria

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Resumo Alargado

Hoje em dia, devido ao elevado crescimento da população mundial, assiste-se ao esgotamento dos recursos, à utilização intensiva dos espaços e à poluição decorrente das atuais atividades humanas, tornou-se necessário procurar novos meios e métodos de produção de bens, nomeadamente alimentares, com vista a minorar o impacto ambiental e económico das atividades humanas.

Uma das respostas pode passar pela produção de microalgas, tais como a *Chlorella vulgaris*. No entanto, apesar de se reconhecer que estes microrganismos apresentam um elevado crescimento e potencial na produção de compostos de grande interesse para a alimentação humana e animal, hoje em dia a sua produção não é ainda, em muitos casos, economicamente viável, ou seja, a sua rentabilidade não compensa os elevados custos de produção associados, nomeadamente o enorme consumo de recursos hídricos e de macronutrientes.

Como tal, um dos objetivos deste trabalho foi utilizar efluentes provenientes de 3 fontes diferentes para fornecer os macronutrientes necessários para o crescimento e paralelamente testar o potencial de biorremediação destas microalgas. Neste contexto, procurou-se ainda avaliar as sinergias entre a microalga e as bactérias, quer do próprio efluente, quer introduzidas propositadamente, de modo a analisar a eficácia desta cultura mista no crescimento algal e na biorremediação do efluente utilizado como substrato. Esta abordagem decorre de se saber que existe uma vasta gama de interações entre estes dois microrganismos que data praticamente desde o seu surgimento na história evolutiva da Terra. Tais interações podem ser benéficas ou não, dependendo da espécie de bactéria em questão dado que muitas são parasíticas e como tal podem levar à morte da cultura de microalgas onde estão presentes. No entanto, o uso correto de uma sinergia entre algas e bactérias pode, decorrente da maior acessibilidade a nutrientes de outra forma inacessíveis às algas existentes em culturas, gerar um melhor crescimento de microalgas que irá aumentar o rendimento em biomassa e simultaneamente proporcionar, o tratamento do efluente utilizado como substrato pela cultura.

Neste trabalho, os substratos utilizados foram três efluentes, um de lagar de azeite, outro lixiviado proveniente de um aterro e ainda águas residuais urbanas. Estes efluentes de origens diferentes apresentam, como é natural, características diferentes entre si, o que foi tido em conta na sua utilização como substrato.

O processo foi avaliado com base nas seguintes monitorizações: concentração de células, concentração de macronutrientes, nomeadamente N e P, COD, entre outros.

O efluente de lagar de azeite (OMWW) foi o primeiro a ser utilizado e devido às suas características físico-químicas foi o que sofreu uma diluição maior para garantir que haveria

condições mínimas de sobrevivência das microalgas na cultura. Foram feitas duas séries de ensaios com este efluente (a segunda com maior diluição), tendo-se verificado que o crescimento das microalgas é afetado negativamente pela presença do efluente; como esperado os ensaios com maior diluição (0.25—0.5% v/v) foram aqueles que apresentaram um crescimento bastante aceitável, comparável com a referência (sem efluente e com nutrientes sintéticos). Nesta série de ensaios verificou-se uma diminuição significativa do COD e um correspondente up-take de N e P, o que representa uma efetiva biorremediação.

O segundo efluente utilizado foi um lixiviado (LWW), tendo sido incorporado a níveis entre 5% (v/v) e 10% (v/v). Tal como com o efluente anterior, foram realizadas duas séries de ensaios. Na 1ª série testou-se a adição ou não de macronutriente, N e P, ao efluente LWW. O crescimento das microalgas foi, em qualquer dos casos, significativamente inferior ao verificado para o controlo (sem efluente e com nutrientes sintéticos). O ensaio com 10% de LWW foi o que apresentou os piores resultados de crescimento. Na 2ª série de ensaios foram introduzidas propositadamente bactérias proveniente de um meio misto (aeróbio/anaeróbio), tendo verificado uma influência positiva, ainda que modesta, sobre o crescimento microalgal. Em contrapartida o seu efeito na biorremediação foi misto, com os valores de CQO a manterem-se elevados e estáveis durante o ensaio, enquanto os valores de assimilação de macronutrientes foram superiores às culturas xénicas utilizadas.

O último efluente utilizado foram as águas residuais urbanas (MWW). De facto, não se ensaiou um efluente, mas sim dois proveniente de duas etapas distintas do processo de tratamento de águas residuais: após crivagem e após tratamento aeróbio. Estes efluentes foram incorporados sem e com esterilização e ainda, no caso do esterilizado, adicionando bactérias aeróbias externas. Os resultados obtidos com estes efluentes foram satisfatórios, com os biorreactores com substrato proveniente dos primeiros passos do tratamento de águas residuais a registarem elevados rácios de crescimento, com particular destaque na cultura axénica onde se encontravam bactérias do próprio efluente; as culturas mistas de algas com bactérias alóctones ao efluente por sua vez registaram picos de crescimento, mas depois entraram numa longa fase de crescimento reduzido. No que respeita à biorremediação, apenas o CQO pôde ser testado e o que se verificou foi um declínio lento, mas constante do valor deste.

Os dados obtidos no final do trabalho levaram-nos à conclusão que apesar de na maioria dos casos os efluentes utilizados resultarem em crescimento de biomassa algal, os melhores resultados ocorreram nas culturas mistas de bactérias e microalgas, particularmente as existentes no efluente MWW, onde as culturas indígenas foram testadas. Isto leva-nos a concluir que de modo a se obter um melhor rendimento global de microalgas, assim como um simultâneo tratamento do efluente, a melhor solução será utilizar culturas mistas destes organismos.

Palavras Chave

Chlorella vulgaris, efluente, CQO, assimilação, sinergia, biorremediação

Abstract

Nowadays, excessive population growth, overexploitation of resources and increased industrial activity has put a huge stress on several ecosystems, with problems such as occupation of arable land and pollution of watercourses endangering human populations and habitats. One of the answers for this is the use of microalgae as it is known that they have huge potential as food and organic compounds source while occupying non-arable land as well as a largely untapped bioremediation potential. However, despite all this potential its cultivation is still largely not cost effective, as such, several methods to augment its potential are suggested. One of these is by using effluents as substrate, as several of them are rich in macronutrients. Still, one underexploited method is the use of synergy between microalgae and bacteria to increase culture growth. To this end, the microalgae Chlorella vulgaris and three different effluents were used, each with different origins and therefore different physicochemical characteristics in a series of essays. The process was evaluated by analysing parameters such as microalgae growth, COD value and macronutrient assimilation in order to depict the cultures potential as a bioremediation and microalgae growth enhancing assets. The results obtained showed that in the effluents studied, microalgae cultures were able to achieve moderate to strong growth notwithstanding problems such as high organic compounds such as phenols or high macronutrients concentration that characterize the effluents OMWW and LWW respectively. The bioremediation results depend on the parameter analysed, as microalgae showed extremely high nutrients assimilation capability while it is not the case for COD. Within these positive results differences were observed between axenic and xenic cultures, as the presence of bacteria generally served as an enhancer of growth. This was especially the case in cultures where a bacterial population indigenous to the effluent was used as it was the case with MWW. Nonetheless, as far as COD bioremediation regards, the presence of these mixed cultures while did not revealed significant divergences from pure microalgae cultures. Thus, it can be said that the use of mixed bacteria-microalgae culture in effluents is a very effective tool to enhance culture growth while simultaneously decreasing associated costs of this kind of industry and possessing good bioremediation capabilities.

Keywords

Chlorella vulgaris, effluent, COD, assimilation, synergy, bioremediation

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Acronyms List

IAA – Indol-3-Acetic Acid

COD – Chemical Oxygen Demand

WW – Wastewater

MWW – Municipal Wastewater

AWW – Agricultural Wastewater

IWW – Industrial Wastewater

WTP – Wastewater Treatment Plant

MPGB – Microalgae Promoting Growth Bacteria

DDT - Dichlorodiphenyltrichloroethane

HAB – Harmful Algae Bloom

HRAP – High Rate Algae Ponds

DNA – Deoxyribonucleic Acid

RNA – Ribonucleic Acid

ATP – Adenosine Triphosphate

FAS – Ferrous (II) Ammonium Sulphate

OMWW – Olive Mill Wastewater

LWW–Leachate Wastewater

Chapter 1 – Introduction and objective

Currently we have been seeing an increasing amount of evidence that the current global population growth will cause massive stress upon the world`s food and fuel supply and consequently social, political and economic instability in both developed and developing worlds. One of the answers to this problem are microalgae. These are photosynthetic microorganism that inhabit both marine and freshwater environments and are among the oldest living organisms on Earth. Its potential as a food and fuel source and their limited impact to the environment and very flexible cultivation make it a target for many companies and researchers [1]. Besides, due to their specific macro/molecular composition several valuable primary and secondary metabolites can be acquired for almost no additional costs [2].

However, we have the problem that the cultivation of this kind of microorganism needs significant initial financial resources and most importantly, of ever more precious hydric resources. In fact, in many cases it is still not economically feasible to make large scale productions of this kind.

As such, several ways are being researched in order to make this culture more accessible; among them, the use of nutrients rich wastewater as a substrate, and the usage of mixed cultures of microalgae and bacteria to enhance growth and reducing costs, are being exploited. Another very important way to reduce costs is in fact the correct choice of microalgae. In our case, *Chlorella vulgaris* was chosen due to its common use among several biotechnological applications thus making the experiment more easily replicable by us in the future or easier to adopt by others. This common usage of *C.vulgaris* is also due to its biochemical and physiological properties that indeed makes it a valuable and interesting choice for axenic and xenic cultures.

In the case of wastewaters, they can be further divided in accordance with their origin; these being industrial, agricultural and municipal which leads **to effluents having different compositions between them such as a higher nitrogen and overall nutrient composition.**

The usage of mixed cultures is however more complex. The history of interactions between these two kinds of microorganisms is an ancient one, going back practically to the first ancestors of microalgae, billions of years ago, an interaction that it is thought dramatically affected the course of Evolution and the chemical composition of Earth´s atmosphere, with all the consequences that followed. To use mixed cultures however, there is a need of more advanced bioreactors, as a matter of fact several of them already exist, but their success largely depends on the culture types [3], as most of bacteria are prejudicial to algae causing competition and subsequent decrease of growth, to death due to parasitism and presence of

pathogenic substances. The use of the correct ones though will increase growth for both microalgae and bacteria; the firsts releasing organic carbon such as proteins and carbohydrates while the later release CO_2 and consume oxygen, easing the photosynthetic oxygen tensions for the microalgae. Bacteria also decompose organic matter into mineral, especially essential macronutrients such as nitrogen (N) and phosphorous (P) and even micronutrients that, due to absence of proper fixing process algae are unable to acquire, and releasing growth hormones such as indol-3-acetic-acid (IAA) and vitamins that are found to further promote growth of microalgae [3]. A careful approach to these interactions will thus increase yield and reduce costs of the cultures with great impact to the industry.

With all these factors in mind, our goal was to analyse the synergy between microalgae and aerobic and mixed cultures of bacteria and their potential for substrates.

Thus, experiments were carried out in batch photobioreactors with light intensity, air flow and pH closely monitored to ensure a relatively stable environment. Culture effectiveness was analysed by following the COD evolution, cell growth and macronutrients consumption.

Chapter 2 – Literature Review

2.1 – *Chlorella vulgaris*

The selected microalgae, the species *Chlorella vulgaris* (Beijerinck,1890) (Fig. 2.1). It is a species of green eukaryotic photolithoautotroph unicellular organism with a high protein content, currently one of the most widely studied algae [4].

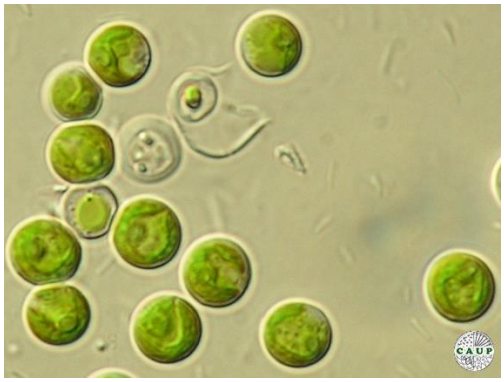

Fig. 2.1 – Microscopic image of *C.vulgaris* taken from Charles University Prague culture collection database

2.1.1 – Taxonomy and General Characteristics

The microalgae per se has a spherical morphology, approximately 5-10μm in diameter. It is composed of a cell wall which serves several biophysiological functions, chiefly recognition of nutrient intake and protection. Inside the cell we have then the cytoplasm that holds the organelles of the microalgae, namely the several vacuoles, the mitochondria, the Gobi complex and the chloroplast; as expected from microalgae it reproduces asexually and rapidly [4]. The following table (Table 1) shows us the taxonomy of the organism in question.

Table 1 – Taxonomy of *Chlorella vulgaris*

Empire	*Eukaryota*
Kingdom	*Plantae*
Phylum	*Chlorophyta*
Class	*Trebouxiophycae*
Order	*Chlorellales*
Family	*Chlorellaceae*
Gender	*Chlorella*
Species	*Chlorella vulgaris* (Beijerinck,1890)

This microorganism has existed on the planet for about 2.5 billion years and has a very large geographical distribution; furthermore, it was revealed that it has a genomic structure that is responsible for cell division similar to one found on *E.coli* as well as segments that are found on terrestrial plants, but at the same time no homologue sequences in red and brown algae were found [5], thus helping to correlate the theory that land vegetation evolved from green algae.

2.1.2 – Reasons for choosing *Chorella vulgaris*

There were several reasons to choosing *C.vulgaris* in our study. On one part it is an organism studied since the 1900s catching attention then due to its high protein content, as attested by Safi et al (2014) [4], with several different experiments being done since its discovery culminating in the sequencing of its chloroplast genome [5]. As such, it is a well-known organism with defined properties and characteristics. The other part is intimately connected to the first one, and it is the cost. Because it is a microalgae used since early 20th century it is one of the cheapest and most widely used in biotechnology which also plays on one of our main objectives, that is increasing the cost-effectiveness of this kinds of treatment.

2.2. - Wastewater

As mentioned above there are several kinds of wastewaters (WW) that can be used, generally divided along its place of origin and each one with different biochemical properties (Fig.2.2) that differently affect the targeted microalgae culture.

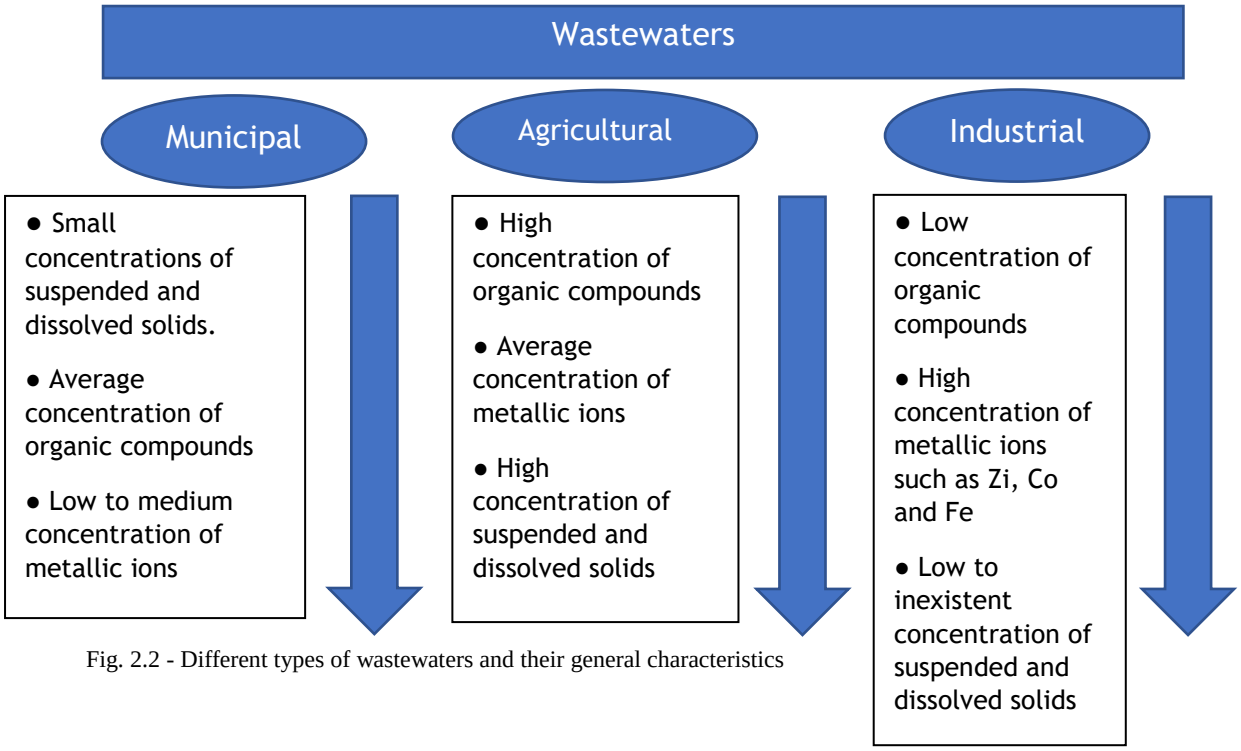

Fig. 2.2 - Different types of wastewaters and their general characteristics

As the above diagram shows, effluents that arrive at watercourses can be generally divided along the lines of Municipal Wastewater (MWW), which usually comes from urban waste in cities, Agricultural Wastewater (AWW), usually originated from agropecuary and agricultural sources, and Industrial Wastewater (IWW), this one originated from the wide range of industries, from refineries to textile and steel mills. As such, all of them have variable chemical and organic properties [6], however, due to the large amount of different sources, even among the same types of WW, differences can be found depending on the source and surrounding environments.

