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Wastewater polishing with microalgae

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Abstract

The need to find alternatives to the conventional methods for wastewater treatment has been growing in order to provide strategies with higher overall efficiency. In this context, there use of microalgal cultures in new methodologies has been implemented steadily in the processes of wastewater treatment, as its use has shown consistent and competitive results with the conventional methods, particularly activated sludge-based treatments. However, it is also known that the composition of wastewater, in particular the C:N:P ratio, affects microalgal growth and the nutrients removal. On the hand, new contaminants of different chemical natures are constantly appearing in wastewaters as the implement methods are not effective for their removal. Thus, the aim of this work was to evaluated the effect of the C:N:P ratio on nutrients removal efficiency by *Chlorella vulgaris*. The microalga, suspended and immobilized, was also tested on the bioremediation of an antibiotic, ciprofloxacin.

Firstly, diverse C:N:P ratios were tested on *C. vulgaris* growth and nutrients (phosphorous and nitrogen) removal. Afterwards, ciprofloxacin was added to the growth medium to evaluate the role of *C. vulgaris* on its bioremediation as well as on nutrients removal. Then, in order to increase the process stability the *C. vulgaris* was immobilized in sodium alginate beads and its efficacy on nutrients and ciprofloxacin removal was assessed under various C:N:P ratios.

The results demonstrated that C:N:P ratio affected microalgal growth and nutrients removal. The ratio causing highest growth and removal efficiencies was 1.8:9:1. On the other hand, it was also found that microalgal immobilization was not only successful at increasing average biomass productivity, from 0.038±0.001 g DW L^{-1} d^{-1} to 0.086±0.004 g DW L^{-1} d^{-1}, and the specific growth rate, from 0.195±0.002 d^{-1} to 0.457±0.013 d^{-1}, but also phosphorous and nitrogen removal efficiencies. Ciprofloxacin removal efficiency was 48.67±0.20% and the removal rate was 0.198±0.002 mg S L^{-1} d^{-1}.

Thus, from the analysis of the results it can be concluded that the *C. vulgaris* cultures used in this thesis were successful at wastewater polishing, including in the bioremediation of ciprofloxacin.

Keywords: Microalgae; *Chlorella vulgaris*; C:N:P ratio; Nutrient removal; Ciprofloxacin.

Resumo

A necessidade de encontrar alternativas aos métodos convencionais de tratamento de águas residuais tem vindo a crescer, de modo a fornecer estratégias com eficiências superiores. Neste contexto, o uso de culturas de microalgas em novas metodologias tem sido progressivamente implementado em processos de tratamento de águas residuais, particularmente no tratamentos de lamas. Contudo, sabe-se também que a constituição das águas residuais, em particular o rácio C:N:P, afeta o crescimento de microalgas e a remoção de nutrientes. Por outro lado, contaminantes de diferentes naturezas estão constantemente surgindo em águas residuais visto que os métodos implementados não são eficazes na sua remoção. Assim, o objetivo deste trabalho foi avaliar o efeito do rácio C:N:P na eficiência da remoção de nutrientes pela *Chlorella vulgaris*. A microalga, suspensa e imobilizada, foi também testada na biorremediação de um antibiótico, a ciprofloxacina.

Primeiramente, o crescimento da *C. vulgaris* e a remoção de nutrientes (azoto e fosforo) foram testados para diversos rácios de C:N:P. Posteriormente, a ciprofloxacina foi adicionada ao meio de cultura de modo a avaliar o papel da *C. vulgaris* na sua biorremediação e na remoção de nutrientes. Em seguida, a fim de aumentar a estabilidade do processo a *C. vulgaris* foi imobilizada em esferas de alginato de sódio e a sua eficácia na remoção de nutrientes e de ciprofloxacina foi avaliada para diferentes rácios C:N:P.

Os resultados obtidos demonstraram que o rácio C:N:P afetou o crescimento da microalga e a remoção de nutrientes. O rácio que obteve maior crescimento e eficiência de remoção foi o de 1.8:9:1. Por outro lado, também foi estabelecido que a imobilização de microalgas não foi apenas bem sucedida no aumento da produtividade média de biomassa, de 0,038±0,001 g DW L^{-1} d^{-1} para 0,086±0,004 g DW L^{-1} d^{-1}, e sa taxa de crescimento específica, de 0,195±0,002 d^{-1} a 0,457±0,013 d^{-1}, mas incrementou também as eficiências de remoção do fósforo e do azoto. A eficiência de remoção de ciprofloxacina foi de 48,67±0,20% e a taxa de remoção foi de 0,198±0,002 mg L^{-1} d^{-1}.

Assim, pela análise dos resultados obtidos pode ser concluído que as culturas de *C. vulgaris* utilizadas durante esta tese foram bem-sucedidas no tratamento de águas residuais, incluindo na biorremediação de ciprofloxacina.

Palavra-chave: Microalga; *Chlorella vulgaris*; C:N:P rácio; Remoção de nutrientes; Ciprofloxacina.

Declaration

I hereby declare, on my word of honour, that this work is original and that all non-original contributions were properly referenced with source identification.

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Nomenclature

Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BOD	Biochemical Oxygen Demand
COD	Chemical Oxygen Demand
DL	Detection Limit
DW	Dry Weight
FAS	Ferrous ammonium sulphate
NH_3-N	Ammonia-nitrogen
NH_4-N	Ammonium-nitrogen
NO_2-N	Nitrite-nitrogen
NO_3-N	Nitrate-nitrogen
OD	Optical Density
OECD	Organization for Economic Co-operation and Development
PCs	Pharmaceutical Contaminants
PE	Population Equivalent
PO_4^{3-}	Inorganic phosphate
PO_4-P	Phosphate-phosphorus
ROS	Reactive Oxygen Species
QL	Qualification limit
UK	United Kingdom
USA	United States of America
Tss	Total suspended solids

Notation

k	Nutrients uptake rate (d^{-1})
M_{FAS}	FAS molecular mass (g mol^{-1})
N	Cell concentration (cells mL^{-1})
N_1	Cell concentration in the beginning of the exponential growth phase (cells mL mL^{-1})
N_2	Cell concentration in the end of the exponential growth phase (cells mL mL^{-1})
P	Average biomass productivity (mg DW L^{-1} d^{-1})
R	Nutrients removal efficiency (%)
R_N	Average nitrogen uptake rate (mg N L^{-1} d^{-1})
R_P	Average phosphorous uptake rate (mg P L^{-1} d^{-1})
R_s	Average nutrients uptake rate (mg S L^{-1} d^{-1})
$S(t)$	Time-course evolution of nutrients concentration (mg S L^{-1})
S_i	Nutrients concentration in the beginning of the cultivation time (mg S L^{-1})
S_f	Nutrients concentration in the end of the cultivation time (mg S L^{-1})
t_1	Beginning of the exponential growth phase (d)
t_2	End of the exponential growth phase (d)
t_i	Beginning of the cultivation time (d)
t_f	End of the cultivation time (d)
$V_{FAS,B}$	Volume of FAS used in the titration of the blank (mL)
$V_{FAS,S}$	Volume of FAS used in the titration of the samples(mL)
VS	Volume of the sample (mL).
X	Biomass concentration (mg DW L^{-1})
X_1	Biomass concentration in the beginning of the exponential growth phase (mg DW L^{-1})
X_2	Biomass concentration in the end of the exponential growth phase (mg DW L^{-1})
X_i	Biomass concentration in the beginning of the cultivation time (mg DW L^{-1})
X_f	Biomass concentration in the end of the cultivation time (mg DW L^{-1})
μ	Specific growth rate (d^{-1})
λ	Lag time (d)

1. Introduction

1.1. Framing and presentation of the work

The increase in population has led to a rise in contamination by anthropogenic sources from a wide range, including domestic agricultural and industrial sources. This had led to an increase in nutrient concentration, specifically nitrogen and phosphorous, which can cause eutrophication of water bodies, loss of key species, degradation of freshwater ecosystems and endanger public health. As such, a growing concern in environmental impact from the conventional treatment processes and wastewater pollution has been arising (Dhote et al., 2012). The microalgal cultures are an alternative to the processes implemented in the tertiary part of wastewater treatments and is generally known as wastewater polishing. It became an attractive process as it has been shown to have a consistent capability to remove nutrients (as nitrogen and phosphorous) and a high ability of resisting changes in environmental conditions. On the other hand, the overall costs of operation can be lowered significantly and the negative effects on the environment can also decrease, while offering the possibility of producing high-energy biomass. However, the microalgal growth and the removal efficiency varies significantly with different factors, such as light intensity, pH levels, temperature, salinity, nutrient concentration and C:N:P ratio (Gonçalves et al., 2017).

Not only there has been a rise in contamination of water bodies, but also there has been an increase in emerging contaminants, such as pharmaceutical products. The main difficulties that conventional treatments face is the ineffective removal of such substances as they were not designed for such purposes (Bolong et al., 2009). The use of microalgal culture also present a solution to this issue, as the substances are incorporated into their metabolism through several mechanisms as biosorption, bioaccumulation and intracellular/extracellular biodegradation (Xiong et al., 2018). As such, the main objectives of this thesis were to evaluate the effects of the C:N:P ratio on *Chlorella vulgaris* growth and on the removal of nutrients and of an emergent contaminant.

1.2. Organization of the thesis

The remainder of this thesis is outlined as follows: in Section 2 the context and state of art; in Section 3 the methods used throughout the course of this work; in Section 4 it is presented and discussed the results obtained; in Section 5 the main conclusions are presented; and in Section 6 suggestions for future work. Section 4 is divided in four trials where different parameters was evaluated: (i) the effect of the C:N:P ratio; (ii) presence of an emergent contaminant, ciprofloxacin, and its effects; (iii) immobilization of *C. vulgaris*; (iv) and C:N:P ratio variation and *C. vulgaris* immobilization.

2. Context and State of the art

The use of algal cultures as an alternative protein source began in the 1950's as it was predicted at the time that the traditional sources would not be sufficient due to the exponential growth of the world population. In the 1960's the first commercially viable large-scale culture of *Chlorella vulgaris* was cultivated in Japan by Nihon Chlorella, while in the 1970's the first aquaculture production with microalgae arose. By the early 1980's a large-scale algal production for human consumption was well-establish in Australia and in the late 1980's the production started in the USA (Spolaore et al., 2006) .

Today, algal cultures are produced for several different areas/applications, such as commercial, industrial and environmental (Rizwan et al., 2018). In the commercial area the most predominant use is as food supplements and nutraceuticals due to their ability of synthesizing various vitamins necessary for a healthy system, for instance vitamin A, various B's, C, D, E and H (Bishop and M. Zubeck, 2012). In the industrial area the most prevalent use is for animal nutrition, such as in aquaculture and for feed of farm animals and pets (Spolaore et al., 2006). On the other hand, the environmental area is focused on the bioremediation of wastewaters and removal of CO_2 from the air (Mata et al., 2010).

Microalgae became an attractive candidate for wastewater treatment due to its capability of decreasing significantly the nutrients concentration that cause eutrophication, such as of nitrogen and phosphorous, in short periods of time as low as one hour (Craggs et al., 1995). The classification of wastewaters can be divided in several categories: (i) industrial; (ii) municipal; (iii) agricultural; and (iv) mixture of industrial with municipal. The composition of most municipal and industrial wastes is comprised of 99.9% of water and the remaining 0.1% is solid material, dissolved or suspended, which is a combination of fecal matter, food scraps, oils and greases, sand and grits, plastics, etc. (Samer, 2015). Traditionally, the removal of nutrients occurs in the secondary and tertiary treatments using biochemical processes, such as precipitations and disinfection with ozone and UV light, the use of lagoons and sand filters (Gray, 2004).

The cost of all the steps in tertiary phase of the treatment adds up to four times the cost of the primary one. However, using bioremediation processes the cost meaningfully lowers, as well as the impact on the environment from the secondary pollution. Moreover, its efficiency competes with the one of traditional processes (Abdel-Raouf et al., 2012). Colak and Kaya (1988) reported in a study about the monitoring of biochemical oxygen demand (BOD) and of chemical oxygen demand (COD) in domestic wastewaters with microalgae a removal of 68.4% and 67.2%, respectively. Beside this, its use has the benefit of disinfecting the waters from the pH increase, the production of oxygen from photosynthesis and the biomass production for animal feed and for the production of biofuel (Abdel-Raouf et al., 2012).

While the removal of pharmaceutical contaminants (PCs) has been carefully studied through the years, this field of study has not been well-established due to in one part of the low efficiency of removal

of such products and in the other due to processes limitations at industrial scale. Thus, the use microalgae in the bioremediation of wastewaters is presented as a good alternative to the standard technologies. In particular, mixotrophic cultures are attractive due to its ability of swapping between photosynthesis and dark respiration, depending on the environmental conditions, which gives great flexibility to the processes (Xiong et al., 2018).