Given the rate of human population growth and their expanding activities, it is common knowledge that the rate of effluents produced will increase, which will cause severe problems, first from an environmental and then from a social point of view. Currently, Wastewater Treatment Plants (WTP), while effective, do not completely satisfy the criteria of having a completely treated wastewater [7]; due to this, several areas, especially in poorer countries, where WTPs are not up to standard, are extremely vulnerable with severe problems such as excessive eutrophication. This is serious, as it will cause a loss of diversity and severely impact the quality of life from human populations in the area. Therefore, finding

ways to ensure a successful and cost-effective treatment of effluents can be seen as crucial factor to ensure a sustainable environment for both human, animal and plant populations.

2.3 – Treatment of Wastewater

To safely discharge treated WW to the aquiferous basin and posing little to no problems to the ecosystem and human life, it must first pass through a rigorous treatment. This treatment consists mainly of 3 steps. Primary treatment, Secondary treatment and Tertiary treatment (fig. 2.3). Occasionally the WW will pass through what is called Preliminary treatment; this treatment involves the removal of contents or material that can easily clog the aerification tanks, tubes and pumps that abound in the latter steps of the process, such as rags, woods and/or fecal matter in the case of Municipal Wastewater [8].

The next phase of the process is known as the Primary Treatment, also known as Clarification, and consists on flowing the effluent through large tanks in which settable solids will be removed by action of gravity, with varying degrees of success depending on the effluent used and its chemical and biochemical composition.

Following Primary Treatment, the effluent will pass to the Secondary Treatment which reduces organic matter by means of a population of heterotrophic bacteria indigenous to the effluent that will digest the organic compounds still present [8]; a variation of this treatment is called activated sludge which is less common tough more effective, albeit vulnerable to toxic wastes.

The Tertiary Treatment consists in removal of organic ions, chiefly ammonium nitrate and phosphate, by either biological or chemical processes as mentioned by Abdel-Raouf et al. (2012) [8]. However, this final process is very expensive, with the biological treatment, being cheaper, and therefore being the most common to find in sewage treatment plants for this reason.

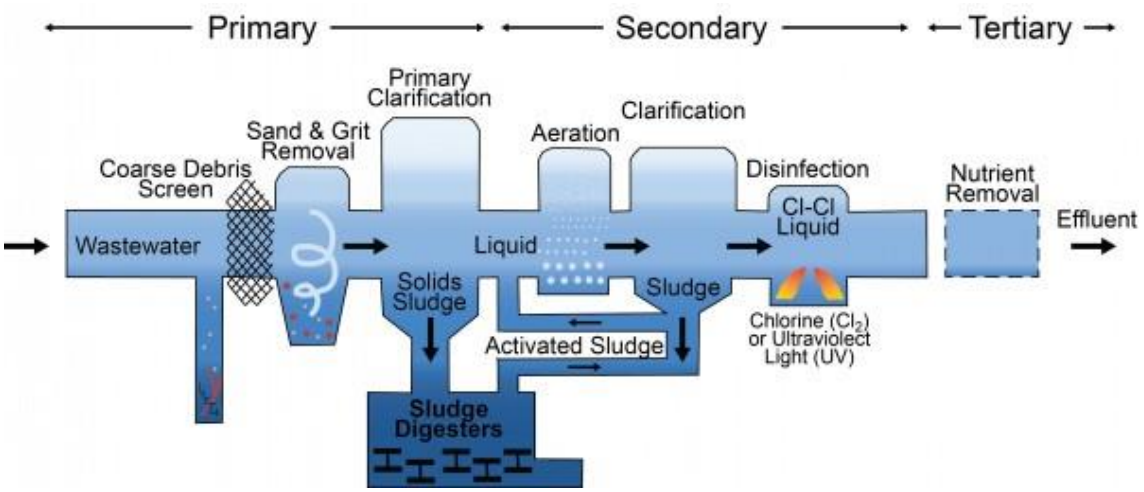

Fig. 2.3 - Scheme of a common Wastewater Treatment Process with Activated Sludge, by University of Michigan

A far more uncommon treatment also exists and is called Quaternary Treatment, whose main function is to remove metallic ions, remaining organic compounds and soluble minerals; thus, it exists mainly in sewage plants close to industrial or other heavy metals rich areas as this is the most expensive treatment, costing as much as 16 times the primary treatment.

2.4. – Sinergy between Bacteria and Microalgae

In terms of total numbers and biomass it is accepted that photosynthetic organisms and other bacterias are the dominant lifeforms on Earth, inhabiting almost every habitat possible, forming the basis of marine and freshwater food webs; as such, its study for understanding and eventual use are of primary interest for research [9]

It is known that in the case of terrestrial organisms, like plants, there are symbiotic relationships between them and bacteria such as *Azotobacter* while in the case of microalgae, recent studies showed than in several cases, bacteria not only help fix nitrogen, but also produce hormones such as Indole-3-acetic Acid (IAA), an auxin whose function is growth and development in plants that can be also produced in bacteria and was shown also have similar effects in microalgae through yet unknown means [10] [11]. In fact, there are fossil records that showed that algae-bacterial interaction is very ancient, and may in fact been responsible for an event called Great Oxygen Event [9], that marks the beginning of biologically induced oxygen in the atmosphere and the rise of aerobic organisms to the detriment of anaerobic ones. Further fossil records indicate that early eukaryotic organisms also evolved from cyanobacteria/early microalgae-bacterial (or Archaea) interactions, thus further contributing to the evolution of Life on Earth (Fig. 2.4) Furthermore, more recent genetic studies indicate that horizontal gene transfer from bacteria and archaea may be responsible for the microalgae survivability in extreme environments [12].

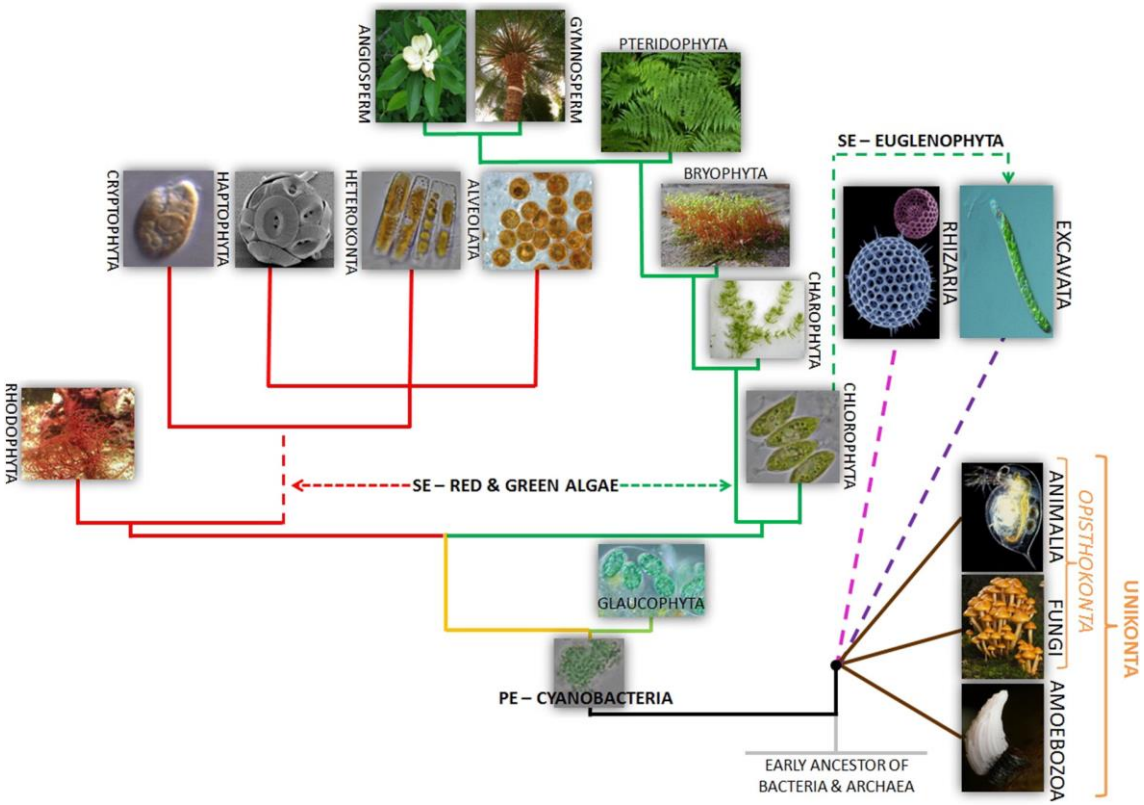

Fig. 2.4 – Diagram with the current evolutionary tree of algae, adapted from Ramadan at al (2016). Note; green lines represent organisms with green pigments (where *C.vulgaris* is included), while red indicates algae known as Rhodophyta that possess red pigments

Besides their importance to the evolutionary pathway, far more important to the current biotechnology industries is understanding the nature, or rather the ecology of microalgae-bacterial relationships. These relationships cover a wide range of interactions, but can be subsided in three major ones: **Mutualism, Commensalism** and **Parasitism** [9]. The first one, Mutualism, broadly refers to interactions between two individuals from different species that benefits both; this interaction goes from the facultative ones, in which they benefit from each other but is not obligatory for survival to as far as symbiotic relationships in which both organisms must obligatory cooperate with each other for their survival. Many cases exist in this relationship, where the bacteria commonly supplies inorganic macronutrients such as Nitrogen (its importance will be explained further ahead), which microalgae, in normal conditions cannot easily access them, as they do not possess nitrogen fixing mechanisms [9]; however, as the name says, mutualism in not a one way road that benefits only one organism, bacteria also benefit from this relationships in a variety of ways, just like microalgae, namely by uptaking the organic carbon compounds produced by them. This enables both of to exist in oligotrophic environments and thus expands the number of habitats both organisms can colonize, thus this bacteria that are recognized as having potential to enhance microalgae growth are known as MPGB (**M**icroalgae **P**romoting **G**rowth **B**acteria). The second relationship

mentioned, commensalism, is one in which only one partner will benefit from the situation, however this puts a thin line between this and either parasitism and mutualism. It is hypothesized that when competing with one another, the algae allowed bacteria to outcompete and eventually outnumbering them in N and P limited environments, as such, this relationship may be described as positively affecting one organism, while the other will have neither benefits nor negatives from such interaction. The third one, parasitism, consists of an interaction in which an organism negatively affects the other to its benefit. This is the most common interaction between microalgae and bacteria, and in it, the parasitic organisms will over time cause cell wall lysis, killing the affected microalgae. Another form of parasitism may simply be competition for nutrients that will slow microalgae growth and can severely affect the economic impact of microalgae cultures [9].

As such, we can see that algae-microbial synergism is very complex in which both the microalgae and the bacteria must be carefully chosen as not all of them are capable of positive synergy between them and even the positive aspects can collide with the final objective of the process. Consequently, it is very important to take in account the presence of indigenous bacteria in microalgae applications as they can dramatically alter the economic and environmental costs of using microalgae for a variety of industrial, environmental and pharmaceutical practices, as will be described below.

2.4.1 – Economical Applications of Microalgae-Bacterial Synergism

2.4.1.1 – Bioremediation and Bloom Control

Due to the fact that microalgae do need micronutrients at very small amounts for an effective growth and metabolism they can be used to effectively degrade metallic compounds [13] and even crude oil [14]. Therefore, they have a great potential for bioremediation, an important aspect through much of the developed and developing world due haphazard industrial growth. A combination of culture from both microalgae and bacterial microorganisms has proven to be even more effective than single strain microalgae. According to Subashchandrabose et al., (2013) [15], such cultures were able to degrade dangerous pesticides such as DDT. This kind of interactions should be viewed as a powerful tool to enhance current attempts to clean several of existent pollutants in areas dangerously affected by them, ensuring a recovery of the previous ecosystems to the benefit of its previous or current inhabitants, including human beings.

Closely related to bioremediation, but standing apart we have Bloom Control, one of the first areas of study in this category, that involves the use of bacteria to control explosive growth of microalgae (Fig. 2.5) that will severely diminish available oxygen to

Fig. 2.5 – Example of a Harmful Algae Bloom (HAB) in Sichuan, China, adapted from Felix Andrews

the populations of the surrounding marine or freshwater habitat thus causing **widespread mortality of animal and plant life and interdiction of consumption of hydric resources by humans in what are referred as Harmful Algae Blooms (HAB)** [9] . By using predatory or competing bacteria it is possible to diminish algae population to safer levels thus precluding the complete collapse of the surrounding ecosystem. However, such acts must be carefully planned, as the increase of microbial populations can contribute to a lowering of available oxygen due to the decomposition of microalgae, additionally contributing to HAB.

2.4.1.2 – Wastewater Treatment

As microalgae do not possess chemolithotrophic properties they are dependent on the environment to supply them with the macronutrients nitrogen and phosphorous for survival and growth; in their absence, microalgae growth will stagnate and eventually die [16].

However, wastewaters, as mentioned in the previous section 2.2, are rich in these macronutrients and thus can damage natural waters where they are discharged. Since the 1950s there are studies that refer to the abilities of a mixed microalgae-bacterial culture to treat wastewaters, however, such works mostly occurred in High Rate Algal Ponds (HRAP), thus being available only on more large-scale works. In newer studies nevertheless the use of bioreactors such as the ones we will be using proved successful in enhancing microalgae growth and reducing costs [17] demonstrating the potential of microalgae-bacterial in lower scale processes.

2.4.1.3 – Biotechnological Applications

As previously said, due to the ability of certain bacteria to release growth hormones typically found in plants such as IAA, they have demonstrated effect on algae and therefore potential to enhance the microalgae cultivation system. According to Ramadan et al. this can increase microalgae growth rate to an average of 10% and in some cases even to 70%. This of course would ensure a higher yield and an easier harvesting process, this way reducing cultivation costs.

Another way that bacteria are useful to the harvesting process is by increasing the flocculation of the algae cells. This may be possible due to the binding of calcium ions (positively charged) of the algae cell wall to the teichoic acid residues (negatively charged) that exist in several Gram-positive microorganisms. Regardless the entire mechanism that bacteria use to increase flocculation is still not totally understood and therefore can be an interesting area for future research, as harvesting is one of the main costs associated for biorefineries [18]. This synergy also has an enormous potential for the production of common primary and the more valuable secondary metabolites [17]. Further research showed that the bioenergy sector stands a lot to gain, with even bacteria such as *Geobacter*, that are capable of electricity production, and coexist synergistically with algae with improved results [19].

Also, another application that is widespread but commonly overlooked in place of the previously mentioned is the aquaculture. Microalgae are used to control pathogenic bacteria with the added bonus of helping in the wastewater treatment.

2.4.1.4 – Importance to the consumption of macronutrients

As previously mentioned, microalgae are photosynthetic organisms that, as all living creatures, need a constant supply of macronutrients, that are needed in large scale, and micronutrients, that are needed in small scale to survive and thrive. Two of the most important macronutrients are Nitrogen and Phosphorous; as such, every experiment with microalgae must take in account the amount of these elements that are available in the substrate to ensure culture growth. Nitrogen (N) is the most common element on the atmosphere, comprising around 80% of the total composition of it, although it is largely inaccessible, so, the one present in the soils and water has huge importance to the normal running of a stable ecosystem as it is constantly interchangeable between several forms. The disruption of this nitrogen cycle (Fig. 2.6) would have dramatic consequences for the targeted ecosystems eventually leading to the destruction of fauna and flora populations in the affected areas.

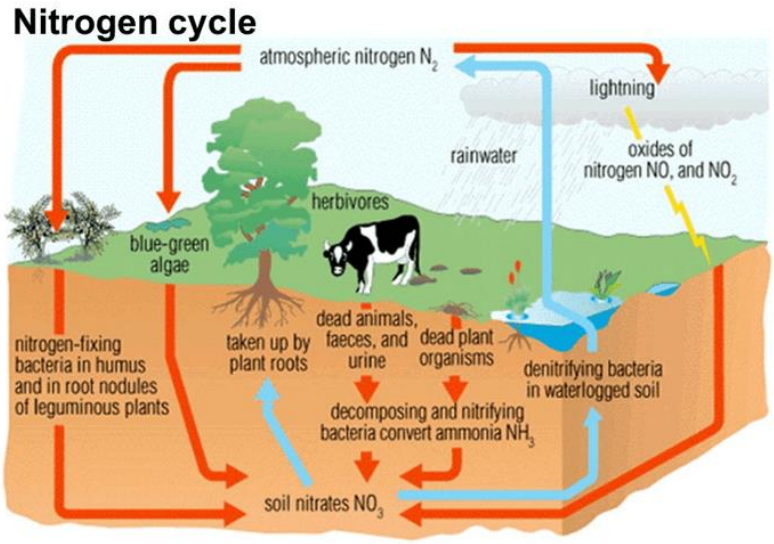

Fig. 2.6 – Scheme of a typical Nitrogen Cycle

Phosphorous (P) meanwhile is never found as a free element on Earth due to its highly reactive nature and it is commonly found as either red phosphorous or white phosphorous. Biologically speaking, it is a key component on known life, as a key element of such basic structures as DNA, RNA, and ATP. Like nitrogen, phosphorous circulates through the several spheres that compose the planet (Fig. 2.7), but unlike the nitrogen, the atmosphere does not play an important part on it.

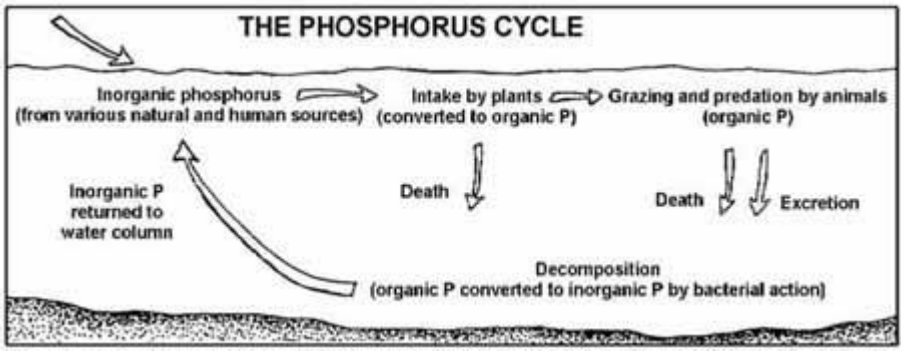

Fig. 2.7 – Scheme of a typical Phosphorous Cycle

That said, despite the extreme importance both nitrogen and phosphorous have for the continued existence of life, both have very negative consequences if there is excessive amount. A clear example of this is the HAB (Harmful Algae Bloom), that is generally caused by an excessive amount of nitrogen in a body of water that left untreated and will severely affect both its immediate habitat and the surrounding system. As mentioned in subsection 2.4.1, algae are known to be a very decent mean of bioremediation by themselves and can remove the nutrients from the aqueous systems. Great care must be taken however, because microalgae are also affected if the nutrient contents are too high. Although several studies have shown that the supposedly ideal Redfield Ratio is 15:1 (N:P ratio), it is not a set rule for

using substrate because several species of algae having better growth rates at different ratios [20], and their removal rates are not affected by the existence of the optimal N/P ratio [21]: However, it is known the concentration of N is serious factor that affects the rate of removal of P from the substrate by microalgae [22].

In addition, the microalgae-bacterial interactions were viewed as a way minimize the risks inherent to the fluctuation of macronutrients in a determined substrate. Indeed, one of the most examples of mutualism between microalgae and several bacteria is in nutrients poor environments, in which, bacteria with a nitrogen fixing mechanism (inexistent in microalgae) supplies nitrogen in exchange of fixed carbon originated from the algae´s photosynthetic mechanism [23].

Based in this information a microalgae-bacterial mixed culture could have a higher yield than a xenic one due to the fact that bacteria may fix nitrogen normally inaccessible in substrates like wastewater and thus guarantying continued growth. However, like it was mentioned before, these are all steps that must be carefully thought as not all bacteria benefit microalgae and even then, many may have different survival conditions such as pH, luminosity and macronutrients level.

Chapter 3 - Materials and Methods

3.1 - Material

3.1.1 - Algae

The microalgae used in these tests was the freshwater *Chlorella vulgaris*. These were obtained from Aqualgae (Viana do Castelo, Portugal). and then grown until we had a sufficient amount that enabled several studies without risk of running out of stock.

3.1.2 - Reagents

The medium we used was the GOLDMEDIUM-FWS, using as reagents, distilled water from the system RIOs^{TM} 3 (Millipore, Waters), and 4 stock solutions as mentioned in table 2. Each solution was prepared and dissolved in distilled water.

Table 2 - Nutrients present in the stock solutions used and their respective concentrations

Nutrient	Concentration(mg/L)	Solution
N in the form of $NaNO_3$	7000	1
P in the form of KH_2PO_4	2046	1
Mg in the form of $MgSO_4.7H_2O$	1480	2
$CaCl_2$	1360	3
Oligomers and vitamins	1450	4

To serve as bioreactors we used several bottles of 500 mL capacity connected to an air pump in order to generate needed air flow to keep stirring conditions in the bioreactors, in addition to supply CO_2. Occasionally we also supplied CO_2 (from pressurised tubes) via gas flow meters in order to balance the bioreactors pH and provide the macronutrient C (carbon).

For the analyses of treated and untreated effluents, several other reagents like Ferrous (II) AmmoniumSulphate (FAS) and Potassium Dichromate were used as well as H_2SO_4 0.5 M and NaOH 0.2 M for controlling the bioreactors pH.

3.1.3 – Installation

The microalgae were grown in 500mL gas washing bottles in shelfs for microalgae growth supplied by Aqualgae (Viana do Castelo, Portugal) comprising an air pump model V-60 (Hailea, Guangdong, China), a control unit for the light-dark cycles (12:12) using 4 fluorescent cylindrical LED. The shelf also possesses 3 air flow meters and 3 flow lines of CO_2, all of them with time controlled electrovalves. (fig. 3.1)

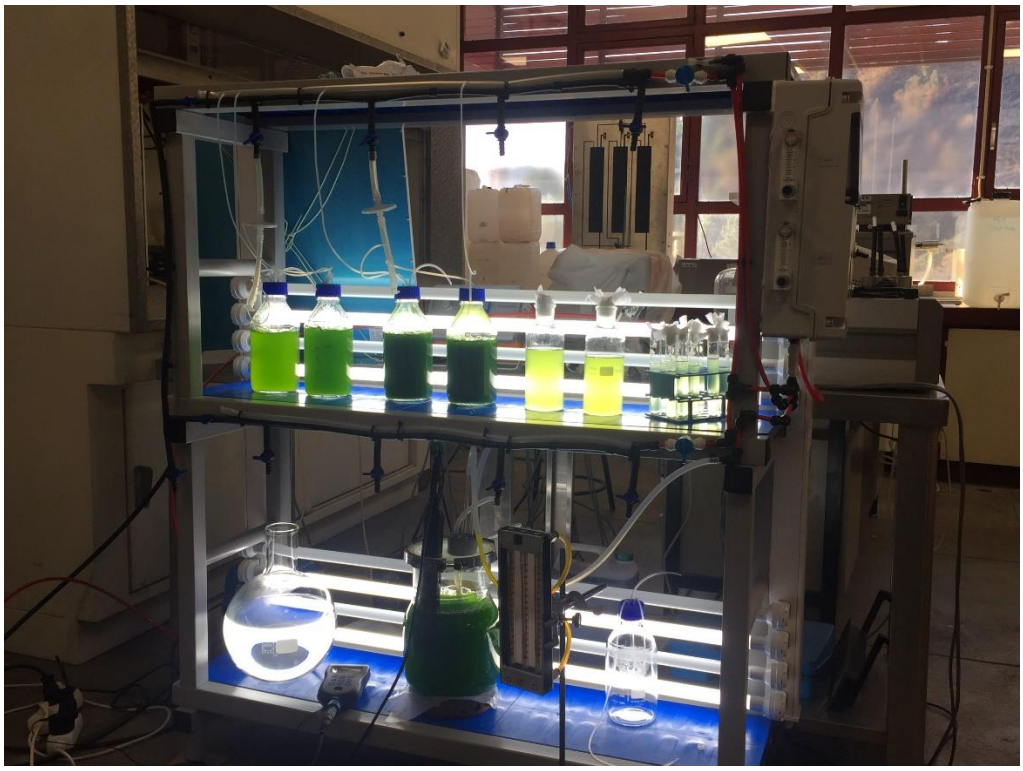

Fig. 3.1 – The installation used for the culture and growth of microalgae for all our essays

3.1.4 - General Equipment

Table 3 resumes other lab required equipment.

Table 3 – Other equipment's used for the duration of the essays

Table of Equipments	
Designation	***Series***
Autoclave	Tuttnauer 2540ML
Spectrophotometer	Helios-Omega UV-VIS
Neubauer Chamber	Zuzi Neubauer Improved
Heat Block	Lovibond ET125
Titrator	Metrohm 682 Titroprocessor
Dosimat	Mettrohm 665 Dosimat
Stirrer	Metrohm 728 Stirrer
Vacuum Pump	GMBH+CO KG

3.2 - Methods

3.2.1 - Growth Evaluation

In order to evaluate the growth of *C.vulgaris* two methods were used to analyse this parameter. These methods were Cell Counting with the use of a Neubauer Chamber and Spectrophotometry, albeit only the first one was deemed efficient enough to get an accurate reading of the microalgae growth; thus only data obtained from the first method will be used in this study. To use the Neubauer Chamber a sample was taken from the bioreactors and diluted (see section 3.5); subsequently 10µL were putted on the Chamber with the help of a 10µL pipette and analysed with a microscope by counting the number of cells present in each square present on a larger square in the Chamber as shown in fig.3.2.

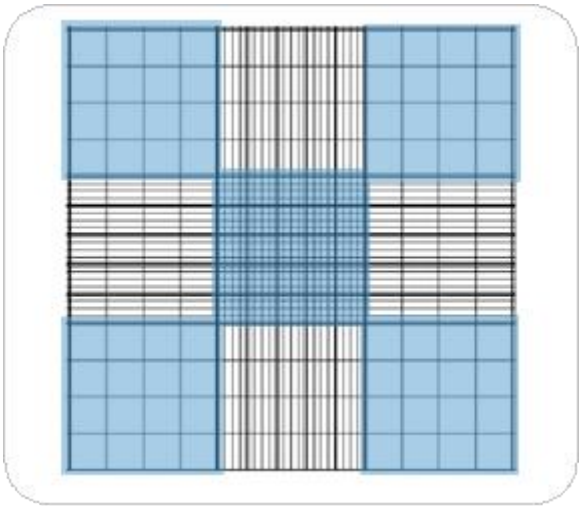

Fig. 3.2 – Neubauer Chamber as seen in microscope. The areas used for counting are the corner ones in blue,(adapted from the site Celoromics)

After counting the number of cells present in each square the following formula was used to estimate the number of microalgae cells present in the bioreactors, assuming that each volume in the larger squares is 0.1µL:

$$\frac{Total\ Number\ of\ Cells}{Number\ of\ larger\ squares\ counted} * Dilution\ Factor * 10000$$

The coefficient of variation of Growth measurement was close to 15%.

3.2.2 – Chemical Oxygen Demand

The effectiveness of this microalgae for effluent treatment was analysed by using Chemical Oxygen Demand (COD) determination, by means of the closed reflux titrimetic method (Fig. 3.3) as described below according to the Standard Methods for the Examination of Water and Wastewater.

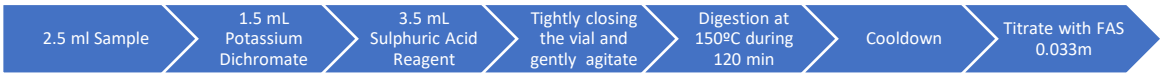

Fig. 3.3 - Protocol followed for COD value analyses

After titration using the Titrator Metrohm 682 Titroprocessor as shown in Fig. 3.4 the COD was calculated with the following formula, as required by the book Standard Methods:

$$\frac{(Blank\ Sample - Sample\ from\ the\ Bioreactor) * Molarity\ of\ FAS * 8000}{Volume\ of\ the\ Sample}$$

Fig. 3.4 – Titrator used for titulation of the samples for COD measurement

The coefficient of variation of the COD measurement was close to 20%.

3.2.3 – Nitrogen and Phosphorous Determination

As mentioned in section 2.4.1.3 N and P consumption is a method to analyse bioremediation potential, as high N and P concentrations are a general indicator of pollution, and its consumption found to be correlated to growth and bioremediation [24]. As such, initial and final N and P concentrations were determined and used to calculate the N and P removal.

To measure N and P concentrations kits LCK 138 and LCK 350 respectively (Fig. 3.5), were used by following the protocol described in the kits instructions and then analysed in a spectrophotometer (Fig.3.6). The coefficient of variation of the N and P measurement was close to 5%.

Fig. 3.5 – The kits used to analyse the Nitrogen and Phosphorous concentrations

Fig. 3.6 – Spectophotometer used for the analysis of the N and P assimilation.

3.2.4 – Production of Algal Biomass in different wastewaters

The algal biomass was cultivated with the main goal of effluent treatment; in addition, the microalgae produced can be used in other industries such as pharmaceutical, bioenergy, food.

Gas washing bottles (500 mL) with gas injection adaption were used as photobioreactors in a batch culture. For an effective volume of 500 mL, the bottle has a surface area of 547.65cm^2/L, but considering that light was supplied only through one side, we consider that its effective area was of 273.83 cm^2/L. Air was supplied through an air pump model V-60 (Hailea, China), filtrated by a nylon membrane with a 0.45µm porosity (Membrane Solutions, USA) with an air flow of 250mL air/ L^{-1}min to each bottle with the help of dispersers with a diameter of 2mm located close to the bottom; when required, CO_2 was supplied through dispersers with a diameter of 1mm in 7 second pulses with intervals of 10 min and a flow of 0.4mL/min,. Luminosity for its turn was supplied through 4 cylindrical fluorescent LED with and intensity of 400 µmol photons/m^2s.

For each series of experiments, several bottles were placed in the microalgae growth shelf, under controlled light intensity and light cycles. In general, replicates were used for each tested condition. Each series had control essays, using artificial nutrients and sterilized distilled water and essays with different effluents under different dilution. In general, 50 mL

of stock microalgae were added to each reactor to obtain a given initial microalgae concentration.