2.1. Microalgae

Microalgae are unicellular photosynthetic organisms with plant-like structures and, on average, a diameter in the range between 6 and 8 µm (Estime et al., 2017). They can grow in fresh or in marine waters, but can be also found in extreme conditions, as in the Sahara Desert or in regions of Scandinavia. However, several factors influence its growth such as nutrient concentrations, light intensity and exposure time, temperature and pH (Abdel-Raouf et al., 2012). On the other hand, the type of growth of most species found in nature is autotrophic but many types grown under controlled conditions can have three types of growth: autotrophic, heterotrophic and mixotrophic (Safi et al., 2014).

2.1.1. Autotrophic Growth

Autotropic growth can occur in open and in closed systems, being the most common open ponds and photobioreactors, respectively. In the former, the systems can be implemented with natural waters or wastewaters in artificial ponds, which are usually built next to factories with a high discharge of CO_2 to the atmosphere (Safi et al., 2014). This type of system is the simplest, but several types were developed over the years, for example raceway and circular ponds as represented in Figure 2.1.

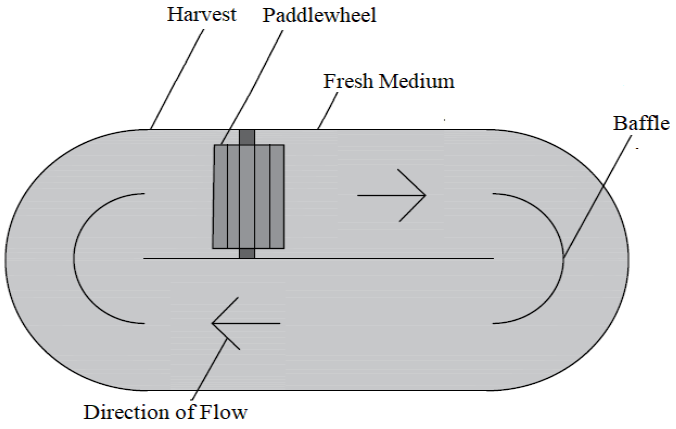

Figure 2.1 - Scheme of an open pond based on Croze et al. (2013).

This type has the advantages of an easier construction and operation and as such a lower cost than the closed systems. However, it faces problems with keeping constant conditions such as temperature, pH,

CO_2 concentration, light intensity, losses through evaporation, risk of contamination with other organisms, etc. (Hoh et al., 2016).

The photobioreactors, as closed systems, present solutions to the disadvantages of the open ones. These include the control of the environmental conditions which results in a higher yield of biomass production, however, with a higher cost. Different models were developed and studied: (i) column photobioreactors; (2) flat-plate photobioreactors; and (3) tubular photobioreactors, which are represented in Figure 2.2. The control of exposure time and intensity of light can be controlled typically with fluorescent lamps, or with Gro-Lux fluorescent lamps, which has a lower intensity, 3.7 to 5.9 W/m^2, but a higher energy emission in the region of 600-700 nm, 37.9% to 20.7%, and a higher lifetime, 15 000 h to 10 000 h duration before degradation of light output along time (Carvalho et al., 2011). On the other hand, the CO_2 necessary for the culture growth can be atmospheric or from flue gases. Presently, there are few projects where factories are built next to industries that have high emissions of CO_2, which allows its capture and employ for microalgae growth (Safi et al., 2014). For instance, the Secil group strategically built Algafarm, located in Pataias (Leiria), Portugal, next to one of its factories, which produces white cement and as result releases 600 kilos of CO_2 to the atmosphere per ton of worked limestone necessary to produced cement.

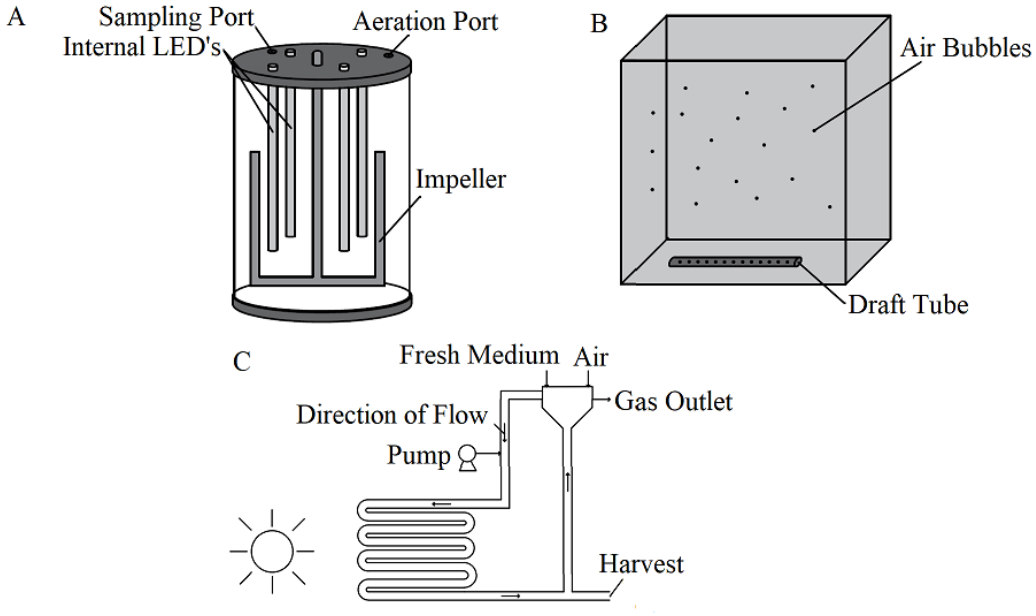

Figure 2.2- Scheme of a vertical-column photobioreactors (A) based on Ugwu et al. (2008); of a flat plate bioreactor (B) based on Croze et al. (2013).; of a tubular photobioreactor (C) based on Kunjapur and Eldridge (2010).

In a laboratory scale, the microalgal species are usually grown in Erlenmeyer flasks or in transparent reagent bottles, with aeration of the liquid medium and usually under agitation. For instance, with an orbital shaker.

It has been reported a lower biomass production for autotrophic growth when compared to the other two types of metabolism, e.g. heterotrophic and mixotrophic. For instance, when Gim et al. (2013) studied the growth of *C. vulgaris* under different types of conditions, it was reported the maximum biomass production values under photoautotrophic, heterotrophic and mixotrophic culture conditions, which were 0.5, 1.7 and 1.8 g L^{-1}, respectively. While, Liang et al. (2009) reported a maximum biomass density of 315 mg L^{-1} and a maximum biomass productivity of 13 mg L^{-1} d^{-1} for *C. vulgaris* under autotrophic culture conditions. The low values on the parameters can be possibly explained by the non-homogenous distribution of nutrients and a less efficient utilization of atmospheric carbon when compared with an organic source (Li et al., 2014).

2.1.2. Heterotrophic Growth

Heterotrophic growth or dark metabolism is a type of metabolism very similar to the one occurring in microorganisms like yeasts. Unlike the autotrophic growth, it does not need a light source and uses an organic source of carbon to form intermediates which are introduced in its central metabolism pathways, such as glucose, fructose, acetate, etc. (Morales-Sánchez et al., 2015; Khan et al., 2016). The system can be composed by a well-stirred tank or a fermenter. As for the biomass yield, the production of fatty acids and removal of nutrients resulting from these systems are substantial. For instance, El-Sheekh et al. (2012) reported a significant difference between the production of proteins in *C. vulgaris* with two different carbon sources, wheat bran and glucose. For a concentration of 1% (v/v) carbon it was determined a decrease of dry weight from 337.1 to 324.0 mg/100 mL, and of protein contents from 337.0 to 253.0 mg g DW^{-1} for wheat bran and glucose, respectively.

2.1.3. Mixotrophic Growth

Mixotrophic growth is a variation of the heterotrophic one, where it is necessary an organic carbon source but the atmospheric CO_2 can be also used in the metabolism of the microalgae, thus ensuing both respiration and photosynthesis (Wang et al., 2016). Many algal strains can display this type of metabolism, such as *C. vulgaris*, *C. sorokinian*, *Spirulina sp.*, *Chroococcus* sp., etc. (Morales-Sánchez et al., 2015). This growth is advantageous has it overcomes the loss of biomass during the phase of dark respiration, while the consumption of organic sources used by the microalgae is diminished (Safi et al., 2014). In the same study mentioned in the heterotrophic growth section (2.1.2), the same parameters were also evaluated but for mixotrophic conditions and it was reported an increment on the value for all parameters. For a concentration of carbon 1% (v/v) it was determined dry weights of 373.7 and 407.8 mg/100 mL, and protein contents of 508.0 and 458.0 mg g DW^{-1} for wheat bran and glucose, respectively (El-Sheekh et al., 2012).

2.2. *Chlorella vulgaris*

C. vulgaris was the algal strain used during the course of this work and is a unicellular spherical autospore which belongs to the *Chlorellaceae* family in the *Chlorella* genus. It has a distinct chloroplast with a cup-shape, a diameter that can vary between 2 and 10 µm. Its growth can occur in autotrophic or heterotrophic culturing conditions but it best potential is achieved in a mixotrophic one (Safi et al., 2014).

When placed in autotrophic conditions some factors can impair its growth, such as lack of nutrients and atmospheric carbon, low light exposure/intensity and variations in pH. However, this microalgal species has shown good biomass production yields and a good removal of heavy metals, even when present in high concentrations. Thus, it presents a considerable capability in the bioremediation area but also in the emerging bioenergy industry (Znad et al., 2018). On the other hand, its growth has been well documented in a wide variety of media, ranging from synthetic, municipal to industrial effluents. For instance, Johnson and Wen (2010) reported a biomass yield of 25.65 g DW m^{-2} with a wastewater from a diary industry as a culture medium. While, Markou (2015) used a synthetic medium to imitate an industrial wastewater rich in ammonia and a removal of volatile fatty acids superior to 90% was achieved in addition to removals higher than 95% for ammonia and phosphorous.

2.3. Wastewater Treatment

The principal objectives of wastewater treatment are the reduction of organic load, removal of pathogens and parasites and removal of nutrients, particularly nitrate and phosphorous. A conventional wastewater treatment process is comprised of three treatments: preliminary, primary and secondary where the first two are physical treatments and the last is of a biological nature. The preliminary treatment begins when the solids of larger size are removed from the raw wastewaters with screens, which are transported to a grit chamber to remove the grit and suspended sand preventing the malfunctioning of later steps. In the primary phase the sediments are withdrawn in a primary sedimentation tank, that removes 40% of the organic matter. This is followed by the secondary phase, of biological nature, where the water is aerated in a tank, lowering its organic load, and a secondary sedimentation tank where the activated sludge flocs sediments are extracted. From the former two streams are obtained, one with the treated wastewater and other with the sludge, which part of it is recycled and re-introduced in the aeration tank and the other part follows to a station for treatment and stabilization (Samer, 2015).

A more complete wastewater treatment process has a tertiary and/or advanced step, of physico-chemical or of biological nature, which removes constituents of the water that cannot be removed with the secondary treatment. So, instead of the effluent being reintroduced in the environment after the secondary sedimentation tank, the stream is directed to lagoons, sand filters or constructed wetlands where the

remaining flocs of smaller dimension are removed. These processes are followed by chemical precipitation or ion exchange in order to remove nutrients such as nitrate, phosphates, heavy metals, etc. As a final step the stream is disinfected with the combination of the addition of chloride and O_3, UV radiation and membrane filtration. As a result, it is obtained a water of enough quality for human consumption (Gray, 2004).

As mentioned above, the tertiary treatment can be outlined with a chemical approach, *i.e.* precipitation, carbon adsorption, reverse osmosis and/or addition of O_3 in order to remove phosphate and nitrate. However, these techniques can provoke eutrophication and are quite costly. As an alternative, systems such as lagoons, sand filters and constructed wetlands which remove very effectively nutrients and fine particles and have a lower impact on the environment (Samer, 2015).

The lagoons, also known as maturation ponds or clarification lakes, can be of shallow or of deep nature and constitute the most effective known tertiary treatment but only when there is sufficient area of cultivation. When the lagoon is shallow the detention and retention times are smaller, 3-4 days and less than 60 h, than the deep lagoons, 17 days and 14-21 days, respectively. However, the removal of suspended solids, coliform bacteria and BOD are significantly better in the second case. For instance, the removal of suspended solids increases from 40% to 80%, as the removal of coliform bacteria from 70% to 99% (Gray, 2004).

When the effluent is of low strength one lagoon is enough for an efficient treatment but if the culture area is small or if the effluent is of high strength, it is prudent to use two or more lagoons in series instead of one with a higher area. Another important factor in the wastewater treatment is a well mixture of the waters, which have a high buffering effect and increase nitrification (Gray, 2004).