Daily samples were taken from each reactor to determine cell and COD concentrations; at specific moments, N and P determination were also carried out.

To choose a wastewater to use as a source substrate, we must place careful attention on the effluents N and P contents, that, as said in chapter 2.4.1.3, are essential to the survivability of organism and whose concentration and ratio influences growth rate.

As a reference, we used GOLDMEDIUM-FWS to serve as our Control against leachate wastewater (LWW), olive mill wastewater (OMWW) and municipal wastewater (MWW) from primary and secondary treatment.

The wastewaters used, OMWW, LWW and MWW, were analysed in respect to COD, pH, phosphorous, nitrogen and other micronutrients to determine its capability to serve as *C.vulgaris* substrate.

3.2.4.1 – Olive Mill Wastewater (OMWW)

In areas of Mediterranean climate were olive trees are autochthonous they have become a main industrial and economic activity. However, this industry is responsible for the production 3.0*10^7 m^3 of OMWW worldwide [25] a staggering amount that consumes a large quantity of resources for its treatment.

From previous work we know that the properties of OMWW depend on a wide range of factors, including, oil extraction process, environmental conditions and cultivation techniques. However, the most serious problem we face in using it as substrate for algae growth, is his very high COD content. As such we were forced to dilute our substrates in order to obtain different concentrations that were then experimented on. These concentrations were decided based on biochemical data determined in a previous studies by the same research group [25]. Table 3 resumes the biochemical composition of the OMWW, after coarse filtration. As we can see, the Chemical oxygen demand (COD) is extremely high (127000 mg/L), whereas the total nitrogen and phosphorus content are 250 mg/L and 211.5 mg/L, respectively, which gives a unfavourable C:N:P of 3051:16:13; the defined recommended ratio is 106 C:16N:1P. In addition, considering the extremely high COD concentration we decided to test OMWW incorporations of 0.5%, 1% and 2% (v/v), which mean dilution of 1:200; 1:100 and 1:50. Despite these dilution factors, the initial COD should be around 635 mg/L, 1270 mg/l and 2540 mg/L. The unbalanced C:N:P ratio was corrected by adding N and P from the artificial stock solution 1, in order to attain an initial concentration of 70 mg N/L and 20.4 mg P/L. Due to the high number of particles present in the effluent, filtration, using a nylon membrane Hydrophobic PTFE Membrane Filter with a diameter of 47mm and pore size

of 0.45µm was used the eliminate the biggest particles that could adversely affect the essays utilising this effluent.

Table 4 – OMWW characteristics

	Concentration (g/L)
COD	127
BOD	62
TN	0.25
TP	0.21

The first series of essays consisted of 8 bottles of 500mL, including:

- 2 Controls (Controlo) (Initial concentrations ($[N]_0$; $[P]_0$) of 70 mg N/L, from $NaNO_3$; 20.4 mg P/L, from KH_2PO_4; 14.8 mg mg/L, from $MgSO_4$.7H20; 13.6 mg Ca/L, from $CaCl_2$.2 H_2O; 14.5 mg/L of oligoelements and vitamins);
- 2 bottles (I and II) 0.5%(v/v) of OMWW and $[N]_0$ = 71.25 mg N/L and $[P]_0$ = 21.6 mg P/L;
- 2 bottles (I and II) with 1%(v/v) of OMWW and $[N]_0$ = 72.5 mg N/L and $[P]_0$ = 22.58 mg P/L;
- 2 bottles (I and II) 2%(v/v) of OMWW and $[N]_0$ = 75 mg N/L and $[P]_0$ = 24.69 mg P/L;

All the bottles above were further supplied with CO_2 in pulses of 7 seconds in intervals of 10 minutes each.

Due to the obtained results in the first series of tests, it was decided to try different concentrations in a new series of essays with the concentrations of 0.5% (v/v) and 0.25% (v/v), the latter corresponding to a dilution of 1:400. This second series of essay consisted of 6 bottles of 500 mL, including:

- 2 Controls (Controlo) (Initial concentrations of 70 mg N/L, from $NaNO_3$; 20.4 mg P/L, from KH_2PO_4; 14.8 mg Mg/L, from $MgSO_4$.7H20; 13.6 mg Ca/L, from $CaCl_2$.2 H_2O; 14.5 mg/L of oligoelements and vitamins);
- 2 bottles (I and II) 0.25%(v/v) of OMWW and $[N]_0$ = 70.63 mg N/L and $[P]_0$ = 21 mg P/L;
- 2 bottles (I and II) 0.5%(v/v) of OMWW and $[N]_0$ = 71.25 mg N/L and $[P]_0$ = 21.6 mg P/L;

Unlike the first series of essays, in this second series no $CO_2$was added to the bottles.

3.2.4.2 – Leachate Wastewater (LWW)

Leachate is generally a type of effluent occurring from leaching in decomposing waste, thus being very common in landfills [26]. These types of effluents, due to their variable origins have similar variable composition, but all of the nonetheless possess very high concentrations of macronutrients as can be seen in the table below (Table 5). Thus, it presents a challenge for the use of microalgae for bioremediation.

This wastewater from a nearby landfill previously was electrochemically treated to reduce its naturally high COD, P and N contents to more manageable levels for *C. vulgaris*.

Table 5 – Leachate wastewater composition.

	Concentration (mg/L)
COD	8753
TN	2665
TP	26.1

The table clearly shows that while the main characteristic of this wastewater is an extremely high Total Nitrogen (2665 mg/L) that by itself leads to a very unfavourable C:N:P ratio of 126:102:1, which together a still high COD concentration led to the need to test LWW incorporations of 5% (v/v) with and without added stock solution 1, and 10% (v/v) with added stock solution 1, corresponding approximately to dilutions of 1:20 and 1:10.

To this end, in the first series of essays, 8 bottles of 500 mL were used:

- 2 Controls (Controlo) (Initial concentrations ($[]_0$) of 70 mg N/L, from $NaNO_3$; 20.4 mg P/L, from KH_2PO_4; 14.8 mg Mg/L, from $MgSO_4$.7H20; 13.6 mg Ca/L, from $CaCl_2$.2 H_2O; 14.5 mg/L of oligoelements and vitamins);
- 2 bottles (I and II) 5%(v/v) (Controlo+5%) of LWW and $[N]_0$=203 mg N/L and $[P]_0$=21.7 mg P/L;
- 2 bottles (I and II) 5%(v/v) (5% EF.) of LWW with no added macronutrients from stock solution 1, which corresponds to $[N]_0$=133.2 mg N/L and $[P]_0$=1.3 mg P/L;
- 2 bottles (I and II) 10%(v/v) (Controlo+10%) of LWW and $[N]_0$=336.5 mg N/L and $[P]_0$=23.0 mg P/L;

The results obtained in the end of the first essay led us to try a new one, this time without added stock solution 1 and using mixed aerobic microbial cultures as a mean to help the microalgae to consume the high N values of LWW.

This second essay contained the following 8 bottles of 500Ml:

- 2 Controls (Initial concentrations of 70 mg N/L, from $NaNO_3$; 20.4 mg P/L, from KH_2PO_4; 14.8 mg Mg/L, from $MgSO_4$.7H20; 13.6 mg Ca/L, from $CaCl_2$.2 H_2O; 14.5 mg/L of oligoelements and vitamins);
- 2 bottles (I and II) 5%(v/v) of LWW with no added macronutrients from stock solution 1;
- 2 bottles (I and II) 5%(v/v) (5% m.) of LWW with no added macronutrients from stock solution 1; 15 Light Expanded Clay Aggregate (LECA) biofilm coated, as a result of several years of cork boiling wastewater treatment under mix conditions aerobic/anaerobic, were also added;
- 2 bottles (I and II) 10%(v/v) of LWW with no added macronutrients from stock solution 1; 15 Light Expanded Clay Aggregate (LECA) biofilm coated, as a result of several years of cork boiling wastewater treatment under mix conditions aerobic/anaerobic, were also added to this samples

3.2.4.3- Municipal Wastewater (MWW)

MWW is the most commonly used wastewater for this kind of studies due to its easiness of acquiring and known properties. It was also used in this work to test the feasibility of effluent treatment and microalgae growth ability. Its composition tends to vary from region to region depending on several factors like the main economic activity and general quality of life of the population.

From the municipal waste water treatment plant (Guarda, Portugal), we received effluent from several stages of treatment identified as A1 (Preliminary Treatment), A2 (Secondary Treatment), A3 (Decanters Exit) and A4 (Filtration and UV Radiation Treatment); its characteristics were then analysed like we did in the previous wastewaters, as showed in table 5.

Table 6 – Municipal wastewater composition

	A1 (mg/L)	A2(mg/L)	A3(mg/L)	A4(mg/L)
COD	518.6	----------	446.8	----------
TN	162.9	396.0	92.7	97.2
TP	3.9	53.8	1.8	1.8

The data obtained showed us that MWW for A1 and A3 are more balanced (C:N:P=50:42:1, and 93:52:1 respectively) than either OMWW or LWW. Indeed, MWW is the most commonly used as a substrate due to that factors as the N:P ratio is far more favourable that either one of the others that were previously used.

Due to the lower values of phosphorous and nitrogen in effluent A3 regarding effluent A1(table 5), it was decided to evaluate the contribution of the aerobic biomass in effluent A3. Use of biomass, as mentioned before (chapter 2.4), can help microalgae by increasing the available nutrients available in an oligotrophic environment. In resume, it was also decided to test the effectiveness of aerobic biomass by supplying them to the sample with the least amount of macronutrients available, that means A3; different initial aerobic biomass concentrations were also tested.

So, in this experiment we used 8 bottles as bioreactors:

- Controls, one 100% A1 (A1 Control) and the other 100% A3 (A3 Control) with no additional nutrients;
- 2 bottles (I and II) 100% A1 sterilised, with no additional nutrients (A1);
- 2 bottles (I and II) 100% A3 sterilised, with no additional nutrients, but with 2mL of aerobic microbial biomass added (A3 + 2mL);
- 2 bottles (I and II) 100% A3 sterilised, with no additional nutrients, but with 4 mL of aerobic microbial biomass added (A3 + 4 mL).

All the bottles were also supplied in CO_2 in pulses of 7s with 10 min of interval between them.

3.2.5 – Recovery of algae biomass

The biomass resultant from the different series of experiments were recovered by centrifuge at 7500 rpm during 30 min to ensure a clean supernatant for COD and macronutrients determination.

For growth evaluation 100µL were withdrawn from the bioreactors. This volume was then diluted 1:30 using distilled water and used for cell counting.

3.2.6 – Preparation of Microbial Biomass

The microbial biomass used for the essays with mixed culture (algae and bacteria) was available in the lab, maintained with sugar under aerobic conditions during the last years.

The aerobic culture was washed 5 times and cultivated during a period of 15 days until nutrient depletion, namely the sugar used to maintain the biomass. The final SST concentration was 5.71 mg/L. Assuming a microalgae/aerobic microbial ratio of 2:1 and considering the initial microalgae concentration of 0.2 g/L, the required volumes of microbial biomass was calculated to be around 1mL. To play safe it was decided to use 2mL for the bottle with higher N levels and 4mL for the one with lower levels, this corresponded to by using the formula described below 0.027 mg/L and 0.055 mg/L respectively for the amount of biomass inserted in the substrates.

$$\frac{(Sample\ Volume * Biomass\ Concentration)}{Volume\ of\ the\ Bioreactor\ in\ the\ beginning\ of\ the\ essay}$$

Chapter 4 – Results and Discussion

4.1. – Olive Mill Wastewater

4.1.1 – Growth

The first experiment had to take in account a very high COD value existent in OMWW [25] that would negatively affect microalgae survivability and growth. As such, dilution was carried out preceded by filtration to remove particulate materials. As during the experiment, the volume change with time due to evaporation, the real volume was always taken in account to guarantee accurate calculation, growth was measured using Neubauer Chamber with a dilution on 1:30.

The first experiment with OMWW lasted for 17 days and consisted of 8 bioreactors of 500 mL operated in batch mode, using as substrate 0.5%(v/v) of OMWW, 1% (v/v) of OMWW, and 2%(v/v) of OMWW, in addition to the blank essay without OMWW (only artificial nutrients and distilled water); 2 replicas for each condition was carried out and 5 samples collected from each replica; despite this procedure, some parameters exhibits high variability. The bioreactors were fed with a continuous supply of air and an intermittent one of CO_2, and the illumination was as described in experimental section. Due to the OMWW natural acidic pH, it was adjusted to around 6.5 in the bioreactors, keeping in mind that in normal growth conditions with *C.vulgaris*, its pH will be around 7 to 7.5, a neutral to slightly alkaline one. As seen in fig. 4.1, the introduction of a very small amount of OMWW had a clear negative effect on the microalgae grow. In fact, the microalgae grow as expected in the control essay, but little growth was seen with different dilutions of OMWW.

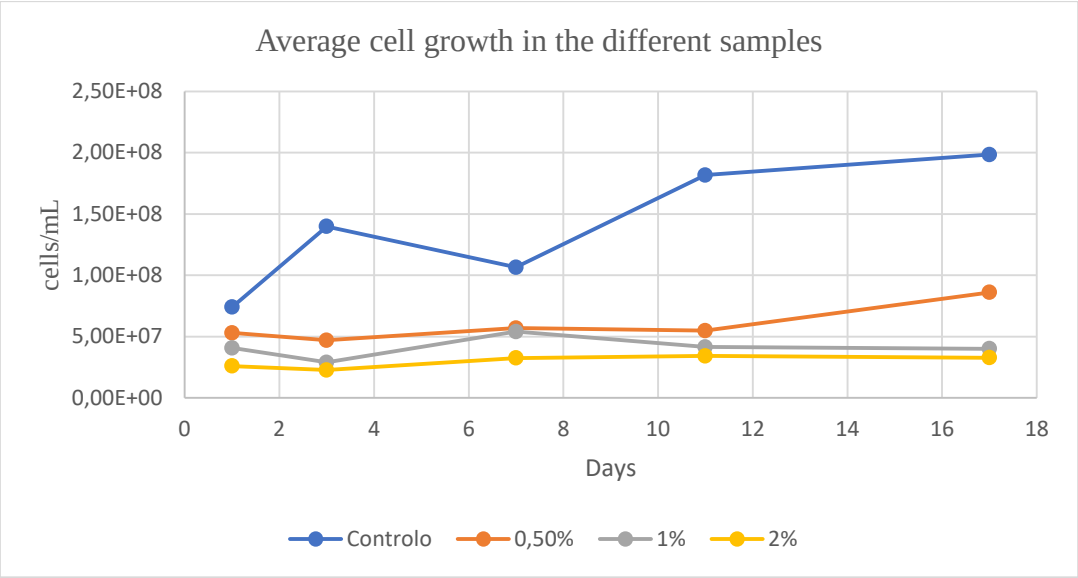

Fig. 4.1 – Average cell growth in the different bioreactors in the first series of essays with OMWW

By analysing the data in Fig. 4.1, there was indeed minimal growth among dilutions 1% and 2%. Di Caprio, Altimari, & Pagnanelli (2018) [26] noted that this behaviour may be due to several factors, mainly the high phenol concentration, that possesses anti-microbial activity. In batch culture, Di Caprio et al. (2018) [26] used around 9% (v/v) of OMWW and reportedly, their cultures stopped growing around days 5-6. Our dilution was greater, but no significant improvement in microalgae grow were observed. As mentioned in table 4 in subsection 3.2.4.1, OMWW has a very unfavourable C:N:P ratio that certainly has affected growth. Indeed while this ratio alone may be a strong inhibitor, the fact that this effluent has a high phenol content that functions as a potent anti-microbial agent can also play a role [28]. Other factors that may have had influence was pH; the effluent has an initial pH of 5.30, but the culture medium was adjusted to 6.5 which is the recommended pH threshold for microalgae survival. However the studies of Rachlin & Grosso (1991) [29] showed that the ideal pH, is around 7.5 to 8.3, which is attained when *C. vulgaris* has a sizable growth rate, like it happened on Control. Other factor that could had had a more significant impact was the reduced light penetration in the culture medium, as OMWW possesses a dark brown colour therefore limiting the available light. Indeed, Hodaifa et al, (2009) [30] in their study also mentioned that the use of this particular effluent leads to a lower light penetration affecting growth.

Considering the experimental results obtained in this first series of essays it was decided to try another series with a higher dilution of OMWW, namely 0.25% (v/v) and 0.5%(v/v) in addition to Control (fig. 4.2). The points in the figure represent the mean value of 5 samples.

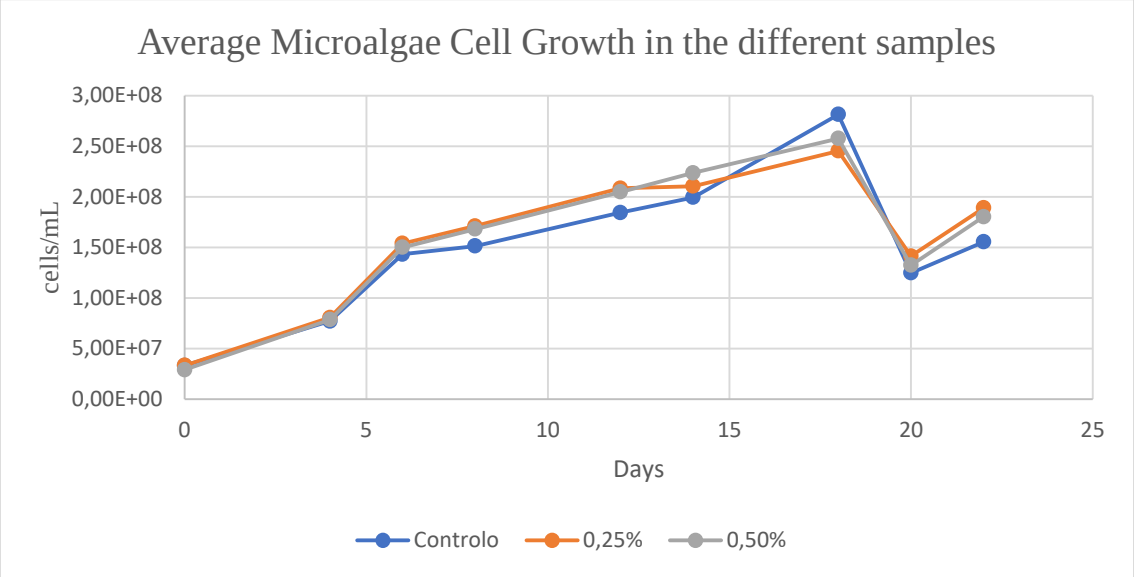

Fig. 4.2 – Average cell growth for the different dilutions in the second series of essays of OMWW

This second series of experiments is different than the previous series, because no significant differences was detected between the control and the essays with OMWW extremely diluted. In the previous one, the differences in growth between Control and OMWW were rapidly noted; here however the growth rates are rather very similar between them. This may be due to the fact that, unlike the first essay, no CO_2 was supplied to the cultures in the second series of experiments. Adding further C to the cultures would further disrupt an already unbalanced ratio that was at least moderately corrected by the addition of stock solution 1 to the cultures with OMWW. These last results were rather similar to the experiment made in batch culture by DiCaprio et al (2018) [27], although, as mentioned, they used a lower dilution value than us. Noteworthy, as Fig. 4.2 shows, is that in day 20 an abrupt decrease in cell/ number was recorded to all the bioreactors although it rebounded in day 22 when the experiment was terminated; we cannot have definitive answers about what caused such an abrupt change, but during that time, pH was slightly acidic, and as mentioned before, *C.vulgaris* grows better in a slightly alkaline environment. pH was thus corrected to neutral levels with NaOH solution because CO_2 was no longer added. This is important as it can be seen as a trade-off. As mentioned, adding C to an already unbalanced C:N:P ratio would disrupt microalgae growth, but not adding it leads to a more uncontrolled pH which must be taken in account in future studies with wastewater where the ratio is very unbalanced. Also, during this time, abnormal weather conditions were recorded, and being in a non-controlled environment, temperatures in the lab varied accordingly which may have affected growth.

Thus, from the data collected from the two series of essays, OMWW can be seen as valuable substrate for microalgae growth. In fact, Sanchezvillasclaras at al. (1996) [31] mention its potential, using lower dilutions than the ones we used here. Nonetheless, for our effluent the

best growth occurs at 0.5% (v/v), indicating that *C.vulgaris* is not capable of withstanding the compounds, especially phenols that are abundant in OMWW.

4.1.2 -COD Content

As one of the means of evaluating the pollution of a water course, it was decided to analyse the COD during the different essays, as COD values are high in effluents its lowering would indicate the bioremediation capacities of the microalgae culture. The COD recorded in our OMWW in the beginning was roughly similar to ones measured by Hodaifa et all. (2009) [30].

In the first series of essay, the same volume (1.5 mL) of medium was centrifuge and then its COD was determinate over a period of 11 days. The differences in volume of the culture medium that occurred during the experiments was considered in the calculations. COD determination is very sensitive to very small imprecisions, even in the best circumstances, and exhibits a high coefficient of variation.

Among the bottles with the same substrate there was not a clear pattern even so, the averages for the different substrates were used to have a clearer picture of the evolution of the COD values. (Fig. 4.3)

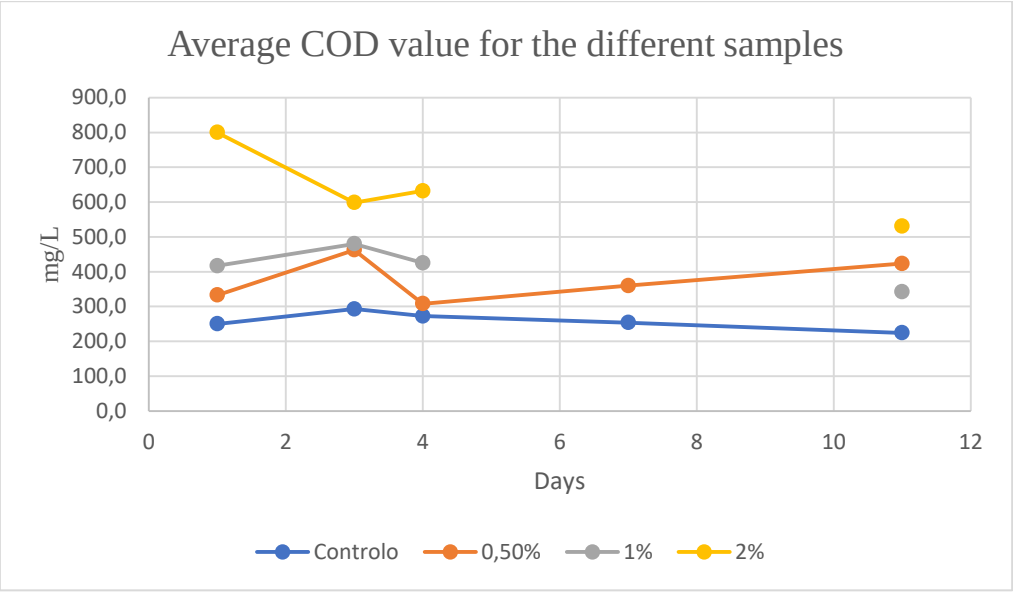

Fig. 4.3- Average COD value for the different substrates used for the first series of essays with OMWW

The figure above shows that the substrate incorporating 2% (v/v) of OMWW has a higher COD value than either one of the other substrates as expected. However, the value is much lower than the expected (2540 mg/L) taking in account the COD of the OMWW, but this can be due to the filtration procedure used to separate de algae biomass from the medium, which can

retain addition effluent particles. In addition, even the microalgae can segregate compounds susceptible to chemical oxygen consumption. In resume, this series of experiments was inconclusive regarding the COD evolution. Noteworthy is that in this experiment, there was little growth in all the bioreactors with the effluent (see 4.1.1) and therefore may have affected the COD.

For the second series of experiment, samples were once again collected, this time for a duration of 21 days and using higher dilution values. (Fig. 4.4)

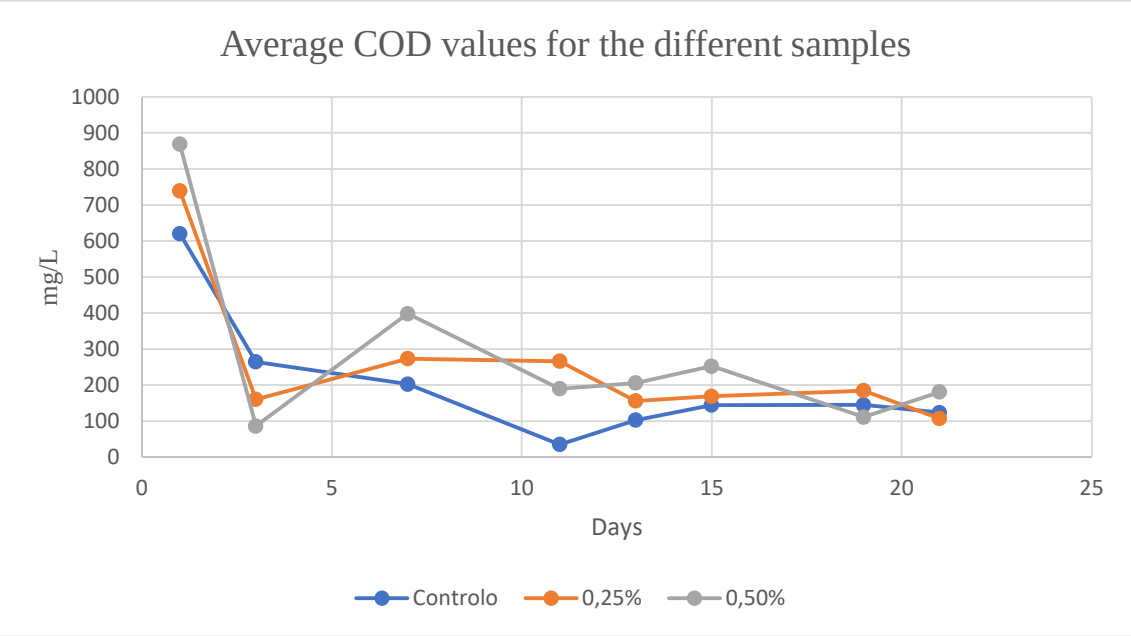

Fig. 4.4 – Average COD values for the different samples used for the second series of essays with OMWW

The initial data from this analysis appears to indicate that the microalgae appeared to have successfully treated COD; nonetheless, there was a wide variability among the reactors with the same substrate. Thus, once again, we must be careful with these data as the initial COD value of 0.50% (v/v) of OMWW is higher than 0.25% (v/v) OMWW although, contrary to the expected, it is not double of the value expected when using 2x more effluent. Still, all samples record a drop of the COD value by a sizable margin; this compares to the article of Fiorentino et al. (2004) that managed to reduce COD through the use of primary producers and Isidori, Lavorgna, Nardelli, & Parrella (2004) that manage to degrade it with the use of microbial cultures. Di Caprio et al. (2015) mentioned that using the algae *Scenedesmus*. sp there was also a decrease in COD, albeit they used non-sterilised effluent in lower dilution.

So, while *C. vulgaris* appears to be effective in treating COD, great care must be taken with the results obtained, because great variability between samples with the same substrate

concentration was observed (data not shown). Nonetheless it appears that microalgae have potential for this type of bioremediation if there is a successful growth as observed in section 4.1.1, as the second series of essays had a higher growth and as the figure above shows, a higher decrease of COD, thus indicating that the number of cells present in the substrate and the consumption of COD are interconnected; even so the addition of a mixed culture of microalgae and bacteria to stimulate further growth and the use of non-sterilised effluent appears to the be best way to ensure an ever better result.

4.1.3 -N and P Assimilation

Another way to analyse the bioremediation potential, is following the macronutrients concentration. It is well known that one of the major sources of water pollution is excessive presence of nitrate and phosphorous compound in the residual waters. As such, the assimilation of the macronutrients N and P was measured in the beginning and in the end of the essays to see if the microalgae culture had any remediating effect on the OMWW effluent.

In the first series of essay the measurements were thus taken at days 0 and 17, when the experiment began and when it ended. Figures 4.5 and 4.6 represent respectively the N and P assimilation.

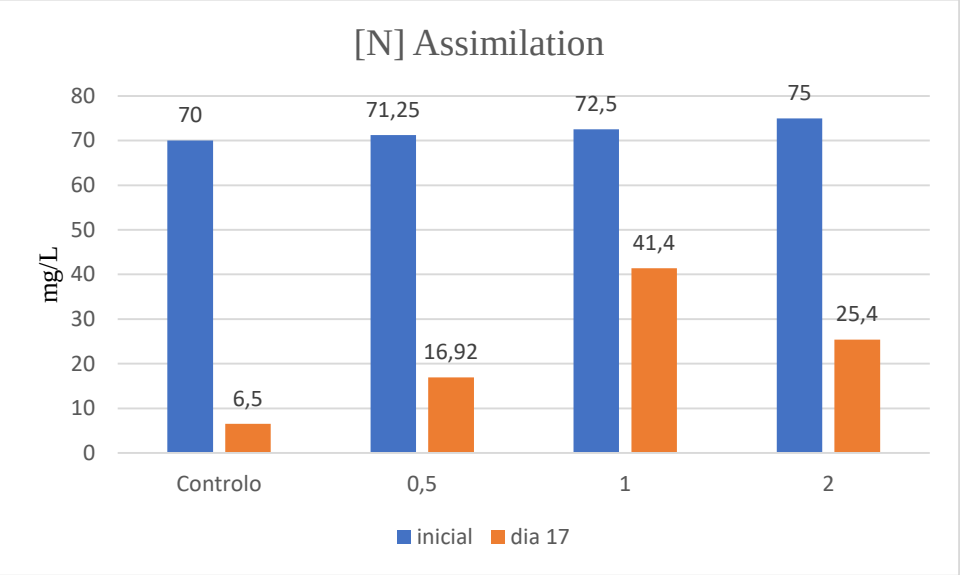

Fig. 4.5 – Nitrogen assimilation in the first series of essay with OMWW

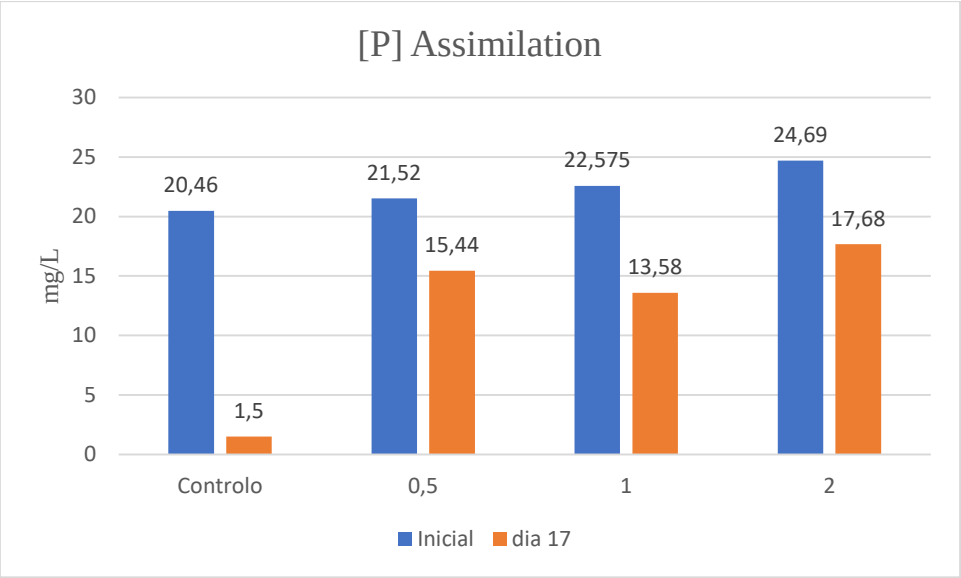

Fig. 4.6 – Phosphorous assimilation in the first series of essay with OMWW

From the figures we can see that the uptakes of N were of 90.71 for Control, 76.25% for 0.5% (v/v), 42.9% for 1% (v/v) and 66.13% for 2% (v/v), while the uptakes of P were of 92.67% for Control, 28.25% for 0.5% (v/v), 39.85% for 1% (v/v) and of 28.39% (v/v) for 2%. These values appear to indicate that there was a significant assimilation of macronutrients, particularly nitrogen by the microalgae culture, being as such capable of bioremediation of the macronutrients existent in the substrate. However, it should be remember that the OMWW has a severe nitrogen deficiency, as already reported previously Hodaifa, Martínez, Órpez, & Sánchez, (2012) and was observed in section 3.3.4.1 table 3, and in our case N and P was mainly provide by the stock solution 1. Comparing the N and P assimilation with microalgae growth and COD evolution, there is, however, a reasonable discernible pattern; Control, which has the highest uptakes, and supposedly the lowest COD is the one with the highest growth; the samples with OMWW substrate exhibit a lower N and P uptake which roughly is in accordance with the microalgae grown.