2.4. Solutions for removal of nutrients

As mentioned above, the tertiary treatment is a very complex process and can be performed with a variety of techniques. Traditionally, it is developed in the presence of autotrophic nitrification associated with heterotrophic denitrification. In the first stage, under aerobic conditions the ammonia oxidizing bacteria, the nitrifiers, oxidize ammonia into nitrite which in turn is oxidized in nitrate by nitrite oxidizing bacteria. Both bacteria use ammonia and nitrite as an electron receptor, respectively, and use CO_2 as a carbon source. On the other hand, the second stage of the process happens in strict anoxic conditions and with the action of nitrite and nitrate reducing bacteria, which reduce the nitrite and nitrate to nitrogen, in its gaseous form, and use the organic matter present in the medium as a source of carbon and energy. The overall process can be described with the following equation (Sri Shalini and Joseph, 2012):

$$NH_4^+ + 2\,O_2 + 0.83\ CH_3OH \rightarrow 0.5\,N_2 + 0.83CO_2 + 3.17\,H_2O + H^+ \qquad (2.1)$$

The first group of bacteria is composed by *Nitrospina*, *Nitrospina*, *Nitrococcus*, and *Nitrocystis*, which belong to the phylum Betaproteobacteria. On the other hand, the second group frequently consists of Gram negative bacteria such as *Pseudomonas*, *Thiobacillus*, *Alcaligenes* and *Paracoccus* (Sheela and Beebi, 2014).

In order to increase the homogenization of the wastewater, the process usually takes place in reactors as the packed bed reactors, rotating biological reactors, membrane biofilm bioreactor (Sheela and Beebi, 2014). Due to an improved contact between the bacteria and the nitrate, there is an increase of the speed of the process. Presently, variations of this process are applied in industrial settings with the interest of decreasing factors such as time delay, aeration and cost.

2.4.1. ANAMMOX

ANAMMOX, or ANAerobic AMMonium Oxidation, was firstly developed in Delft University of Technology in the 1990's decade and is defined as a continuous process carried in a single reactor, as a fluidized bed reactor suitable to the treatment of wastewaters with a high concentration of ammonia and low concentrations of carbon and nitrogen (Samer, 2015). It combines aerobic nitrification of ammonia and anaerobic ammonium oxidation in the presence of microbial species of the phylum *Planctomycetes*, for instance the genera *Anammoxoglobus*, *Scalindua*, *Kuenenia*, *Jettenia* and *Brocadia*. Thus, the production of a nitrate intermediate is skipped as the nitrite and ammonium form nitrogen gas. The overall reaction of the process can be described by the Equation 2.2 (Quoc Anh et al., 2015):

$$NH_4^+ + 1.32NH_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.0066CH_2O_{0.5}N_{0.15} + 2.03H_2 \qquad (2.2)$$

This process compared to the conventional ones produces significant lower amounts of sludge, does not entails an organic carbon source and requires up to 65% less oxygen supply and as such needs up to 60% less energy. Furthermore, it is a process whose robustness and nitrogen removal efficacy has been well established and leaves a small footprint on the environment. However, the relationship between the temperature of the process and its performance has been studied and the best achieved results were at approximately 29 ºC (Dosta et al., 2008). For instance, Laureni et al. (2015) obtained an Anammox activity of 465 mg N L^{-1} d^{-1} and doubling times of 18 days at this temperature. However, when it was lowered to 12.5 ºC it was attained a decrease in the activity to 46 mg N L^{-1} d^{-1} and an increase of doubling time to 79 days.

2.4.2. SHARON

The SHARON process, or Single reactor system for High activity Ammonia Removal Over Nitrite, is suitable to wastewaters rich in ammonium and like Anammox was also first developed in Delft University of Technology. However, its objective is the partial nitrification of the sludge obtained from the anaerobic digester and is characterized by two steps. The first being the production of the intermediate hydroxylamine by the oxidation of ammonia nitrogen and the second the formation of nitrite from the oxidation of hydroxylamine with oxygen as an electron acceptor. The overall process can be described by Equation 2.3 (Sri Shalini and Joseph, 2012):

$$NH_4 + 0.75\ O_2 + HCO_3^- \rightarrow 0.5\ NO_2^- + 0.5\ NH_4^+ + CO_2 + 1.5\ H_2O \quad (2.3)$$

The most common ammonia oxidizing bacteria are *Nitrosomonas europaea* and *Nitrosomonas eutropha* and species from the phylum *Proteobacteria* such as *Nitrosovibrio*, *Nitrosopira* and *Nitrosolobus*. This method does not need retention of the sludge, but it is performed temperature above room temperature and pH, between 30-40 ºC and 7-8, respectively (Hellinga et al., 1998). It has been shown that an increment in the pH the concentration of ammonium declines hastily but for values inferior to 6.5 the process simply does not occur. Thus, techniques have been studied and developed to adjust the pH. These include the addition of caustic agents, denitrification and CO_2 removal, which have been shown to work in an industrial scale (Sri Shalini and Joseph, 2012).

2.4.3. Combined SHARON-ANAMMOX

The combined SHARON-ANAMMOX process is suitable for ammonium rich wastewaters as the SHARON process, with an ideal NH_4:NO_2 ratio of 1:1.32 that allows the formation of water and nitrogen gas. The combined process can be defined by Equation 2.4 (Sri Shalini and Joseph, 2012).

$$2.3\ NH_4^+ + 2.95\ O_2 + 1.2\ CO_2 \rightarrow N_2 + 0.3\ NO_3^- + 1.2\ CH_{1.8}O_{0.2} \quad (2.4)$$

The system can be composed of one or two reactors, where in the first case the SHARON and ANAMMOX occurs in the same reactor and in the second the SHARON occurs on the first reactor and the ANAMMOX occurs on the second. The type of reactors used need to have a good capacity of biomass retention and support a high density of cells. As such, the bioreactors mostly used are of immobilized cells or of fixed films.

This technique has the advantages of not needing an external carbon source and a reduction up to 60% of its oxygen necessity. Thus, the energy demand and cost of operation are greatly diminished. Furthermore, the resulting sludge and the emissions of nitrous oxide are lower than those of conventional processes (Sri Shalini and Joseph, 2018).

2.5. Microalgae and the removal of nutrients

The use of algal cultures presents a good solution for the reduction of nutrients, as phosphate and nitrate, and of heavy metals, as lead or cadmium, due to its consumption on the metabolism of the microalgae. The biomass produced in the process adds value to this approach as it, depending on the treated wastewater, can later be sold as food supplements, feed of farm animals or to the biofuel industry.

The principal problem of an excess of nutrients in water is its eutrophication, which in turn encourages the growth of plants such as aquatic macrophytes and algae. On the other hand, high concentrations of nitrogen inhibits the disinfection, as the process requires residuals of free chloride (Abdel-Raouf et al., 2012). However, these types of nutrients are important to the microalgal mechanisms, as it enables the production biomass and necessary bi-products to their metabolism.

The available sources of nitrogen can vary, as nitrite-nitrogen (NO_2-N), nitrate-nitrogen (NO_3-N), ammonia-nitrogen (NH_3-N) and urea, being the ammonia the preferred one as is the one which requires the lowest energy for its intake, thus being the most energetically efficient (Delgadillo-Mirquez et al., 2016). In the case of NO_2-N and NO_3-N, the nutrients enter the intracellular medium through active transport after two reactions of reduction. The first reaction produces NO_2-N from NO_3-N with nitrate reductase and NADPH as reducing agent (Equation 2.5). The second one produces ammonium-nitrogen (NH_4-N) from NO_2-N and uses nitrite reductase and ferredoxin (Equation 2.6). After the formation of NH_4-N, amino acids are synthesized via the glutamine synthetase-glutamate synthase pathway (Equation 2.7), in which glutamine is produced in the presence of glutamine synthase (Cai et al., 2013).

$$NO_3^- + 2H^+ + 2e^- \xrightarrow{\text{Nitrate reductase}} NO_2^- + H_2O \quad (2.5)$$

$$NO_2^- + 8H^+ + 6e^- \xrightarrow{\text{Nitrite reductase}} NH_4^+ + 2H_2O \quad (2.6)$$

$$\text{Glutamate} + NH_4^+ + \text{ATP} \xrightarrow{\text{Glutamine synthase}} \text{Glutamine} + \text{ADP} + PO_4^{3-} \quad (2.7)$$

In the case of phosphorus, the available sources are $H_2PO_4^-$ and HPO_4^{2-} and are utilized in the microalgal growth and metabolism through active transport by the process of phosphorylation across the plasma membrane. In this process adenosine diphosphate (ADP) produces adenosine triphosphate (ATP), in the presence of an energy input and is described by Equation 2.8 (Cai et al., 2013).

$$\text{ADP} + PO_4^{3-} \xrightarrow{\text{Energy}} \text{ATP} \quad (2.8)$$

The energy input can be originated from different sources, the electron transport system from the mitochondria, oxidation of respiratory substrates, or more relevantly in this case from light, through photosynthesis. For the algal growth, some species are able not only to use inorganic forms of phosphate but also organic ones, as phosphorous from organic esters. It should be pointed out that the external

conditions, as pH and dissolved oxygen, also can affect the removal of phosphorous. Contrary to nitrogen, it does not exists in a gaseous state and so when the pH and the concentration of dissolved oxygen of the medium increases it causes a precipitation of phosphorous (Cembella et al., 1984; Cai et al., 2013).

Lastly, as mentioned previously carbon sources can be organic or inorganic depending on the type of algal growth. In autotrophy, the photosynthetic mechanism integrates the atmospheric CO_2 in the Calvin cycle, through active transport or diffusion and in heterotrophy an organic carbon source is used directly. In mixotrophy both mechanisms are used by the microalgae depending on the environmental conditions (Kang et al., 2004).

C. vulgaris has been widely used for the treatment of different types of effluents, at the laboratorial scale, and its biomass productivity and nutrient removal has been well reported. Table 2.1 presents values of *C. vulgaris* performance using different effluents.

The removal of nutrients can vary significantly depending on several factors such as the origin of wastewater, the concentration of nutrients, the scale of the process, etc. Lim et al. (2010) studied the removal of phosphorous, nitrogen and COD from a wastewater of a textile industry, using a volume system of 36 L. These authors reported a low removal of such nutrients, between 44.4% and 45.1% for nitrogen, between 33.1% and 33.3% for phosphorous, and between 38.3% and 62.3% for COD. In contrast, Gouveia et al. (2016) used a primary municipal wastewater under 150 L process conditions and reported a higher removal of nitrogen (84%) and phosphorous (95%), but a lower removal of COD (36%).

The biomass productivity usually decreases with the increase of the volume of the system, mainly due to the difficulty in maintaining homogeneous conditions inside the reactor (La et al., 2016). Markou (2015) used modified BG-11 medium and reported high biomass productivity (1.526 g L^{-1} d^{-1}) with a system of 400 mL. In contrast, Mayhead et al. (2018) reported a lower biomass productivity (0.190 g L^{-1} d^{-1}) using F2P medium when operating a system with a volume of 4 L.

The removal of other substances such as potassium, magnesium and iron has also been reported. For instance, Markou (2015) used modified BG-11 medium and reported a removal of such micronutrients with efficiencies of 22%, 75% and 70%, respectively. N and P removal was more than 99%.

Table 2.1 - Reports on *C. vulgaris* action on nutrient removal and biomass productivity.

Type of effluent	System and operation mode	V (mL)	Removal efficiency			Biomass Productivity	Reference
			N (%)	P (%)	Others (%)		
Dairy wastewater treatment	Immobilized system, batch mode	200	61-79	73-93	n.a.	2.57 g DW L^{-1} d^{-1}	Johnson and Wen (2010)
Primary municipal wastewater	Closed suspended system, batch mode	250	82.4	83.2	COD: 51	n.a.	Wang et al. (2010)
Modified BG-11 medium	Closed suspended system, fed-batch mode	400	>99	>99	K: 22; Mg: 75; Fe: 70	1.526 g L^{-1} d^{-1}	Markou (2015)
Synthetic secondary effluent	Closed suspended system, batch mode	500	44.4-45.1	33.1-33.3	COD: 38–62	0.0214 g DW L^{-1} d^{-1}	Gonçalves et al. (2016b)
Synthetic wastewater medium	Closed suspended system, batch mode	1,000	n.a.	99.2	COD: 71	n.a.	Salgueiro et al. (2016)
Synthetic wastewater medium	Closed suspended system, batch mode	1,000	23-100	46-94	n.a.	n.a.	Aslan and Kapdan (2006)
Secondary municipal wastewater	Closed suspended system, batch mode	3,000	74.3	70.2	n.a.	n.a.	Ruiz-Marin et al. (2010)
Primary municipal wastewater	Closed suspended system, batch mode	3,200	95.22	96.63	n.a.	0.164 g L^{-1} d^{-1}	Mayhead et al. (2018)
F2P medium	Closed suspended system, batch mode	4,000	n.a.	89.64	n.a.	0.190 g L^{-1} d^{-1}	Mayhead et al. (2018)
Synthetic wastewater medium	Closed suspended system, batch mode	4,000	30-95	20-55	n.a.	n.a.	González et al. (1997)
Textile industry wastewater	Open suspended system, batch mode	36,000	44.4-45.1	33.1-33.3	COD: 38–62	0.061 g L^{-1} d^{-1}	Lim et al. (2010)
Primary municipal wastewater	Closed suspended system, semi-continuous mode	150,000	84	95	COD: 36	0.050 g L^{-1} d^{-1}	Gouveia et al. (2016)

2.6. The role of C:N:P ratio

The chemical composition of the deep ocean was first documented in 1934 and its C:N:P ratio was 106/16/1, which was denominated as the Redfield ratio and was considered as the optimal conditions for the growth of phytoplankton (Redfield, 1934). Later, this ratio was revisited and it was found to be closer to 117:14:1 (Benitez-Nelson, 2000).