For the second series of experiment the results are indicated in figs. 4.7 and 4.8.

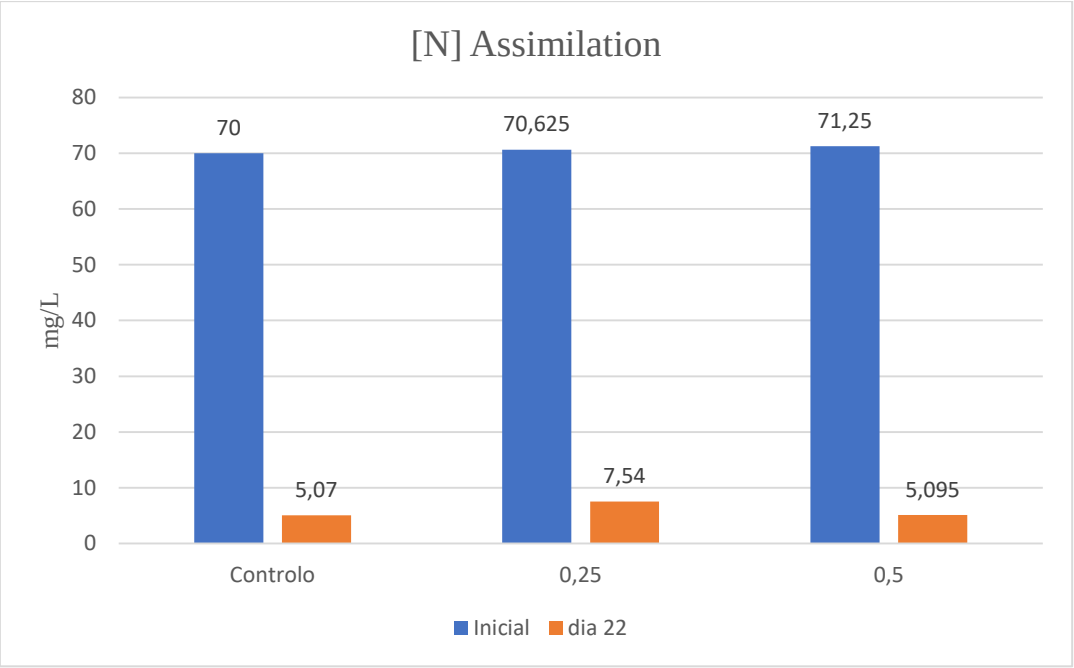

Fig. 4.7 – Nitrogen assimilation in the second series of essay with OMWW

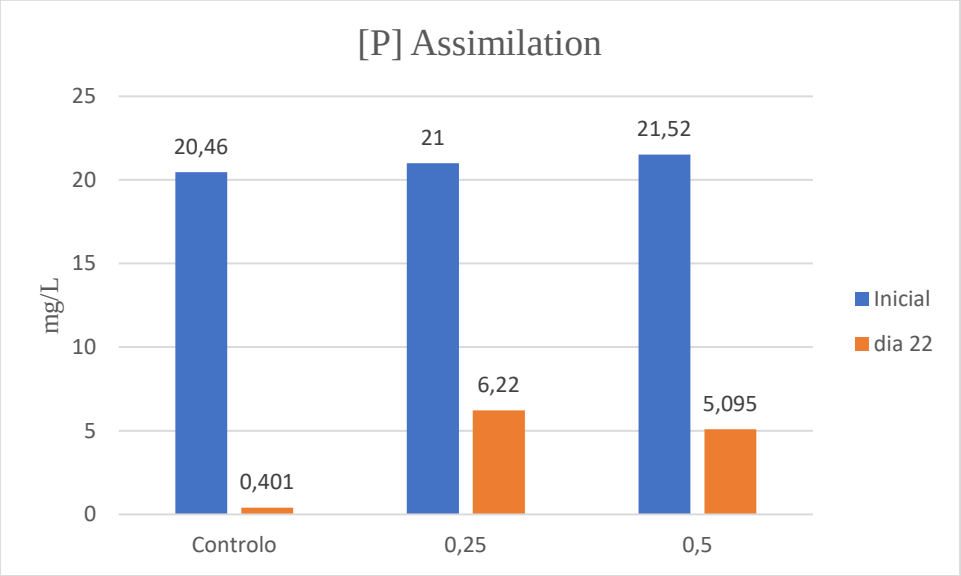

Fig. 4.8 – Phosphorous assimilation in the second series of essay with OMWW

In these essays the uptakes for N are of 92.76% for Control, 89.32% for 0.25% (v/v) and 92.85% for 0.5% (v/v), while for P the values are 98.04% for Control, 70.38% for 0.25% (v/v) and 76.32% for 0.50% (v/v). These are higher uptake values than in the first essays. These essays lasted more time, but the main reason for the higher assimilation is the fact that there was greater microalgae growth in the samples with OMWW and similar one in Control. However, once again, here the fact is that most of the nutrients assimilated were not present in the effluent, but rather added through stock solution 1, nonetheless the bioremediation potential of microalgae in OMWW is attested as they have similar assimilation uptakes to Control. In

this particular essay the culture was successful in also lowering COD, which seems to indicate that *C.vulgaris* has great potential in treating OMWW but this occurs only at higher dilution values and with the addition of macronutrients N and P to compensate for their low presence in the effluent.

4.2 - Leachate Wastewater (LWW)

4.2.1 - Growth

As mentioned in subsection 3.2.4.2, Leachate is a type of wastewater originated from landfills that is rich in macronutrients.

Like the previous wastewater, growth was analysed by means of Neubauer Chamber with a dilution of 1:30 and 2 essays were made. However different characteristics had to be taken in account with this new wastewater; unlike OMWW, this effluent has a more balanced ratio of 126:102:1, this means that there is almost equal amounts of C and N, typical of leachates that as mentioned have a very high nutrient content. Fig. 4.9 shows the average growth of the different samples.

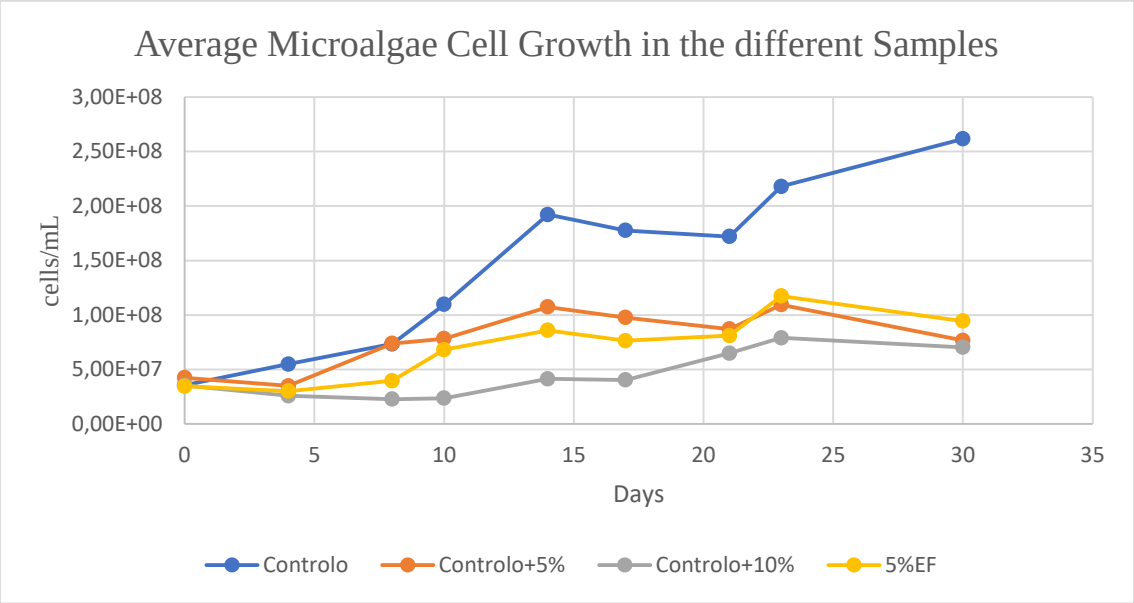

Fig. 4.9 – Average cell growth for the different samples of the first series of essay with LWW

From this figure we can then see that from a rather similar initial concentration, the Growth rates of the different samples rapidly began to differ between them around day 4, with Control with a faster growth. Controlo+5% began to widen their differences with the

remaining LWW substrates after day 10. From that day forward, we clearly see that Control had a much higher growth rate that any of the WW samples, as expected, as the microalgae in those bioreactors weren´t in the same stress conditions as the others due to the chemical composition which affected their growth.

In the bioreactors with the samples of LWW, Control+10% had the lowest growth rate among the three. Meanwhile, Controlo+5% and 5% EF without additional nutrients had a rather similar growth rate, and despite beginning to have a decline in the cell concentration for the end of the experiments it still registered the triple of the original microalgae concentration, in the meantime, Controlo+10% registered the lowest growth among the substrates tested in this essay.

The results obtained, especially the similar data between Controlo+5% and 5% EF. encouraged us to perform another experiment with LWW. Thus this time, the samples would consist only of diluted LWW at the same concentrations as before, but with one of the samples (Controlo+5%) having been added mixed-cultures of microorganisms, as according to Qi et al. (2018) co-cultures of microalgae and bacteria have a better performance than pure cultures. A previous study by Nordin, Yusof, & Samsudin (2017) showed that *C. vulgaris* growth rate in LWW was better at 20% (v/v), while Paskuliakova et al. (2018a) [37] cite that growth was recorded even at 30% (v/v). Here we did not used more than 10% (v/v), even so, our growth was higher at 5% EF. (v/v) which does not have stock solution 1.

In this second experiment, that lasted for about 33 days, we tried to ensure that there was again a relatively similar initial microalgae concentration between the different bioreactors. Like in the previous experiment by the fifth day there was already a notorious difference between the different samples, with Controlo (two reactors) having a growth rate clearly above the rest of the samples. However, also by the fifth day, the microalgae in the bioreactors 10% I and II died, due to a very high pH increase. This unexpected increase in pH killed the microalgae in both replicas. This effect of pH is in accordance with the mentioned by Paskuliakova et al (2018a) [37]. As fig. 4.10 shows, until day 13, there was a high similarity with the growth of samples 5% and 5% m. (Mix culture)

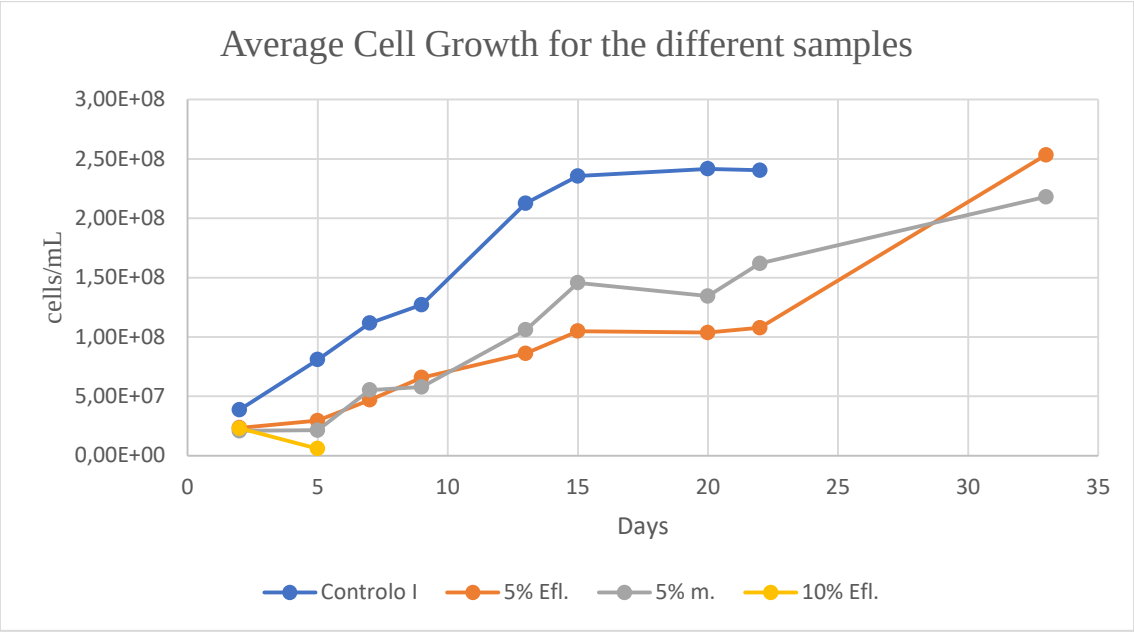

Fig. 4.10 – Average cell growth in the different samples in the second series of essays with LWW

As it is observed before, 5% Efl. and 5% m. had a similar growth until day 13, when 5% m. started to have a greater rate, although by the final stages of this experiment it had a slightly lower concentration of cells than the 5% Efl. essay, but both with cell concentrations far higher than the first series of experiment made.

These series of experiments clearly show this effluent can be used as a source of nutrients for C. vulgaris growth, because the cell concentration increased 752.3% in 11 days. In resume, LWW has great potential for biomass growth, as mentioned in literature [36] and [37]. These authors reported that LWW demonstrated to be a reliable substrate for microalgae growth during an extended period of culture, albeit dilution values and pH proved to be a more influencing factor than the N:P ratio.

On the other hand, in our particular case, a microalgae-bacterial synergy was not as effective as expected in 5% Efl. (v/v), but this can be partly explained due to factors already explained in subsection 2.4, such as competition and parasitism. In fact, the microbial biomass introduced in the microalgae cultures had a unknown content apart from the fact that it was a mixed aerobic culture, as such, these factors must carefully be taken in account in the results obtained.

4.2.2 – COD Content

Several samples were collected during the duration of the essay to analyse the evolution of Chemical Oxygen Demand (COD) present in the culture medium. In this case, our initial value was of 8753 mg/L, as such the values of 5% (v/v) and 10% (v/v) would be of 437.5 mg/L and 875 mg/L respectively, and with more or lesser degree, all our samples had approximate initial values to these.

For the first experiment with LWW once again the averages of the different samples were calculated as shown in Fig. 4.11.