The composition of different wastewaters varies significantly depending of its source, which in turn affects the growth and nutrients removal capability of the microalgae. Algal growth is characteristic to decrease when the supply of the source of inorganic carbon is low. On the other hand, it has been reported a stimulation of bacterial growth for high concentrations of an organic carbon source (Cho et al., 2017). Wastewaters from food industries, such as dairy and brewery, have high COD levels. The high organic strength present in the culture medium may cause substrate inhibition, thus inhibiting the microalgae growth (Gupta et al., 2017). For instance Qin et al. (2016) reported a high COD between 2,000 and 20,213 mg L^{-1} in a wastewater from a dairy industry, while Hernández et al. (2013) reported a COD of 745 mg L^{-1} for an agricultural wastewater from a potato processing industry.

Regarding the N:P ratio it has been established that for microalgae growth in freshwater the optimum level is between 8:1 and 45:1 (Whitton et al., 2016). For ratios lower than a 8:1 ratio, nitrogen limitation is induced. As for ratios superior to 45:1 ratio, phosphorous limitation is induced in turn. Regarding the nutrient concentration, it has been reported that its increase leads to a superior nutrient removal rate. For instance, Raposo et al. (2010) compared the microalgal growth in a wastewater from a brewery industry diluted to 1:1 with a OHM culture medium and a modified BG medium. The nutrient load for the BG medium was superior to the brewery wastewater, e.g. there was an increase of $NaNO_3$ and total phosphorous from 0.201 to 1.61 g L^{-1} and from 17.8 to 22.4 mg L^{-1}, respectively. Thus, the nutrient load increase caused an increment on the removal rate of nitrogen (from 103 to 225 g kg^{-1} d^{-1}) and phosphorous (from 23 to 70 g kg^{-1} d^{-1}).

2.7. Pharmaceutical contaminants

One of the biggest challenges in wastewater treatment is the presence of numerous pharmaceuticals contaminants (PCs). Their presence in wastewaters is a recognized source of ecotoxicity and potential public health issues. Therefore, there is a critical need to implement solutions for their effective treatment when present in a wastewater. In this context, microalgal-based bioremediation is gaining attention as a strategy of interest. Currently, the main integrated processes used are oxidative processes, constructed wetlands, lagoons and sand filters which can be used with just microalgae but also with microbial consortia of microalgae and/or bacteria (Xiong et al., 2018). The mechanisms on the removal of PCs by microalgae

has been well studied and can be differentiated in three categories: (i) bioadsorption; (ii) bioaccumulation; and (iii) intracellular/extracellular biodegradation (Xiong et al., 2018).

Bioadsorption is defined as the process of integration of dissolved substances in a medium on a appropriated interface of a microorganism, which is possible due to an affinity between the substance and the microorganism (Romera et al., 2006). Unlike bacteria and fungi, microalgae present a more complex cell wall which is negatively charged and with assemblages of polymers very similar to pectins, cellulose, hemicelluloses lignin, extension and arabinogalactan proteins, allowing an efficient bioadsorption of PCs.

On the other hand, bioaccumulation is an active metabolic process driven by energy with the consumption of substances that already demonstrated to be effective in the removal of organic compounds, as trimethoprim, triclosan, and sulfamethoxazole. This process is initiated by the formation of reactive oxygen species (ROS) and its production is activated by the accretion of PCs in the microalgal cells. ROS activates metabolic paths that eliminate the external substances, as it acts on the control of cellular metabolism and on defense of pathogens.

Lastly, biodegradation is the defined as a disruption of substances by enzymatic process of the microalgae metabolism and can be divided in extracellular and intracellular, being the intracellular one the most effective process that microalgae present to remove contaminants dissolved in a liquid medium, e.g. wastewaters (Xiong et al., 2018). The former occurs through a complex algal enzymatic system that is divided in two families: (i) phase I; and (ii) phase II. Firstly, the PCs get attacked by the phase I enzyme, in this case cytochrome p450, through a series of reactions of reduction, oxidation and hydrolysis in order to turn the PCs more hydrophilic. In the second stage, the phase II enzyme opens the epoxide ring, to prevent oxidation that will provoke damage to the cell. Xiong[1] et al. (2017) studied the biodegradation of levofloxacin by *C. vulgaris* and reported a removal of 12%. In another study, they also studied the removal of ciprofloxacin by *Chlamydomonas mexicana* and reported a removal of 13% with a growth inhibition between 36 and 88% (Xiong[2] et al., 2017).

3. Materials and Methods

3.1. Microalgae and cultivation conditions

In all the trials it was used stock solutions of the microalgae *C. vulgaris* 0002 CA (from CMP-Algafarm collection). This microalgal species was chosen for several reasons: (i) it has been well studied and documented throughout the years which provides enough scientific background to support the present study; (ii) it can growth in many culture medias and under different environmental conditions, giving flexibility to the settings of the cultivation; (iii) it has a low doubling time, around 19 hours, allowing the collection of a significant amount of results in a short period of time; (iv) its effectiveness on the removal of nutrients and other emerging contaminants has been widely demonstrated; and (v) it has been used in many different biotechnological sectors such as wastewater treatment, biodiesel production and Co_2 capture (Lv et al., 2010; Liu and hu, 2013; Minhas et al., 2016).

Stock solutions were prepared by incubating the cells for twelve days in 2 L glass flasks, agitated with an inlet at the bottom of the flask connected to a pump which introduced bubbles of atmospheric air, under an irradiance of 24 μE m^{-2} s^{-1} using Gro-Lux fluorescent light (Sylvania, Germany) with a ligh:dark ratio of 16:8 h, and at room temperature (approximately 24.6 ± 1.6 °C). The air continuously introduced was filtered thought 0.20 μm syringe filter (Fisher, China). In order to perform the experiences, on the twelve day of culturing, samples were collected and centrifugated at 3,050 *g* for 15 min in an Eppendorf 5810 R centrifuge (Eppendorf, Germany). Afterwards, it was diluted with sterilized water and inoculated in the OECD test medium previously autoclaved (description in the Section 3.2) in order to obtain an OD of 0.05 (at 750 nm), corresponding to a cell concentration of 3.71 ×10^6 cells mL^{-1}.

The Gro-Lux fluorescent light was specifically developed for the growth of plants under artificial conditions. It was chosen to be used in this work instead of the traditional cool-white fluorescent light not only due to the aspects presented in the Section 2.1.1. (higher energy emission with a lower intensity, higher lifetime, higher duration before degradation of light output along time), but also because it provides a higher energy in the blue and red regions of the light spectrum. These are the areas most active on the photosynthesis process, thus increasing biomass production and productivity (Helson, 1965).

In Figure 3.1 it is showed the schematic representation of the apparatus. The microalgae were incubated in glass-flasks of 2 L exposed to a Gro-Lux fluorescent light (Sylvania, Germany). To allow the entrance and the exit of air flow, it was made two holes on the lids of the flasks where it was inserted two silicone tubes, one connect to an air 550 R plus pump (sera, Germany) with an air pressure of 140 mbar which in turn was attached to 0.20 μm syringe filter (Fisher, China) to allow the entrance of atmospheric

air at the bottom of the flask, thus aerating the medium and promoting medium agitation. Another one was connected to a filter to allow the exit of the O_2 produced by the microalgae.

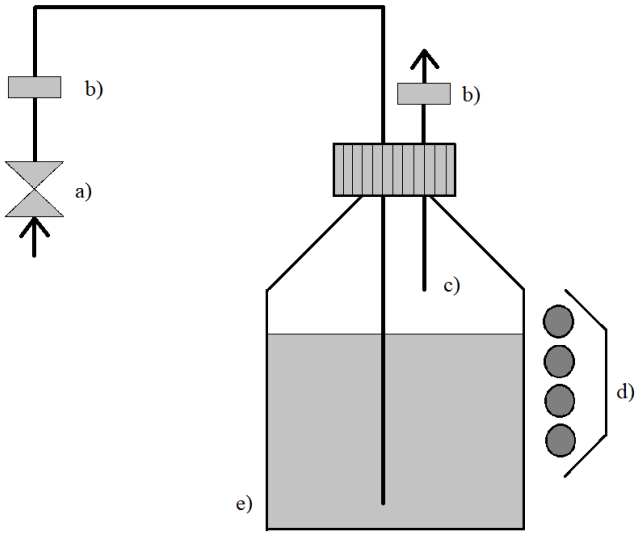

Figure 3.1- Schematic representation of the reactor used for the removal of nutrients from wastewater by *C. vulgaris* adapted from Gonçalves et al. (2016a) : (a) air pump; (b) filters; (c) silicone tubes for aeration of the medium and O_2 output; (d) fluorescent lamps and (e) cultivation flask.

3.2. Culture media

In order to test the effect of the C:N:P ratio on biomass production and on the removal of nitrogen and phosphorous, variations of the levels of the standard Organization for Economic Co-operation and Development (OECD) test medium were used. OECD test medium has C:N:P ratio of 1.8:9:1 and its composition, per liter, is the following: 250 mg $NaNO_3$ (Merck, Germany); 12 mg $MgCl_2 \cdot 6H_2O$ (Merck, Germany); 18 mg $CaCl_2 \cdot 2H_2O$ (VWR, EC); 15 mg $MgSO_4 \cdot 7H_2O$ (VWR, EC); 45 mg KH_2PO_4 (Merck, Germany); 0.08 mg $FeCl_3 \cdot 6H_2O$ (VWR, EC); 0.1 mg $Na_2EDTA \cdot 2H_2O$ (Merck, Germany); 0.185 mg H_3BO_3 (Merck, Germany); 0.415 mg $MnCl_2 \cdot 4H_2O$ (Merck, Germany); 3 µg $ZnCl_2$ (Merck, Germany); 1.5 µg $CoCl_2 \cdot 6H_2O$ (Merck, Germany); 0.01 µg $CuCl_2 \cdot 2H_2O$ (Riedel-de Haen, Germany); 7 µg $Na_2MoO_4 \cdot 2H_2O$ (May & Baker, England); and 50 mg $NaHCO_3$ (Merck, Germany).

On the first trial different variations of C:N:P were tested. These are described in Table 3.1.

Table 3.1 – C:N:P ratio in each sample at time zero on Trial 1.

Culture	Ratio		
	C	**N**	**P**
1A	10	1	1
1B	1	10	1
1C	1	1	10
1D	10	10	1
1E	10	1	10
1F	1	10	10
1G	1	1	1
1H	1.8	9	1

On the second trial, the objective was to evaluate the effect of the presence of an emerging contaminant, ciprofloxacin. The C:N:P ratio of the sample 1F was the variation which produced the best results on the first trial. Consequently, this ratio was chosen to be repeated on the second trial in the presence and absence of the antibiotic. In another OECD medium the concentration of carbon was increased in order to assess its effect on the algal growth and nutrient removal, also with and without the antibiotic. The compositions of the media are described in Table 3.2.

Table 3.2 – C:N:P ratio and ciprofloxacin concentration (in μg L^{-1}) in each sample at time zero on Trial 2.

Culture	[Ciprofloxacin] ($\times 10^3$ μg L^{-1})	Ratio		
		C	**N**	P
2A	0.00	20	9	1
2B	0.00	1	10	10
2C	2.50	20	9	1
2D	2.50	1	10	10
2E	0.00	1.8	9	1

As in the previous trials (Trial 1 and 2), the ratio of 1.8:9:1 produced the best results it was chosen to be further evaluated on Trial 3. As such, the productivity and nutrient removal of immobilized microalgae, with and without ciprofloxacin, in the OECD medium was tested. The compositions of the samples are described in Table 3.3.

Table 3.3 – C:N:P ratio and ciprofloxacin concentration (in μg L^{-1}) in each sample at time zero on Trial 3.

Culture	[Ciprofloxacin] ($\times 10^3$ μg L^{-1})	Ratio		
		C	**N**	P
3A	0.00	1.8	9	1
3B	2.50	1.8	9	1
3C*	0.00	1.8	9	1
3D*	2.50	1.8	9	1

* C. vulgaris immobilized in sodium alginate beads.

On the fourth trial, the effects of microalgal immobilization was once more tested but for a different concentration of antibiotic and for two different C:N:P ratios. The compositions of the samples are described in Table 3.4.

Table 3.4 – C:N:P ratio, ciprofloxacin concentration and identification of immobilization in each sample at time zero on trial 4.

Culture	[Ciprofloxacin] ($\times 10^3$ µg L^{-1})	Ratio		
		C	N	P
4A	0.00	1.8	9	1
4B	0.00	20	9	1
4C	5.00	20	9	1
4D*	0.00	20	9	1
4E*	5.00	20	9	1
4F	0.00	1.8	9	10
4G	5.00	1.8	9	10
4H*	0.00	1.8	9	10
4I*	5.00	1.8	9	10

* *C. vulgaris* immobilized in sodium alginate beads.

The sterilization of all the media was carried out by autoclaving at 121ºC, at 1.2 bar for 15 min in an Uniclave 88 (Vidrolab, Portugal).

3.3. Microalgae immobilization

To immobilize the microalgae it was used an adaptation of the method proposed by Larsson et al. (1979), which focus on a live immobilization in a sodium alginate gel. Collected samples were centrifuged in an Eppendorf 5810 R centrifuge (Eppendorf, Germany) and resuspended in sterilized water in order to have an OD of approximately 1.00 (at 750 nm), corresponding to a cell concentration of 6.87×10^7 cell mL^{-1}. For a volume of 1,500 mL of OECD medium it was used 2 g of sodium alginate (VWR, Belgium) which were added to 88 mL of distilled water and heated in order to dissolve it. Then, 70 mL of the resuspended sample was added and homogenized. With the help of a syringe the mixture was dropped to a solution of calcium chloride (Merck, Germany) with a concentration of 40 g L^{-1} which was kept at room temperature and under constant agitation with a magnetic stirrer. The beads were kept in the agitated solution for 15 min to complete the gelation process. Then, they were washed in calcium chloride (Merck, Germany) in pH 4.5 acetate buffer (Carlo Erba, France) with a concentration of 10 g L^{-1} and dried in filter paper (Whatman, China). After drying they were transfer to a 2,000 mL glass flask with a volume of 1,500 mL of OECD medium previously autoclaved at 121 ºC for fifteen minutes.

3.4. Calibration curves

For the biomass concentration calibration curve vs. optical density (OD), samples of 10 mL were first placed in dried and weighed porcelain crucibles. Then they were dried at 105ºC for twenty-four hours and placed in a glass vacuum desiccator until it was obtained a constant weight. At that point, the samples were transferred to a furnace (Salfor, Portugal) at 550 ºC for two hours, in order to oxidize the dried biomass. After that time period, they were placed in the desiccator and weighed again. The ratio of the difference of the mass before and after furnace and the volume of the sample is the ash free dry weight (X, in mg DW L^{-1}), as described in Equation (3.1).

$$X = \frac{m_2 - m_1}{V_s} \quad (3.1)$$

where the m_2 and m_1 are the sample weights after and before the muffle (in mg), respectively, and V_s is the volume sample (in L). On Annex I, Figure I.1, the calibration curve is presented.

For the cells concentration calibration curve, the number of cells from 10 µL samples were counted in a Neubauer counting chamber under a Leica DM LB microscope (Leica Microsystems, Germany). On Figure I.2 (Annex I) the calibration curve is presented.

3.5. Growth monitoring

In order to monitor the microalgal growth the OD of the culture was measure daily. To correlate the results to the biomass concentration and to the number of cells two experimental calibration curves were developed: (i) OD vs. biomass concentration; and (ii) OD vs. number of cells.

For the measurement of the OD it was used a V-1200 spectrophotometer (VWR, Portugal) at an wavelength of 750 nm, as it was proposed by Pegallapati and Nirmalakhandan (2013). This wavelength is the desirable for the detection of free chlorophyll.

In order to monitor the growth of the immobilized cultures, it was followed the method proposed by Lau et al. (1998) with adaptations. At the end of the monitoring period of twelve days, the sodium alginate beads were separated from the medium through decantation and dried in filter paper (Whatman, China) and then weighed. A sample of five beads were dissolved in 2 mL of 0.1 M of tri-sodium citrate under constant agitation. After dissolving, the biomass was retrieved by filtration through a paper filter (Whatman, China) and transferred and dried in porcelain crucibles at 105 ºC until for twenty-four hours. After that time period, the porcelain crucibles were transferred to a glass vacuum desiccator until achieving a constant weight. Then the samples were transferred to a furnace (Salfor, Portugal) at 550 ºC for two hours, in order to oxidize the dried biomass, and placed in the desiccator and weighed again. As mentioned

previously, the difference of the mass before and after furnace and the volume of the sample indicates the ash free dry weight of the sample.

3.6. Growth inhibition

As ciprofloxacin is an emerging contaminant, its effects on algal growth are not well studied (Norvill et al., 2016). Thus, to study inhibition factor on the microalgae growth it was used the paper-disc agar method where a collected microalgae sample of 100 mL (OD = 1, at 750 nm) inoculated OECD agar plates with two paper-discs: (i) one with a sample of 10 µL of H_2O for negative control; and (ii) one with a sample of 10 µL of ciprofloxacin solution. The plates were incubated for seven days at room temperature (approximately 24.6 ± 1.6 ℃), with a light:dark ratio of 16:8 h and with a light irradiance of 24 µE m^{-2} s^{-1} (Sylvania, Germany).

3.7. Nutrients quantification

The nutrients evaluated through the course of this work were NO_3-N and PO_4-P. However, it was also evaluated the soluble chemical demand, COD. Samples were taken daily in duplicate and the tests were made in triplicates.

3.7.1. Nitrate quantification

The quantification of nitrate was based on the method proposed by Collos et al. (1999), where the absorbance of the sample was measured at 220 nm. It was chosen as it is an easy and quick method and does not require the addition of reagents, such as cadmium.

Thus, the samples were firstly collected, centrifuged at 16,500 *g* for 10 min and the supernatants were separated and stored at -20 ºC until analysis. When the monitoring period was over and all the samples were collected, the samples were removed from storage, thawed, diluted twenty times with distilled water and filtered through a 0.20 µm syringe filter (Fisher, China) in order to remove interferences from algal exudates. Subsequently, in a T80 UV/VIS spectrophotometer (PG Instruments, UK) the absorbance of the filtered samples was measured in a quartz cuvette (Starna, US) at 220 nm and as blank it was used distilled water. For the calibration curve, presented in Figure I.3 (in Annex I), dilutions with concentrations ranging from 5 to 25 mg L^{-1} were prepared from a stock solution of $NaNO_3$ (Merck, Germany) with a concentration of 100 mg L^{-1}.

3.7.2. Phosphorous quantification

The quantification of phosphorous was based on the method proposed by Lee et al. (2009), which is centered on the reaction of ammonium molybdate with the inorganic phosphate, P_i with the formation of a phosphomolybdate complex. The procedure to prepare the samples for the method was the same as it is described on the previous section (Section 3.7.1). Where the samples were collected, centrifugated at 16,500 *g* for 10 min, the supernatants were separated and stored at -20 ºC until the analysis. When the monitoring period was over and all the samples were collected, they were thawed and 60 µL of each sample and 140 µL of a reaction mix was pipetted to a well of a 96-well microtiter plate. The reaction mix was composed of 1 part of a reagent A, 10% w/v of ascorbic acid (Acros, USA), and 6 parts of a reagent B, 0.42% w/v $(NH_4)_2MoO_4 \cdot 4H_2O$ (Merck, Germany) in 1 N H_2SO_4 (Fluka, Germany). The microtiter plate was then incubated at 37 ºC for one hour and its absorbance was measured at 820 nm in a SPECTROstar^{Nano} spectrophotometer (BMG LABTECH, Germany). For the calibration curve, presented in Figure I.4 (Annex I), dilutions were prepared at concentrations ranging from 1 to 60 mg L^{-1} using a stock solution of KH_2PO_4 (Merck, Germany) with a concentration of 100 mg L^{-1}.

3.7.3. Ciprofloxacin quantification

For ciprofloxacin quantification it was necessary to find a method that its execution was quick and simple and preferably did not require the addition of reagents. Since the ciprofloxacin molecule has an aromatic ring, its detection is possible in the UV region of the spectrum. Thus, in order to validate the quantification method and to find the absorbance with the maximum intensity on its emission spectrum, it was done a sweep in a T80 UV/VIS spectrophotometer (PG Instruments, UK), with samples of ciprofloxacin with concentrations ranging from (1.00 to 10.00)x10^3 µg L^{-1}. For the preparation of the samples, it was made a stock solution of ciprofloxacin in distilled water with a concentration of 10.00x10^3 µg L^{-1}. In turn, the samples were placed in quartz cuvettes (Starna, US) to read the absorbance at 272 nm in a T80 UV/VIS spectrophotometer (PG Instruments, UK), as it was found that this was the maximum absorbance of the emission spectrum of ciprofloxacin. In Annex II it two of the emission spectrums where it displays the maximum absorbance are presented (Figures II.1 and II.2). As for the calibration curve, it is presented in Figure II.3 (Annex II), while several parameters, such as detection and quantification limits are shown in Tables II.1 and II.2 (Annex II).

For the analyzes of the collected samples, each sample was placed directly in a quartz cuvette (Starna, US) and its absorbance was read at 272 nm. As a blank it was used a culture with the same C:N:P ratio of the sample but without ciprofloxacin.

3.8. Soluble chemical oxygen demand quantification

The quantification of the soluble chemical oxygen demand (COD) was based on the procedure described in APHA (1999) were the collected samples were digested in a strong acidic solution with a known excess of potassium dichromate ($K_2Cr_2O_7$), followed by a titration of the unreduced $K_2Cr_2O_7$ with ferrous ammonium sulphate (Merck, Germany), FAS, which allowed to determinate the amount of consumed $K_2Cr_2O_7$. The organic matter was calculated in terms of oxygen equivalent, in mg O_2 L^{-1}, which was calculated through the Equation 3.2:

$$\mathrm{COD} = \frac{(\mathrm{V_{FAS,B}} - \mathrm{V_{FAS,S}}) \times \mathrm{M_{FAS}} \times 8000}{\mathrm{V_S}} \quad (3.2)$$

where $V_{FAS,B}$ is the volume of FAS used in the titration of the blank (in mL), $V_{FAS,S}$ is the volume of FAS used in the titration of the samples (in mL), M_{FAS} is the molecular mass of FAS (in g mol^{-1}) and V_S is the volume of the sample (in mL). The method was initiated with the addition of 1.5 mL of standard $K_2Cr_2O_7$ solution 0.01667 M, prepared by dissolving approximately 4.903 g of $K_2Cr_2O_7$ (VWR, EC) in 500 mL of distilled water, followed by the addition of 167 mL of H_2SO_4 (Fluka, Germany) (VWR, Belgium) and dilution to 1 L, and 3.5 mL of sulphuric acid reagent, prepared by the addition of 5.5 g Ag_2SO_4 (VWR, Belgium) to 1 kg H_2SO_4 (Fluka, Germany), to 2.5 mL of the collected samples in culture tubes of 16 x 100 mm. The tubes were transferred to the block heater and the digestion was performed at 150 °C for two hours. After the vessels were cooled to room temperature, each sample was moved to a glass beaker in order to perform the titration with the titrant 0.10 M FAS solution, previously prepared by dissolving 39.2 g of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (Merck, Germany) in distilled water, with the addition of 20 mL of H_2SO_4 (VWR, Belgium) and dilution to 1000 mL, and the indicator ferroin (Fluka, Germany). When the color changed from blue-green to red it was reached the endpoint. As a blank it was used distilled water which was submitted to the same procedure.

3.9. Kinetic growth parameters

In order to evaluate the algal kinetic growth, two parameters were calculated: average biomass productivity and specific growth rate. For the calculation of the average biomass productivity (P, in mg DW L^{-1} d^{-1}) Equation 3.3 was used. This considers the variation in biomass concentration between the end and beginning of the microalgae cultivation.

$$\mathrm{P} = \frac{\mathrm{X_f} - \mathrm{X_i}}{\mathrm{t_f} - \mathrm{t_i}} \quad (3.3)$$

where the X_f and X_i represent the final and initial biomass concentration (in mg DW L^{-1}) and the t_f and t_i the end and beginning of cultivation time (in d).

For the specific growth rate (μ, in d^{-1}) it was used the Equation 3.4 which can be determined from the variation of biomass or cell concentration throughout the trial.

$$\mu = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} = \frac{\ln N_2 - \ln N_1}{t_2 - t_1} \quad (3.4)$$

where X_2 and X_1 represent biomass concentration (in mg DW L^{-1}) and N_2 and N_1 are cell concentration (in cells mL^{-1}) for the times t_2 and t_1 (in d), respectively.

3.10. Nutrients removal kinetics

In order to evaluate the nutrients removal, it was first determined two parameters: (i) the average removal rate; and (ii) the nutrient removal efficiency. These parameters take in consideration the nutrients concentration at the end and the beginning of the algal culturing. For the average removal rate (R_s, in mg S L^{-1} d^{-1}) Equation 2.5 was used:

$$R_s = \frac{S_i - S_f}{t_f - t_i} \quad (3.5)$$

where S_i and S_f represents the concentration of nutrients (in mg S L^{-1}) at times t_i and t_f (in d), respectively.