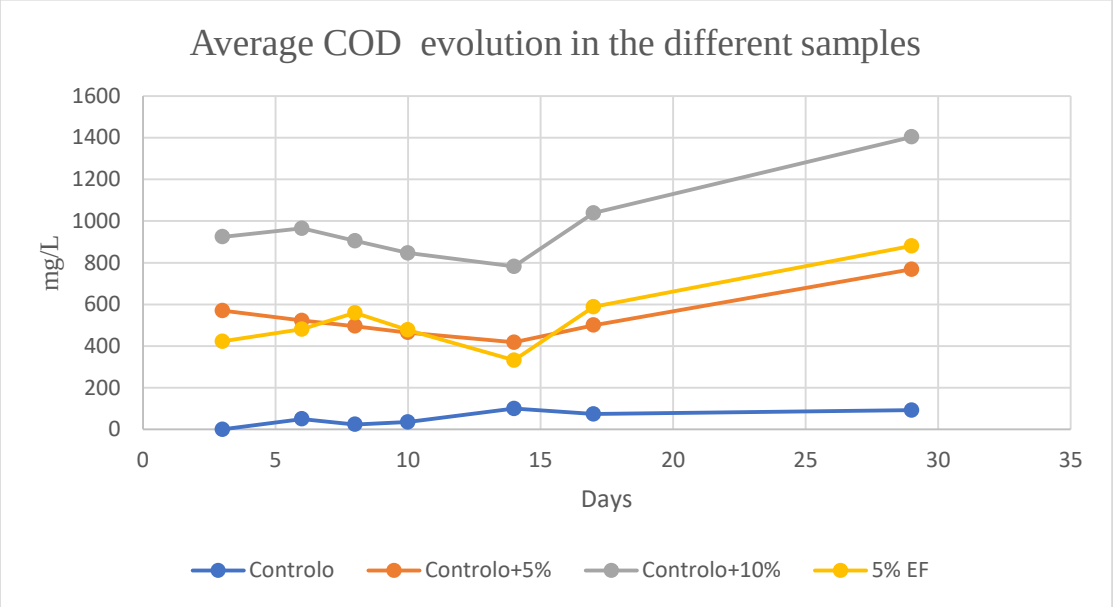

Fig. 4.11 – Average COD values for the different samples during the first series of essays with LWW

Due to the sensible nature of COD, generally the obtained results should be taken in account in combination with other factors despite their importance to treatment of effluents. In fact most of the articles, such as Paskuliakova et al (2016) [38] rarely mention it, with more importance being given to the consumption of macronutrients. In addition, it is known that the COD from a leachate wastewater (LWW) is very recalcitrant and therefore with low biodegradability.

Our data from the first essay shows that the COD present in the samples with LWW show no signs of decreasing, instead in all the samples it slightly increases. Nordin et al. (2017) [36]

however mentioned that COD decreases during the course of its essay, although the decrease is not significant.

For the second series of experiment the same process was repeated, with samples collected and analysed, except for the bioreactors that contain the 10% sample. The data is thus shown in Fig. 4.12.

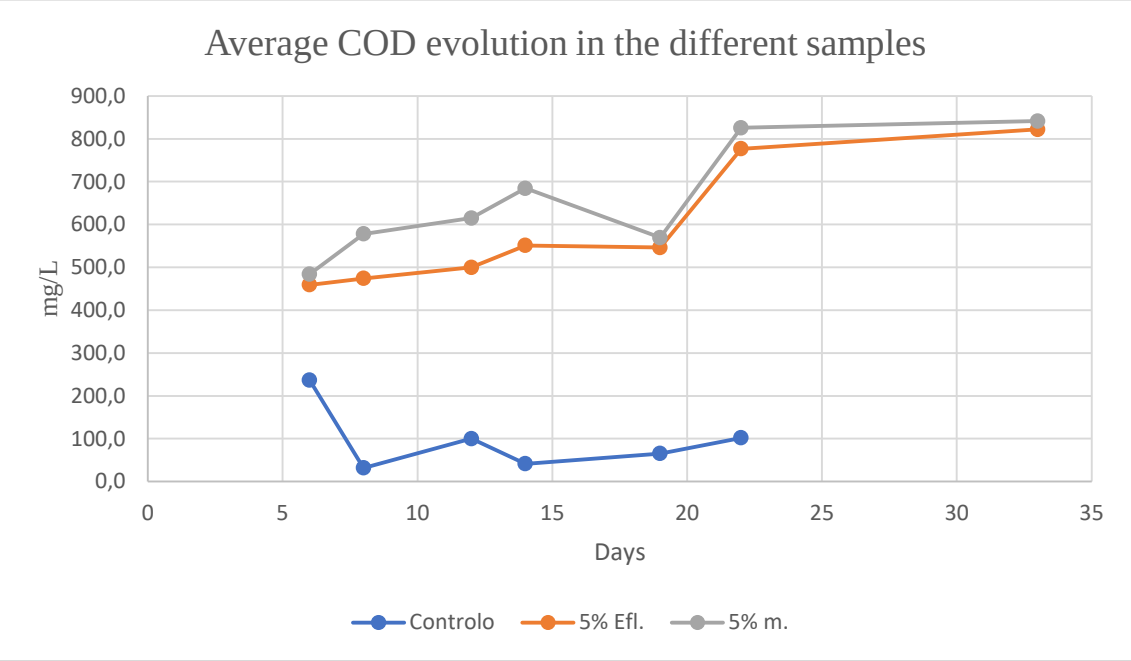

Fig. 4.13 – COD averages for the different samples in the second series of essays with LWW

As expected COD in Control is very low, while in the LWW samples have around the expected values of 437.65 mg/L for 5% Efl. (v/v) for this effluent. However, the values for the replicas with LWW increase over the duration of the experiment, although it stabilizes around the 22nd day. The addition of a mixed aerobic culture of microorganisms to test the effects of synergy between microalgae and bacteria did not showed any differences from the axenic cultures.

Thus, from the data assembled from our essays as well as comparing with data from Nordin et al. (2017) [36] it appears that COD may be not the best parameter used to follow the bioremediation. Instead, as seen in subsection 4.2.3, it is the assimilation of macronutrients, in high concentration in this type of effluents, that should be used as references for analysing the effectiveness of bioremediation by microalgae.

4.2.3 – N and P Assimilation

Like it was the case in OMWW, the N and P consumption was measured, as like the COD content, it is a sign of bioremediation. Particularly in this case, due to the very high concentration of macronutrients recorded in the different types of leachates. As such, unlike COD, assimilation of macronutrients is seen as the main indicator of effluent treatment in LWW. As mentioned in subsection 3.2.4.2, there is a very unfavourable N:P ratio of 102:1. Mandalam & Palsson (1998) [39] mention that a favourable or rather a balanced ratio increases growth, so as mentioned in 3.2.4.3 and 4.2.1, two of the samples in the first experiment, 5%(v/v) and 10% (v/v) had stock solution 1 added to them to try to create a more favourable ratio.

In the first series of essays, the initial and final concentrations of nutrients were calculated. Keeping in mind that despite the original concentration being 2665 mg/L of TN and 26.1 mg/L of P, since the dilutions used were 5% (v/v) and 10% (v/v) the initial concentrations in the different samples were actually 133.25 mg/L and 1.305 mg/L for the first and 336,25 mg/L and 2.61 mg/L for the second; however, since stock solution 1 was added in 2 of our samples, the final initial concentrations were higher (see fig. 4.13 and 4.14).

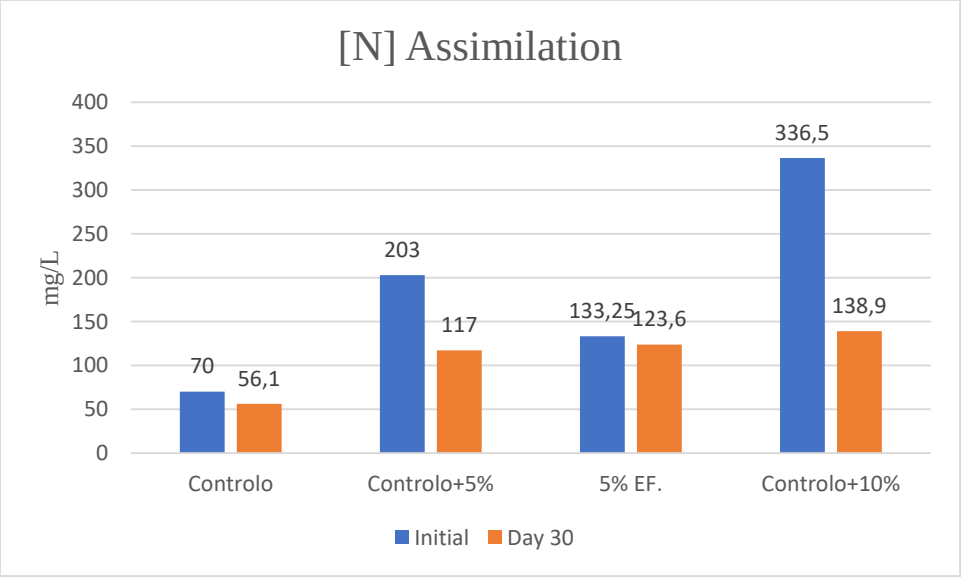

Fig. 4.13 – Nitrogen assimilation in the first series of essays with LWW

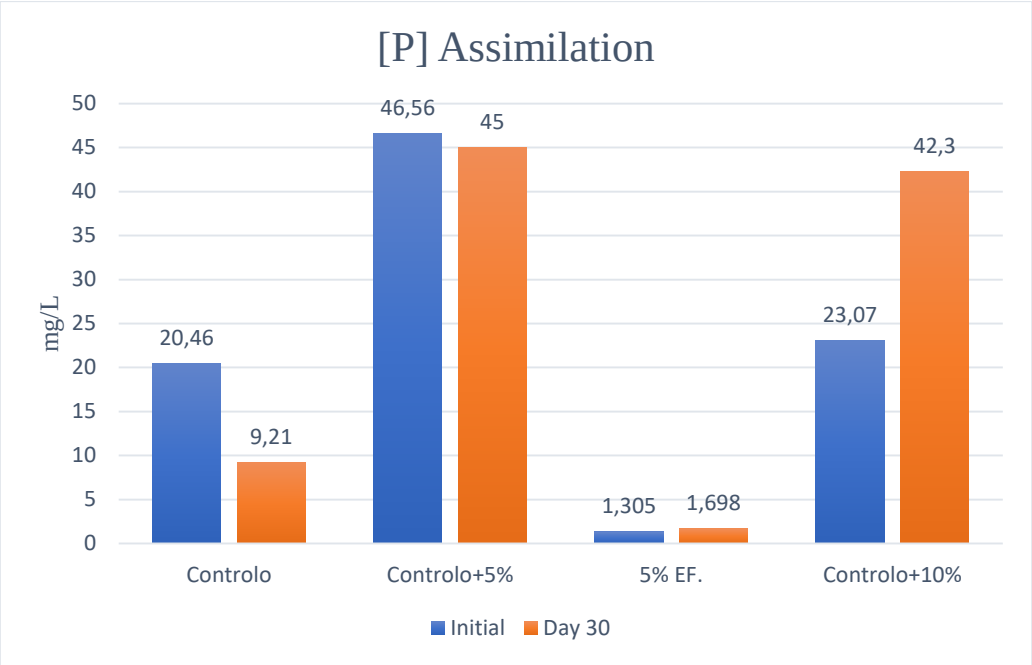

Fig. 4.14 – Phosphorous assimilation in the first series of essays with LWW

The results for assimilation of N and P in this first series of essays were unexpected. Even for the control essay, particularly the N assimilation was lower than that observed in the series with OMWW. In fact, the mg of N/cell is much lower in these essays compared with those for OMWW ($0.7x10^{-10}$ vs $3.5x10^{-10}$). For the essays with 5% (v/v) of LWW the experimental were unexpected, particularly for P and require further investigations. However, it should be noted that the cell increase was also very limited.

For the second series of essays with LWW the initial and final concentrations were calculated taking in account the dilutions used (Fig 4.15 and 4.16).

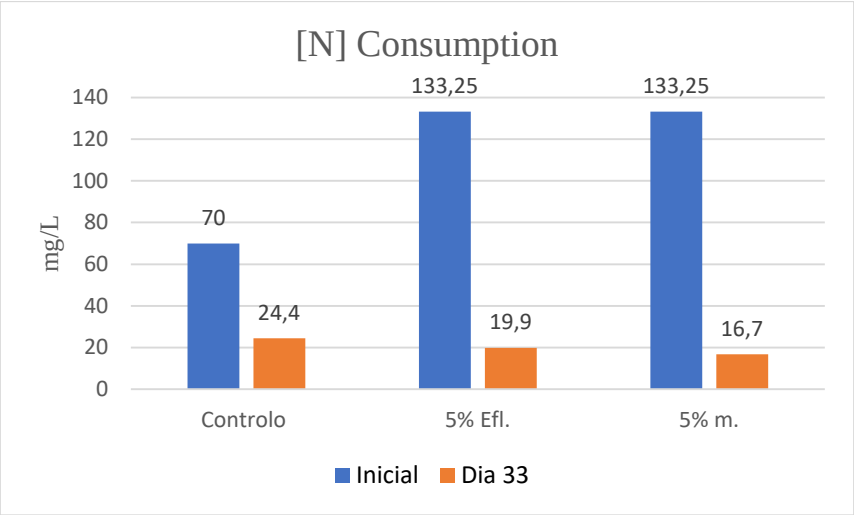

Fig. 4.15 – Nitrogen assimilation in the second series of essays with LWW

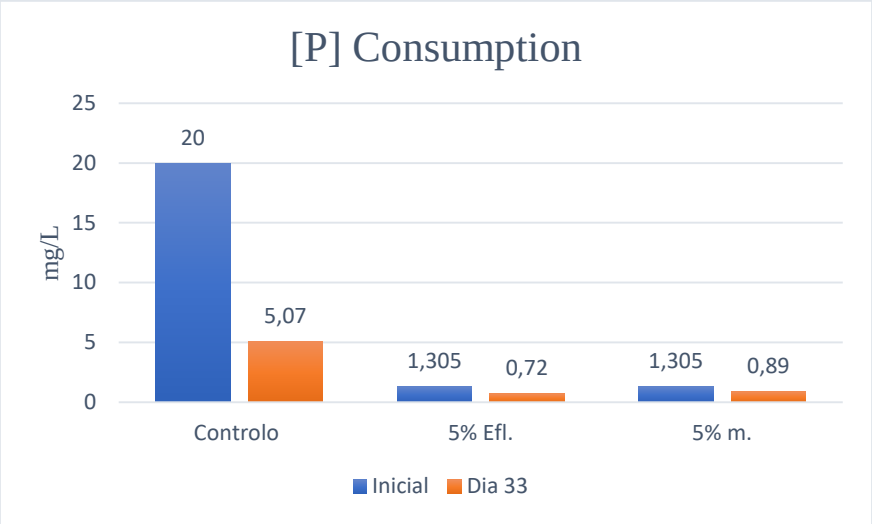

Fig. 4.16 – Phosphorous assimilation in the second series of essays with LWW

The data shows us that the assimilation of N and P in 5% Efl.(v/v) was 85.06% and 44.83% while 5% m. (v/v) was of 87.47% and 31.8%. By contrast, Paskuliakova et al (2016) [38] reported that phosphate assimilation was higher than the nitrogen assimilation.

Interestingly, the cell growth was very significantly despite the very low initial P content for the essays with LWW. According to Pereira et al (2016) [40] phosphorous is critical and enhances uptake of macronutrients, that means that a more balanced N:P may in fact be important to assimilation. Mandalam & Palsson (1998) [39] using another medium already demonstrated that balancing the elements enhances biomass growth. In our case, however, a very good microalgae growth occurs despite the extremely low content of P in the medium. One possible explanation for this abnormal behaviour can be the P present in particulate

matter that the measurement procedure does not take in account because the medium is submitted to a filtration step before determination.

In resume, the data demonstrated that *C.vulgaris* has the ability to uptake N and P from a LWW and growing at a sizable enough rate to be considered worthy of extraction.

Another important note to be taken from here is that the potential synergetic use of bacteria and microalgae in this effluent appears to not have had a significant impact on in either of the parameters studied. The reason for this is unknown to us, but due to the high availability of macronutrients in this effluent, even after being electrochemically treated precludes one of the main positive effects of a microalgae-bacterial relationship; another reason for this may be simply the inability of the used bacterial culture to survive in such nutrient rich environments, thus their impact would naturally be smaller as they do not have the necessary biomass to have a positive effect on microalgae.

4.3 – Municipal Wastewater (MWW)

4.3.1 - Growth

The third wastewater used is Municipal Wastewater (MWW) and for these experiments two collection points were used: effluent from the beginning of the process of treatment, that means, raw wastewater before the primary treatment, and from the end of the secondary treatment.