For the nutrient removal efficiency (R, in %) Equation 3.6 was used:

$$\%R = \frac{S_i - S_f}{S_i} \times 100 \quad (3.6)$$

Secondly, the modified Gompertz model was used to determine two kinetic parameters of the microalgal growth: (i) the uptake rates (k, in d^{-1}); and (ii) the lag time (λ, in d). This model uses the experimental data of the nutrients concentration throughout the cultivation. The model is presented in Equation 3.7:

$$S(t) = S_i + (S_f - S_i) \cdot \exp[\exp\{-k \cdot (\lambda - t) + 1\}] \quad (3.7)$$

where $S(t)$ represents the values of nutrients concentration corresponding throughout the time-course evolution (in mg S L^{-1}). The estimation of both parameters was made from the minimization of the sum of squared residuals with the use of Solver supplement of Microsoft Excel 2016.

3.11. Statistical analysis

To evaluate the statistically significance of the results obtained from different conditions ANOVA test was used considering a significance level of 0.05. The statistical software used was SPSS 25.0 (SPSS Inc., Chicago, USA).

4. Results and Discussion

4.1. The effect of C:N:P ratio

4.1.1. Microalgal growth

The microalgal growth curve of a batch culture can be divided in four distinct phases (Forget et al., 2010): (i) the lag phase, where the synthesis of cellular components occurs. This is necessary for the basal metabolism and adaptation of the microalgae to the medium and as such there is virtually no observation of growth; (ii) the exponential or log phase, where there is a doubling of the number of cells per unit of time and as such an exponential increase of the population; (iii) the stationary phase, where the rate of cell division is equal to the rate of cell death which is caused by a lack of nutrients and accumulation of wastes; and (iv) the death or decline phase. In Figure III.1, III. 2, III.3 and III.4 (Annex III) it is represented the growth curve for each C:N:P ratio variation of Trial 1, 2, 3 and 4, where it is possible to detect for the various cultures the lag phase, of approximately one day, the exponential and the beginning of the stationary phase, around the eleventh day. Due to time limitations the trial was stopped at the twelve day which coincided with the starting of the stationary phase and as such the death phase was not observed. Blair et al. (2014) reported entering the stationary phase at the tenth day of culturing for *C. vulgaris* with a variation of the Bold basal medium, e.g. the overall nutrient concentration was reduced by 50% from the original composition.

On Trial 1, it was evaluated the effect of the variation of the C:N:P ratio on the growth of *C. vulgaris.* In this trial, a suspended culture with a C:N:P ratio of 1.8:9:1 and in the absence of ciprofloxacin was used as a control. In Table 4.1 the kinetic growth parameters obtained for each C:N:P ratio, the average biomass productivity and the specific growth rate are presented.

Table 4.1 – Average biomass productivities (in g DW L^{-1} d^{-1}) and specific growth rate (in d^{-1}) for each C:N:P ratio.

C:N:P ratio	P (g DW L^{-1} d^{-1})	μ (d^{-1})
10:1:1	0.035±0.001	0.204±0.001
1:10:1	0.026±0.002	0.185±0.002
1:1:10	0.017±0.007	0.154±0.002
10:10:1	0.040±0.002	0.217±0.001
10:1:10	0.030±0.009	0.192±0.002
1:10:10	0.046±0.005	0.224±0.002
1:1:1	0.043±0.003	0.223±0.001
1.8:9:1	0.050±0.002	0.235±0.002

Values are presented as mean ± standard deviation of two independent experiments.

The obtained values for the average biomass productivity showed a minimum for the C:N:P ratio of 1:1:10, of 0.017±0.007 g DW L^{-1} d^{-1}, which was statistically different from the control of a C:N:P ratio of 1.8:9:1 (0.050±0.002 g DW L^{-1} d^{-1}; p<0.01). The maximum was of 0.046±0.005 g DW L^{-1} d^{-1} which was achieved for the C:N:P ratio of 1:10:10 and was not statistically different from the control (p=0.128). Gonçalves et al. (2016b) also reported similar results for the average biomass productivity (0.0214±0.0040 g DW L^{-1} d^{-1}) of *C. vulgaris* grown in a synthetic secondary effluent.

In Figure VII.1 (Annex VII) both kinetic growth parameters are presented in order to facilitate comparison between the experimental results. The ratios of 10:1:1, 1:10:10 and 1:1:1 presented three out of the four highest average biomass productivities and all had in common a N:P ratio of 1:1. However, the control, with a N:P ratio of 9:1, showed a high productivity of 0.050±0.002 g DW L^{-1} d^{-1} and was not statistically different from all the three samples (p=0.371, p=0.375 and p=0.373, respectively). On the other hand, the ratio of 1:1:10 and 1:10:1 presented the lowest productivities, which indicates that a limitation of nitrogen inhibited the microalgal growth. This was expected as the nitrogen concentration is normally parallel to the cellular protein content (Tam and Wong, 1996).

Similarly to the average biomass productivity, the values of the specific growth rate presented a minimum for the ratio of 1:1:10 with an experimental value of 0.154±0.002 d^{-1}, which was statistically different from the control (p<0.01). As for the maximum, it was of 0.224±0.002 d^{-1} for the ratio of 1:10:10 but was closely accompanied by the ratio 1:1:1 (0.223±0.001 d^{-1}). Both samples were not statistically different from the control (p=0.336 and p=0.333, respectively). Similar results for the average biomass productivity (between 0.054 and 0.236 d^{-1}) were reported by Filali et al. (2011) for *C. vulgaris* cultivated in a continuous photobioreactor.

4.1.2. Nutrients removal

As mentioned in Section 3.10 it was evaluated four nutrient removal kinetic parameters for nitrogen and phosphorous: (i) uptake rate (k, in d^{-1}); (ii) lag time (λ, in d); (iii) nutrient removal efficiency (R, in %); and (iv) average removal rate (Rs, in mg L^{-1} d^{-1}). Table 4.2 and Figure VIII.1 (Annex VIII) presents such parameters relatively to nitrogen.

The values obtained for the uptake rate of nitrogen showed a minimum of 0.298±0.002 d^{-1} for the C:N:P ratio of 1:10:1 and of 0.298±0.003 d^{-1} for the C:N:P ratio of 10:10:1. Both values were superior to the control (0.270±0.004 d^{-1}) and not statistically different (p=0.440 and p=0.445, respectively). The maximum value was of 0.351±0.005 d^{-1} for the C:N:P ratio of 1:10:10, which was statistically different from the control (p=0.004). The obtained values were lower than of those previously reported. For instance, Gonçalves et al. (2016b) studied the nitrogen removal of OECD medium by *C. vulgaris* and reported a higher uptake rate (1.42 ± 0.23 d^{-1}).

Table 4.2 – Uptake rates (k, in d^{-1}), lag time (λ, in d), nutrient removal efficiency (R, in %) and average removal rate (Rs, in mg N L^{-1} d^{-1}) of nitrogen for each C:N:P ratio.

C:N:P ratio	k (d^{-1})	λ (d)	R (%)	Rs (mg N L^{-1} d^{-1})
10:1:1	0.312±0.004	0.578±0.019	67.07±0.17	1.265±0.005
1:10:1	0.298±0.002	1.357±0.049	43.15±0.01	11.463±0.001
1:1:10	0.328±0.002	0.217±0.146	46.78±0.68	0.886±0.022
10:10:1	0.298±0.003	0.998±0.032	55.18±0.24	14.675±0.049
10:1:10	0.322±0.007	0.936±0.067	57.05±0.21	1.069±0.005
1:10:10	0.351±0.005	1.062±0.012	58.99±0.30	15.688±0.124
1:1:1	0.329±0.002	0.845±0.033	75.85±0.16	1.423±0.005
1.8:9:1	0.270±0.004	0.684±0.097	83.48±0.71	17.692±0.339

Values are presented as mean ± standard deviation of two independent experiments.

As for the lag time, the obtained values presented a minimum of 0.217±0.146 d for the C:N:P ratio of 1:1:10, which was statistically different from the control (p=0.021). The maximum value was 1.357±0.049 d^{-1}, obtained for the C:N:P ratio of 1:10:1, which was also statistically different from the control (p=0.002). In this case, the lag time values were similar to the previously reported. In the study mentioned above, Gonçalves et al. (2016b) reported an alike lag time for nitrogen, of 1.27 d. The highest value for the average removal rate was of 15.688±0.124 mg N L^{-1} d^{-1} for the C:N:P ratio of 1:10:10, which was lower than removal rate of the control (17.692±0.339 mg N L^{-1} d^{-1}). Both values were statistically different (p<0.001). Delgadillo-Mirquez et al. (2016) also reported a similar value for the removal rate of nitrogen for *C. vulgaris* at a temperature of 25 ºC, of 0.32 mg N L^{-1} h^{-1} (7.68 mg N L^{-1} d^{-1}).

The EU has established limits for nitrogen concentration and imposed minimum percentage load reductions (Directive 1991/271/EEC, 1991; Directive 1998/15/EC, 1998). According to the EU Directives, the limits for effluent discharge per population equivalent (PE) are 15 mg N L^{-1} (10 to 100 thousand PE) or 10 mg N L^{-1} (more than 100 thousand PE) for total nitrogen with a minimum percentage of reduction of 70-80%. Only the control and culture with C:N:P ratios of 1:1:1 achieved a reduction higher than 70%. On the other hand, the time-course evolution of the nitrate concentration for the different C:N:P concentrations of the Trial 1 can be analyzed in Figure IV.1 and IV.2 (Annex IV). It can be assessed that only the cultures with the ratios 10:1:1, 1:1:10, 10:1:10 and 1:1:1 presented a final concentration lower than 15 mg L^{-1}. However, it must be taken into account that the initial concentration of nitrogen was ten times lower than for the rest of the cultures. From the analysis of Figure XI.1 (Annex XI), Figure XII.1 (Annex XII) and Table 4.2, it can be established that, apart from the control, the best results on nitrogen removal were from the culture of 1:1:1, as it presented the highest removal efficiency and one of the highest uptake rates.

The removal kinetic parameters relatively to phosphorous are presented in Table 4.3 and in Figure IX.1 (Annex IX).

Table 4.3 – Uptake rates (k, in d^{-1}), lag time (λ, in d), nutrient removal efficiency (R, in %) and average removal rates (Rs, in mg P L^{-1} d^{-1}) of phosphorous for each C:N:P ratio.

C:N:P ratio	k (d^{-1})	λ (d)	R (%)	Rs (mg P L^{-1} d^{-1})
10:1:1	0.263±0.028	1.511±0.511	23.13±6.35	0.811±0.232
1:10:1	0.366±0.013	1.368±0.246	42.79±1.54	1.505±0.027
1:1:10	0.506±0.053	0.600±0.600	16.71±0.40	6.260±0.065
10:10:1	0.335±0.023	1.083±0.171	40.70±2.48	1.426±0.154
10:1:10	0.294±0.047	0.615±0.615	33.91±0.49	13.166±0.387
1:10:10	0.281±0.035	0.198±0.198	44.39±5.77	16.892±2.290
1:1:1	0.271±0.001	0.845±0.445	49.52±4.80	1.689±0.246
1.8:9:1	0.270±0.004	0.684±0.097	79.89±0.72	2.909±0.056

Values are presented as mean ± standard deviation of two independent experiments.

The values obtained for the uptake rate of phosphorous showed a minimum of 0.263±0.028 d^{-1} for the C:N:P ratio of 10:1:1, which was not statistically different to the control (p=0.546). The maximum value was 0.506±0.053 d^{-1} for the C:N:P ratio of 1:1:10, which was statistically different from the control (p<0.001). The maximum value of the lag time was 1.511±0.511 d for the C:N:P ratio of 10:1:1, which was also statistically different from the control (p=0.021). Gonçalves et al. (2016b) reported a slightly higher uptake rate of phosphorus of *C. vulgaris* of 0.593 d^{-1}. However, it reported a lag time of 0.869 d^{-1}, which is in the range of the values obtained on this trial.

As for the average removal rate, it showed a minimum of 0.811±0.232 mg P L^{-1} d^{-1} for the C:N:P ratio of 10:1:1, which was statistically different from the control (p<0.005). The maximum value was 16.892±2.290 mg P L^{-1} d^{-1} for the C:N:P ratio of 1:10:10, which was also statistically different from the control (p<0.001). Delgadillo-Mirquez et al. (2016) with a culture of *C. vulgaris* in modified Bristol medium at a temperature of 25 ºC reported a lower value for the removal rate of phosphorous, of 0.024 mg P L^{-1} h^{-1} (0.576 mg P L^{-1} d^{-1}).

The EU also has established limits for phosphorus, which for effluent discharge per PE are 2 mg P L^{-1} (10 to 100 thousand PE) or 1 mg P L^{-1} (more than 100 thousand PE) for total phosphorus with a minimum percentage of reduction of 80% (Directive 1991/271/EEC, 1991; Directive 1998/15/EC, 1998). In Figure V.1 and V.2 (Annex V), it is represented the time-course evolution of the phosphorous concentration for the different C:N:P concentrations of the Trial 1. From its analysis, it is clear than only the control reached a concentration lower than that imposed by the EU regulation. Furthermore, as the maximum value for the nutrient removal efficiency, apart from the control (79.89±0.72%), was of 49.52±4.80% for the C:N:P ratio of 1:1:1, none of the variations of C:N:P achieved the minimum percentage of reduction of 80%. Aside from the control, the best results on phosphorous removal were achieved with 1:10:10 ratio as it presented the highest average removal rate, the second highest removal efficiency and a low lag time.

Thus, it can be assessed that the C:N:P ratio of 1:10:10 presented the best results as it presented the highest kinetic growth parameters and in overall high removal kinetic parameters for nitrogen and phosphorous.

4.1.3. COD

The chemical oxygen demand was measured for all the cultures. However, as the detection limit of the method was too low, the obtained results were not coherent. As this work had strict time restrictions, there was not enough time to validate another method to properly quantify the COD and as such this quantification was not performed in the subsequent trials.

4.2. Trial 2: Presence of an emerging contaminant and its effects

4.2.1. Microalgal growth

In the first trial, it was not possible to assess the influence of the carbon on the microalgal growth and on the removal of nutrients. Thus, its concentration was increased to a C:N:P ratio of 20:9:1. Apart from the control, the C:N:P ratio of 1:10:10 was the one which produced the best results. As such, it was chosen to be reproduced in the presence of ciprofloxacin.

Similarly to the first trial, as a control it was used a suspended culture with a C:N:P ratio of 1.8:9:1 and in the absence of ciprofloxacin. Table 4.4 and Figure VII.2 (in Annex VII) present the average biomass productivity and specific growth rate for each C:N:P ratio in the presence or absence of ciprofloxacin.

Table 4.4 – Average biomass productivity (P, in g DW L^{-1} d^{-1}) and specific growth rate (μ, in d^{-1}) for each C:N:P ratio.

C:N:P ratio	[Ciprofloxacin] ($\times 10^3$ µg L^{-1})	P (g DW L^{-1} d^{-1})	µ (d^{-1})
1.8:9:1	0.00	0.053±0.001	0.235±0.003
20:9:1	0.00	0.047±0.003	0.242±0.002
20:9:1	2.50	0.041±0.001	0.235±0.006
1:10:10	0.00	0.046±0.002	0.224±0.002
1:10:10	2.50	0.039±0.004	0.237±0.009

Values are presented as mean ± standard deviation of two independent experiments.

When comparing the average biomass productivities of the controls for Trials 1 and 2, they were not statistically different (p=0.682) and the same occurred for the specific growth rate (p=0.085). As for the samples with the C:N:P ratio of 1:10:10 for Trials 1 and 2 were also not statistically different (p=0.134 and p=0.507 for average biomass productivity and specific growth rate, respectively).

The average biomass productivity presented a maximum for the control (0.053±0.001 g DW L^{-1} d^{-1}). However, the second highest productivity was of 0.047±0.003 g DW L^{-1} d^{-1} for 20:9:1 ratio in the absence of ciprofloxacin. Both values were not statistically different (p=0.317). The minimum value of the average biomass productivity was of 0.039±0.004 g DW L^{-1} d^{-1} for the 1:10:10 culture with ciprofloxacin, which was statistically different from the control (p=0.018). The decrease in the productivity from the increase of carbon concentration was unexpected, as it has been reported an increase in productivity with the increase of carbon concentration when using sodium bicarbonate as a carbon source. For instance, Mokashi et al. (2016) reported an increment in productivity from 0.237 to 0.996 g d^{-1} L^{-1} for a sodium bicarbonate concentration of 0 and 1 g L^{-1}, respectively. On the other hand, El Jay (1996) reported an inhibitory effect for *C. vulgaris* when using carbon sources of methanol and ethanol.

When comparing both samples with the ratio 20:9:1, a decrease in the productivity from the presence of ciprofloxacin was found, from 0.047±0.003 to 0.041±0.001 g DW L^{-1} d^{-1}. However, the values were not statistically different (p=0.134). The same took place for the sample with the ratio of 1:10:10, a decreased from 0.046±0.002 to 0.039±0.004 g DW L^{-1} d^{-1}. These values were also statistically different (p<0.005). Nie et al. (2008) also reported a low growth inhibition rate (7.2%) for a ciprofloxacin concentration of 2.00 × 10^3 µg L^{-1}, which is due to ciprofloxacin being a weak inhibitor of *C. vulgaris*.

Concerning the specific growth rate, the sample using 20:9:1 without ciprofloxacin allowed a maximum of 0.242±0.002 d^{-1}. The control presented a slightly lower result, 0.235±0.002 d^{-1}, which was not statistically different from the previous culture (p=0.497). When comparing the 20:9:1 ratio in absence and in presence of ciprofloxacin, there was a decrease from the addition of the antibiotic, from 0.242±0.002 to 0.235±0.006 d^{-1}. Nevertheless, the samples were not statistically different (p=0.957). However, when comparing the 1:10:10 ratio in absence and in presence of ciprofloxacin, there was an unexpected increment in specific growth rate, from 0.224±0.002 to 0.237±0.009 d^{-1}. However, the samples were not statistically different (p=0.613).

As mentioned in Section 3.6 it was used the paper-disc agar method in order to assess the algal growth inhibition induced by the ciprofloxacin. In Figure XIV.1 is presented a plate for a ciprofloxacin concentration of 2.50x10^3 µg L^{-1}. From its analysis it can be assessed that this concentration of ciprofloxacin did not have any inhibition effect on *C. vulgaris*.

4.2.2. Nutrients removal

Similarly to the Trial 1, nutrient removal kinetic parameters for nitrogen and phosphorous were determined but in the presence and absence of ciprofloxacin. Table 4.5 and Figure VIII.2 (Annex VIII) presents the kinetic removal parameters related to nitrogen.

Table 4.5 – Uptake rates (k, in d^{-1}), lag time (λ, in d), nutrient removal efficiency (R, in %) and average removal rate (Rs, in mg N L^{-1} d^{-1}) of nitrogen for each C:N:P ratio.

C:N:P ratio	[Ciprofloxacin] (×10^3 µg L^{-1})	k (d^{-1})	λ (d)	R (%)	Rs (mg N L^{-1} d^{-1})
1.8:9:1	0.00	0.321±0.002	1.147±0.019	56.12±0.31	11.476±0.070
20:9:1	0.00	0.339±0.004	2.372±0.070	42.33±0.60	7.520±0.133
20:9:1	2.50	0.385±0.003	1.155±0.030	57.13±0.48	13.056±0.131
1:10:10	0.00	0.310±0.008	1.564±0.192	53.03±0.34	9.433±0.068
1:10:10	2.50	0.329±0.013	1.828±0.133	44.21±0.19	9.935±0.028

Values are presented as mean ± standard deviation of two independent experiments.

The values obtained for the uptake rate showed a minimum of 0.310±0.008 d^{-1} for the C:N:P ratio of 1:10:10, in the absence of ciprofloxacin, which was not statistically different from the control (p=0.451). The maximum value was 0.385±0.003 d^{-1} for the C:N:P ratio of 20:9:10 in the presence of ciprofloxacin, which was also not statistically different from the control (p=0.272). When comparing the values obtained in the absence and in the presence of ciprofloxacin there was a small increase in both C:N:P ratios. However, as all values were not statistically different it should be concluded that the uptake rate was not affected by the presence of the antibiotic.

The values obtained for the lag time showed a minimum of 1.155±0.030 d for the C:N:P ratio of 20:9:1 in the presence of ciprofloxacin, which was higher than the control (1.147±0.019 d). However, both values were not statistically different (p=0.062). The maximum value was 2.372±0.070 d for the C:N:P ratio of 20:9:1 in the absence of ciprofloxacin, which was statistically different from the control (p=0.032). As the addition of ciprofloxacin caused the lag time to increase with the 1:10:10 ratio but it also caused it to decrease with the 20:9:1 ratio, the contaminant effect on the parameter could not be assessed. These previous values were statistically different from value of the control (p<0.005 and p=0.035, respectively).

As for the average removal rate, it showed a minimum of 7.520±0.133 mg N L^{-1} d^{-1} for the C:N:P ratio of 20:9:1 in the absence of ciprofloxacin, which was statistically different from the control (p=0.042). The maximum value was 13.056±0.131 mg N L^{-1} d^{-1} for the C:N:P ratio of 20:9:10 in the presence of ciprofloxacin which was not statistically different from the control (p=0.399). Escapa et al. (2015) reported that the presence of pharmaceuticals in the culture medium caused an increase in biomass and in the removal of nutrients for a culture of *Chlorella sorokiniana* in the presence of salicylic acid and of paracetamol.

None of the cultures achieved a final concentration below the EU legislation of a minimum 15 mg L^{-1}, as it can be assessed by Figure IV.3 (Annex IV). Furthermore, none of the cultures achieved the minimum removal efficiency of 70% as the maximum obtained value was 57.13±0.48% for the C:N:P ratio of 20:9:1 in the presence of ciprofloxacin, which was statistically different from the control (p<0.001). The minimum value was 42.33±0.60% for 20:9:1 ratio in the absence of ciprofloxacin, which was also statistically different from the control (p<0.001). Figure XI.2 (Annex XI) and of Figure XII.2 (Annex XII) present the removal efficiencies and average removal rates for the different cultures of trial 2. Apart from the control, the culture using the 20:9:1 ratio in the presence of ciprofloxacin produced the best results in terms of nitrogen removal, as it had the highest removal efficiency and average removal rate.

As mentioned previously, the same parameters estimated for nitrogen were estimated for phosphorous. As such, Table 4.6 and Figure IX.2 (Annex IX) presents the kinetic removal parameters for phosphorous. The values obtained for the uptake rate presented a minimum of 0.248±0.003 d^{-1} for the C:N:P ratio of 1:10:10 in the absence of ciprofloxacin, which was not statistically different from the control (p=0.360). The maximum value was 0.476±0.011 d^{-1} for the C:N:P ratio of 20:9:10 in the absence of ciprofloxacin, which was statistically different from the control (p=0.001). Similarly to the uptake rate of nitrogen, the effect of the presence of ciprofloxacin could not be assessed as the two ratios had different responses.

Table 4.6 – Uptake rates (k, in d^{-1}), lag time (λ, in d), nutrient removal efficiency (R, in %) and average removal rate (Rs, in mg P L^{-1} d^{-1}) of phosphorous for each C:N:P ratio.

C:N:P ratio	[Ciprofloxacin] (×10^3 µg L^{-1})	k (d^{-1})	λ (d)	R (%)	Rs (mg P L^{-1} d^{-1})
1.8:9:1	0.00	0.270±0.004	0.684±0.097	80.38±0.72	3.038±0.058
20:9:1	0.00	0.476±0.011	1.437±0.056	27.36±0.67	1.009±0.028
20:9:1	2.50	0.431±0.107	1.620±0.138	39.73±4.51	1.506±0.179
1:10:10	0.00	0.248±0.003	1.313±0.194	12.02±0.52	4.541±0.241
1:10:10	2.50	0.305±0.089	(1)	12.97±1.54	4.741±1.019

Values are presented as mean ± standard deviation of two independent experiments.
(1) It was not possible to calculate the parameter as the software could not fit the experimental values to the Gompertz model since the nutrient removal was too low.

On the other hand, as the removal efficiency of the culture with a 1:10:10 ratio in the presence was too low, it was not possible to determine its lag time. However, the minimum obtained lag time was of 1.313±0.19 d for the C:N:P ratio of 1:10:10 in the absence of ciprofloxacin, which was superior to the value of the control (0.684±0.097 d). Both values were statistically different (p=0.004). The maximum value was of 1.620±0.138 d for the C:N:P ratio of 20:9:1 in the presence of ciprofloxacin, which also was statistically different from the control (p<0.001). Contrary to the lag times of nitrogen with the cultures of a ratio of 20:9:1, the lag time of phosphorous showed a tendency to increase with the addition of ciprofloxacin. However, this increase was not statistically different (p=0.455).

As for the removal efficiency, only the control achieved a superior value of removal efficiency than the indicated by the EU legislation (80.38±0.72%). While, none of the cultures had a final concentration of phosphorous below 2 mg L^{-1} (Figure V.3, Annex V).

As for the average removal rate, it showed a minimum of 1.009±0.028 mg P L^{-1} d^{-1} for the C:N:P ratio of 20:9:1 in the absence of ciprofloxacin, which was statistically different from the control (p<0.001). While, the maximum value was 4.741±1.019 mg P L^{-1} d^{-1} for the C:N:P ratio of 1:10:10 in the presence of ciprofloxacin, which was statistically different to the control (p<0.001).