This WW is one of the most commonly used for microalgae growth due to the fact that it is the most readily available. In this last series of experiments once again we decided to use biomass to enhance microalgae growth [9], however, unlike the biomass used in LWW, in this particular instance we decided to use full aerobic microorganism.. Samples A1 (raw WW) and A3 (after Secondary Treatment), without sterilisation, were used as Control. The same samples were sterilised and in the A3 sample aerobic bacteria were added in different proportions, leading to aerobic biomass concentrations of 0.027 mg/L and 0.055 mg/L. Due to limited supply of WW, the total volume of the culture was 400 mL instead of the 500 mL previously used.

Occurring during a period of 14 days, the experiment began the same way of the previous ones, by measuring pH value and due to its alkaline values, its pH was adjusted to around 6.5 for the different bioreactors, as well as trying to ensure a similar initial concentration of microalgae to all the eight bioreactors involved.

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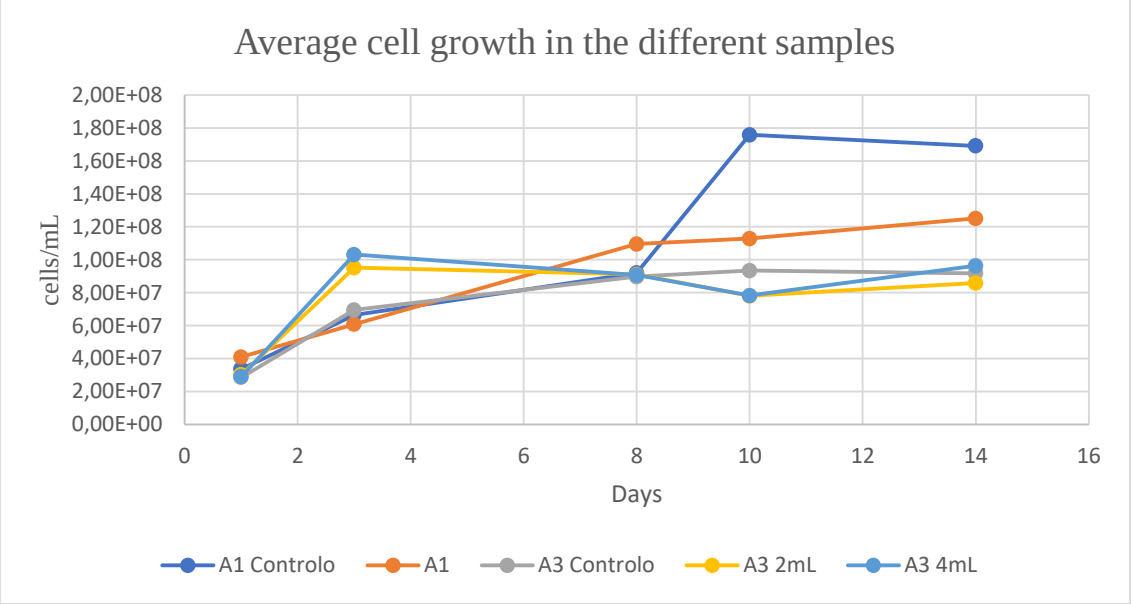

Fig. 4.17 – Average cell growth in the essay with MWW

As can be seen in the figure above, there is no significant difference between A1 Control (without effluent sterilisation) and A1 (with effluent sterilisation) until 8th day. Later, the no sterilised effluent samples enable additional microalgae growth, which suggest that the native bacteria of the effluent have the ability to liberate nutrients that potentiate the microalgae growth; bacteria would had helped microalgae to access more nutrients after the assimilation of the easier ones. These results are in accordance with studies of Cho et al (2015) [17] that demonstrated strong synergistic actions between *C. vulgaris* and several other microorganism; albeit the study was made in a different type of wastewater, our data nonetheless appears to indicate that in MWW this actions also exist and are a worthwhile way to enhance microalgae cell growth. To evaluate this hypothesis, the COD and the N and P should be monitored, but only COD was determined which not enable definitive conclusion. It should be also noted that the duplicate reactors (A1) exhibited a slightly different behaviour (data not shown).

A3 (sterilised effluent) exhibits a profile similar to A1, but with lower microalgae growth in accordance with the lower concentration of nutrients. A3 was further downstream of a wastewater treatment plant, thus several characteristics of A1 were already lost. Regardless it is one of the most commonly used substrates for microalgae cultures. However, as mentioned above it has lower N and P that A1, thus being more vulnerable to nutrient

scarcity after some time. Compounded this, as mentioned in subsection 2.3, the Secondary Treatment involves the reduction of indigenous microorganism present in the effluent which further reduces the available macronutrients for *C. vulgaris* as some of them are beyond the algae capacity to assimilate, thus, by the time the effluent reaches Tertiary Treatment, its indigenous bacterial population was already mainly eradicated.

Aerobic bacteria (2 ml and 4 ml of bacterial biomass suspension, corresponding to 0.035mg/L and 0.065mg/L of biomass respectively) were added to the sterilised A3 effluent (A3 2ml and A3 4 ml). Despite a higher microalgae growth in the first days (day 3) the microalgae concentration stagnated thereafter. For the corresponding A3 sample (no sterilised), the microalgae growth starts slower, but reach at the end similar biomass (cell) concentration. In resume, the added bacteria and the indigenes bacteria led to the same microalgae production. In addition, after 14 days, all the A3 samples had an inferior yield to A1, sterilised, but with higher initial nutrient concentration.

Therefore, growth was far higher in A1 Control and A1 than in either of their A3 Control, A3 2mL and A3 4mL counterparts despite biomass being added in A3 to enhance assimilation. In fact, there was no significant difference between the different reactors using A3 as substrate; this may indicate that the microalgae present did not managed to assimilate enough nutrients to ensure a higher growth or that the bacterial biomass added was detrimental to its survivability (see section 2.4). However, the bacteria present do not explain the fact that A3 Control had similar concentration to A3 2mL and A3 4mL at the end of the essay. While the reason for this is unknown as the concentration of macronutrients used were similar to other used in previous effluents, the fact remains that these may have not been the necessary amount (in contrast to the ones in A1 Control and A1) and/or were not easily accessible to the microalgae and the bacterial biomass did not had the ability to supplement this deficiency, which may have thus led to the stagnant growth observed for this particular substrate.

By contrast, the bottles A1 Control and A1 reported very high growth. As reported, the difference between these bottles was that the first one was not sterilised and therefore maintained the indigenous bacteria of the effluent (it must be reminded that A1 is MWW **before** any kind of treatment to remove bacteria and macronutrients). Previously, studies by Gonzalez & Bashan (2000) [11] using alginate beads with the bacteria *Azospirillum brasilense* demonstrated the potential of axenic cultures and recently, studies by Toyama et al. (2018) [41] in wastewaters with *C. vulgaris* tested Secondary Treatment MWW as both autoclaved as Control and with the indigenous bacteria.

Their results notwithstanding the fact that the effluent was from a part of the wastewater treatment that we did not used, were remarkably similar in effect with the cultures without indigenous bacteria also reporting stagnating growth compared with the ones with bacteria. A1 Control, as the one that was not sterilised and thus had the original indigenous bacterial

population had the highest growth rate among the bottles used in this essay; as mentioned, this correlates to the previous studies mentioned above. By contrast, the A1 replicas were sterilised; comparing with the A3 essays the growth by A1 was higher tough not as high as A1 Control. The reason for this maybe lies with the fact that this stage of the effluent had higher concentrations of macronutrients than A3 (see Table 5 in subsection 3.2.4.4) thus compensating the lack of bacteria for helping with nutrient assimilation with the sheer amount of available macronutrients in the substrate, ensuring a higher growth. By comparison of A1 Control and A1, the fact that the first had indigenous population clearly appears to be the main reason for the differences in growth, thus being classified as MGPB as no other reasons were determined, such as Ph, and C distribution that could influence the results.

4.3.2 – COD Content

For this experiment COD was measured during a period of 16 days, using once again the Closed Reflux Tithrimetric Method using samples of 1 mL and 1.5 mL in the last measurement.

Noteworthy in this series of experiments was the fact that, COD in general diminished over time. For the A3 2mL and A3 4 mL (with aerobic bacteria) there are some fluctuation but it is not easy to relate these fluctuation with a possible microalgae decomposing after the initial growth or microalgae and bacteria die due to age or pH increase (Fig. 4.18).

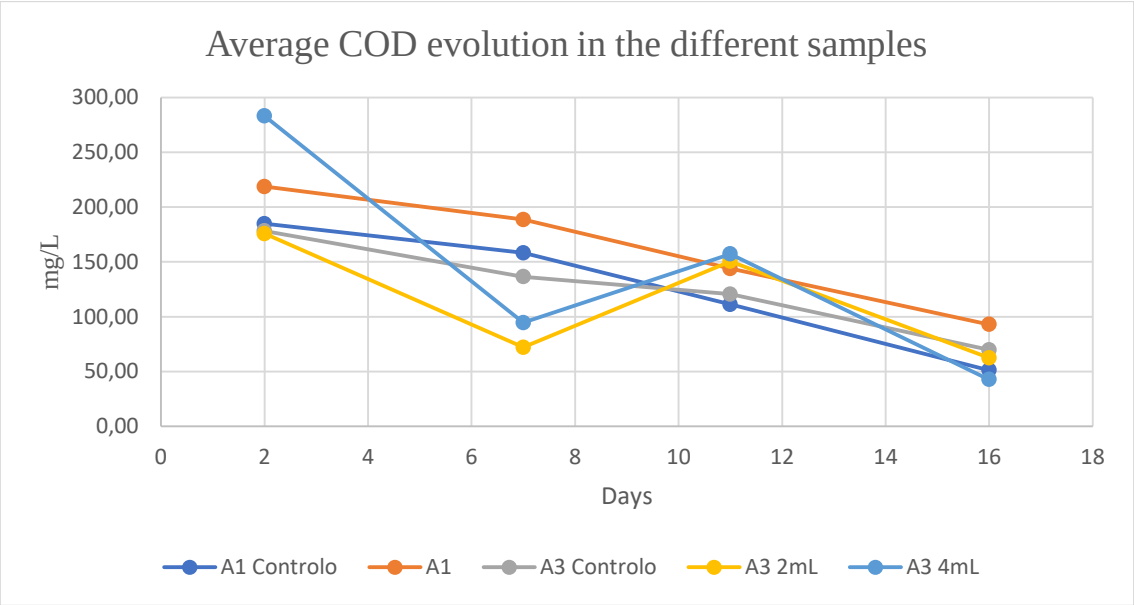

Fig. 4.18 – COD evolution for the duration of the essay with MWW substrate

The general decrease of COD suggests that microalgae alone or in combination with bacteria may play a significant role in MWW effluent treatment. However, the similar trend observed for A1 Controlo and A1 (sterilized) deserves additional research in order to clarify the individual role of each microorganism.

Compared with subsection 4.3.1 there is not a large variance between the several different substrates used. While initial COD values should be similar to the ones mentioned in table 6 in subsection 3.2.4.3, the fact that COD measurements were only made in the 2nd day of this essay and because COD is known to drop and varying rapidly, may indicate us that there was a decrease in this parameters value from the start, especially taking in account the fact that C was supplied to this essay at regular intervals. Nonetheless, there appears to be a sudden drop in COD value around day 11, for the samples A3 2mL and A3 4mL that by the next measurement was higher and similar to the other bottles. This may be due to the fact that in the case of A1 Control, the concentration was stabilising, thus until the existing population adapted to the current conditions and as mentioned, due to the supplying of C through CO_2 may have led to its stabilisation, in fact the stabilisation of population in practically all our samples around the same time and like mentioned, the fact that C was still continuously being supplied may answer the reason for this until the microalgae existent in the cultures adapted to the current conditions and once again began to consume and thus lowering the value of COD. However, most likely the reason for this was due to an experimental error by our part, it is also needed to say once again that the results obtained by COD measurement tend to be difficult to read over a long period of time, thus making COD a parameter seldom

used in the bibliographical references used in comparison to the other parameters discussed in the course of this work.

Another interesting note is that the samples were COD value was lower appeared to the ones were aerobic biomass was added to the substrate. This, compared with growth appears to indicate that due to being indigenous lifeforms to the effluent, these bacteria present have adapted to become more tolerable to higher values of COD and therefore it appears that the presence of these bacteria have little practical difference with the foreign ones in regards to lowering the COD with their greatest difference being their different effectiveness in helping microalgae growth (see subsection 4.3.1). This leads to an interesting scenario where the samples with the largest yield do not necessarily correspond to the ones where the effluent treatment was more effective, therefore creating a problem if the objective is maximising both. Yet, the fact that A1 still recorded a diminishing COD value indicate that while microalgae may not be as effective in its treatment, judging by their values, it may nonetheless present the best all-around option if the objective is **simultaneously** treat the MWW and guarantee the highest yield.

Chapter 5 – Conclusions and Future Perspectives

Throughout this work the essays made led to a deeper understanding of the properties of different effluents and their potential as substrates for microalgae. Another important take was the synergetic potential of a mixed bacterial-microalgae culture both for enhancing growth for microalgae, which, in conjunction with effluents may reduce the high costs associated with large scale microalgae cultivation in industry as well as the cultures potential as a bioremediation process for the effluents tested here with the objective of finding new and more cost effective ways of treatment of polluted watercourses that would otherwise be very difficult to return to pre-pollution levels. The main conclusions of the work were as followed:

● The effectiveness of the effluent OMWW as a substrate was tested in two series of essays using four different dilution values (2% (v/v), 1% (v/v), 0.5% (v/v) and 0.25% (v/v) with added macronutrients and in the case of the first series CO_2 to compensate the very unbalanced C:N:P ratio. By the end of the essays it was observed that the dilution 2% and 1% (v/v) were unsuitable, with little growth occurring at these concentrations, while 0.5% and 0.25% (v/v) registered moderate growth that was very similar between them. This indicates that OMWW, due to its unbalanced C:N:P ratio and high phenol and other organic compounds contents is a moderately effective substrate at high dilution and after being previously filtrated.

● The bioremediation potential of microalgae was measured using COD and nutrients (N and P) assimilation. In the case of OMWW C. vulgaris showed promising results in uptake rates, assimilating most of the macronutrients present in the substrate. COD however was inconsistent due to the moderate growth registered in this effluent and therefore should be used as a nonspecific global parameter for analysing the results, nonetheless the general trend was for continuous, albeit slow decline of the COD value.

● LWW, was tested in two series of essays with one of them using a mixed bacterial-microalgae culture to test the synergetic potential in growth and bioremediation. The values obtained in growth showed that lower dilution values than the ones used in OMWW can be used, even with no added macronutrients to compensate the C:N:P ratio, and growth rate are higher than the ones in OMWW tough the difference between the xenic and axenic cultures was insignificant.

● The bioremediation of this effluent, known for its very high macronutrients concentration, was once again tested with COD and nutrient assimilation with the COD values showing little

to no sign of decreasing, thus little conclusions can be taken from this parameter. The nutrient assimilation however, despite a certain experimental error in the first series of essays showed great potential in the second series, with large assimilation values that are critical in such a nutrient rich effluent, suggesting great potential in bioremediation of these types of effluents.

● The third effluent tested, MWW, was where the majority of studies using synergy between bacteria and microalgae was made. The work showed that the use of bacteria indigenous to the effluent in question has the greatest potential for microalgae growth whit added bacterial cultures having a more limited impact.

● For bioremediation only, COD was tested and showed a slow but steady decline of its value for the duration of the essay, but no significant differences were reported between the substrates with the indigenous bacteria and the ones with foreign ones, nonetheless, the fact that the first ones clearly had a better growth rate indicates that they should be persecuted as a better all-around synergetic model.

Therefore, in this way, the bioremediation and growth potential of xenic and axenic bacteria-microalgae cultures was tested in different types of effluents showing that attention is needed to factors such as pH, organic compounds and macronutrients concentration and the correct bacterial culture.

Nonetheless, with the correct use of the factors mentioned above, a C. vulgaris culture in general, and synergy between indigenous bacterial-microalgae cultures in particular showed enormous prospects for further research in order to ensure a more cost-effective and environmental friendly approach to several industries, particularly the ones involving microalgae culture or heavy use of water-based pollutants.

Chapter 6 – Bibliographical References

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