The presence of ciprofloxacin appears to have stimulated the removal of nutrients by the microalgae, as it increased significantly the value of the uptake rate, the nutrient removal efficiency and the average removal rate of most cultures. While, it decreased significantly the value of the lag time for both nitrogen and phosphorous. This is an unexpected effect, as ciprofloxacin is an third generation antibiotic and is designed to kill microorganisms, specifically aerobic Gram-positive and Gram-negative bacteria (Norvill et al., 2016). However, Grimes (2016) reported a similar response to a culture *Scenedesmus dimorphus* in the presence of high concentrations of ciprofloxacin.

In order to evaluate the bioremediation of ciprofloxacin it is presented in Table 4.7 and Figure X.1 (in Annex X) the kinetic removal parameters for the two C:N:P ratios tested.

Table 4.7 – Uptake rates (k, in d^{-1}), lag time (λ, in d), nutrient removal efficiency (R, in %) and average removal rate (Rs, in mg S L^{-1} d^{-1}) of ciprofloxacin for each C:N:P ratio.

C:N:P ratio	k (d^{-1})	λ (d)	R (%)	Rs (mg L^{-1} d^{-1})
20:9:1	0.255±0.020	1.139±0.700	32.12±0.17	0.133±0.001
1:10:10	0.302±0.001	0.735±0.294	22.42±1.21	0.100±0.005

Values are presented as mean ± standard deviation of two independent experiments.

As for the uptake rates and lag times, they were similar to the values obtained by nitrogen and phosphorous. Which, suggests that the nutrients were assimilated by the algal metabolism at the same rate. As ciprofloxacin is an emerging contaminant there are not studies with the evaluation of their uptake rate and lag time. However, the concentration evolution of ciprofloxacin of both cultures can be analyzed in Figure VI.1 (Annex VI) and it can be assessed that both finals concentrations were superior to $1.50x10^3$ µg L^{-1}.

From further analysis of Table 4.7, it can be also assessed that there was a significant removal efficiency of ciprofloxacin in both cultures. The 20:9:1 ratio presented a higher removal efficiency of 32.12±0.17%, while the 1:10:10 ratio presented a respectful efficiency of 22.42±1.21%. Both values were statistically different from each other (p=0.032). On the other hand, from the analysis of the Figure XII.3 (Annex XII) the average removal rate of ciprofloxacin can be evaluated. Its value ranged between 0.133±0.001 and 0.100±0.005 mg L^{-1} d^{-1}, for the 20:9:1 and the 1:10:10 ratios, respectively. However,

Hom-Díaz et al. (2017) reported higher values for both parameters of the removal of ciprofloxacin in the treatment of a secondary domestic wastewater, in which they reported a removal efficiency and a volumetric removal rate of 59±9% and 0.29 mg L^{-1} d^{-1}, respectively.

4.3. Trial 3: Immobilization of *C. vulgaris*

4.3.1. Microalgal growth

The controls of Trial 1 and 2 was the one which presented consistently the highest nutrient removal efficiencies and rates. As such, its C:N:P ratio (1.8:9:1) was chosen to be tested on the third trial. On the other hand, *C. vulgaris* was immobilized in sodium alginate in order to mimic the behavior of sessile cells, as described in Section 3.3. In Table 4.8 and in Figure VII.3 (in Annex VII) are presented the average biomass and specific growth rate for each C:N:P ratio and ciprofloxacin concentration.

Table 4.8 – Average biomass productivity (P, in mg DW L^{-1} d^{-1}) and specific growth rate (μ, in d^{-1}) for each C:N:P ratio and ciprofloxacin concentration (in μg L^{-1}).

C:N:P ratio	[Ciprofloxacin] ($\times 10^3$ μg L^{-1})	P (g DW L^{-1} d^{-1})	μ (d^{-1})
1.8:9:1	0.00	0.053±0.001	0.205±0.002
1.8:9:1	2.50	0.041±0.001	0.196±0.002
1.8:9:1*	0.00	0.079±0.007	0.253±0.007
1.8:9:1*	2.50	0.079±0.003	0.246±0.004

Values are presented as mean ± standard deviation of two independent experiments.
* *C. vulgaris* immobilized in sodium alginate beads.

As in the previous trials, the control used was the suspended culture in the absence of ciprofloxacin and with a C:N:P ratio of 1.8:9:1. When comparing the average biomass productivities of the controls of the Trial 2 and Trial 3 they were not statistically different (p=0.941) and the same occurred for the specific growth rate (p=0.085).

The values for the average biomass productivity showed a minimum of 0.041±0.001 g DW L^{-1} d^{-1} for the suspended biomass in the presence of ciprofloxacin, which was not statistically different from the control (p=0.983). The maximum was shown by the immobilized cultures with and without ciprofloxacin, 0.079±0.007 and 0.079±0.003 g DW L^{-1} d^{-1}, respectively. Both values were statistically different from the control (p=0.08 in both cases). The increase between the suspended and immobilized biomass productivities in the absence of ciprofloxacin was quite significant, from 0.043±0.001 to 0.079±0.007 g DW L^{-1} $d^{-}1$, which shows an enhance on microalgal growth. Mujtaba and Lee (2017) also reported a similar enhancement of on the productivity from the immobilization of *C. vulgaris* in an alginate polymer, from 0.03 to 0.05 g L^{-1} d^{-1}.

Relatively to the specific growth rate, a minimum of 0.196±0.002 d^{-1} was observed for the suspended microalgae with ciprofloxacin, which was not statistically different from the control (p=0.497). The maximum specific growth rate was shown by the immobilized microalgae without ciprofloxacin (0.253±0.007 d^{-1}), which was statistically different from the control (p=0.004). As expected, there was a small decrease in the growth rate with the addition of ciprofloxacin with the suspended microalgae, from 0.205±0.002 to 0.196±0.002 d^{-1}. Both values were not statistically different (p=0.497). The same happened for immobilized cultures, a decrease from 0.253±0.007 to 0.246±0.004 d^{-1} due to the addition of antibiotic. Both values were also not statistically different (p=0.721).

4.3.2. Nutrients removal

As in the previous trials the kinetic removal parameters were calculated for to nitrogen and are presented in Table 4.9 and Figure VIII.3 (in Annex VIII).

Table 4.9 – Uptake rates (k, in d^{-1}), lag time (λ, in d), nutrient removal efficiency (R, in %) and average removal rate (Rs, in mg N L^{-1} d^{-1}) of nitrogen for each C:N:P ratio.

C:N:P ratio	[Ciprofloxacin] ($\times 10^3$ µg L^{-1})	k (d^{-1})	λ (d)	R (%)	Rs (mg N L^{-1} d^{-1})
1.8:9:1	0.00	0.305±0.001	1.093±0.026	44.38±0.33	9.329±0.085
1.8:9:1	2.50	0.355±0.007	2.223±0.069	42.61±0.31	8.946±0.081
1.8:9:1*	0.00	0.280±0.012	1.032±0.031	34.52±0.20	7.023±0.042
1.8:9:1*	2.50	0.395±0.002	3.276±0.028	49.30±0.27	10.343±0.067

Values are presented as mean ± standard deviation of two independent experiments.
* *C. vulgaris* immobilized in sodium alginate beads.

The addition of ciprofloxacin to the culture medium increased the values of the uptake rate, being the maximum of 0.395±0.002 d^{-1} for the immobilized culture in the presence of ciprofloxacin, which was statistically different from the control (p<0.001). However, the immobilization of the microalga did not have an effect on the uptake rate.

As for the lag time, it also presented an increment in value from the addition of antibiotic to the medium. This was unexpected as generally when the uptake rate increases there is a decrease on the lag time value. However, there also was no visible effect on the lag time from the microalgal immobilization. Nevertheless, a minimum of 1.032±0.031 d for the immobilized culture in the absence of ciprofloxacin, which was not statistically different from the control (p=0.534), and a maximum value of 3.276±0.028 d for the immobilized culture in the presence of ciprofloxacin, which was statistically different from the control (p=0.002).

Figure IV.4 (Annex IV) presents the time-course evolution of nitrogen and it shows than none of the cultures achieved the minimum concentration of 15 mg L^{-1} require by the EU regulation. As for the nutrient removal efficiency, also none of the cultures achieved the minimum of 70% as the maximum value

was 49.30±0.27% for the immobilized culture in the presence of ciprofloxacin, which was statistically different from the control (p<0.001).

Similarly to the removal efficiency, the minimum value of the removal rate (7.023±0.042 mg N L^{-1} d^{-1}) was for the immobilized culture in the absence of ciprofloxacin, which was statistically different from the control (p<0.001). While, the maximum value (10.343±0.067 mg N L^{-1} d^{-1}) was for the immobilized culture in the presence of ciprofloxacin, which was statistically different from the control (p=0.001). It appears that the addition of ciprofloxacin to the suspended cultures inhibited the assimilation of nitrogen. However, the addition of antibiotic in the immobilized cultures heightened the nitrogen removal.

Thus, from the analysis of Figure XI.3 (Annex XI) and of Figure XII.4 (Annex XII) it can be concluded that the best results were obtained by the immobilized culture in the presence of ciprofloxacin as it had the highest removal efficiency and average removal rate.

In Table 4.10 and in Figure IX.3 (in Annex I) it is presented the kinetic removal parameters relatively to phosphorous.

Table 4.10 – Uptake rates (k, in d^{-1}), lag time (λ, in d), nutrient removal efficiency (R, in %) and average removal rate (Rs, in mg P L^{-1} d^{-1}) of phosphorous for each C:N:P ratio.

C:N:P ratio	[Ciprofloxacin] (×10^3 µg L^{-1})	k (d^{-1})	λ (d)	R (%)	Rs (mg P L^{-1} d^{-1})
1.8:9:1	0.00	0.392±0.030	1.756±0.170	49.43±0.97	1.856±0.053
1.8:9:1	2.50	0.542±0.042	2.516±0.108	46.81±1.02	1.731±0.071
1.8:9:1*	0.00	1.061±0.012	0.837±0.044	95.50±0.21	3.428±0.119
1.8:9:1*	2.50	1.132±0.032	0.410±0.007	96.79±0.07	3.424±0.038

Values are presented as mean ± standard deviation of two independent experiments.
* *C. vulgaris* immobilized in sodium alginate beads.

The immobilization of the microalgal produced a significant increase on the uptake rate in the presence and in the absence of ciprofloxacin. On the other hand, the addition of ciprofloxacin also caused an increase of the value of the uptake rate, which was not evident in the previous trial. As such, the maximum value (1.132±0.032 d^{-1}) was for the immobilized culture in the presence of ciprofloxacin, which was statistically different from the control (p<0.001).

As for the lag time, it was shown an expected decreased in its value from the immobilization of *C. vulgaris*. Secondly, in the case of the immobilized cultures there was also a decrease of the lag time value due to the presence of ciprofloxacin. As such, the minimum (0.410±0.007 d) was presented by the immobilized culture in the presence of ciprofloxacin, which was statistically different from the control (p<0.001). In the case of the suspended cultures, the addition of the antibiotic to the medium caused an increase in value, which was statistically different from the control (p<0.039).

Figure V.5 (Annex V) presents the time-course evolution of phosphorous and it shows that the immobilized cultures achieved the minimum concentration of 2 mg L^{-1} require by the EU legislation, while the suspended ones did not. The 80% minimum in the removal efficiency was also only achieved by both immobilized cultures.

As for the average removal rate, it did not show a significant variation from the addition of ciprofloxacin (p=0.721 and p=0.983 for the suspended and immobilized cultures, respectively). This was unexpected as in the previous trial (Trial 2) this addition caused a significant increase in the average removal rate of phosphorous. However, the immobilization of the microalga caused an expected increase in the parameter. The maximum value was 3.428±0.119 mg P L^{-1} d^{-1} for the immobilized culture in the absence of ciprofloxacin, which was statistically different from the control (p<0.001).

Thus, the phosphorous results were far more satisfactory in this trial as the immobilization of the *C. vulgaris* produced removal efficiencies higher than 95%, as it can be assessed by the analysis of Figure XI.3 (Annex XI) and of Figure XII.4 (Annex XII).

In Table 4.11 and in Figure X.2 (in Annex X) the kinetic removal parameters for ciprofloxacin for the suspend and immobilized cultures are presented.

Table 4.11 – Uptake rates (k, in d^{-1}), lag time (λ, in d), nutrient removal efficiency (R, in %) and average removal rate (Rs, in mg L^{-1} d^{-1}) of ciprofloxacin for each C:N:P ratio.

C:N:P ratio	k (d^{-1})	λ (d)	R (%)	Rs (mg L^{-1} d^{-1})
1.8:9:1	0.023±0.003	0.099±0.012	26.17±0.19	0.0542±0.001
1.8:9:1*	0.215±0.002	0.078±0.020	58.84±0.29	0.122±0.009

Values are presented as mean ± standard deviation of two independent experiments.
* *C. vulgaris* immobilized in sodium alginate beads.

The immobilization of the microalgae greatly improved the removal of ciprofloxacin, as it significantly increased the uptake rate from 0.023±0.003 d^{-1} to 0.215±0.002 d^{-1}, it decreased the lag time from 0.099±0.012 d to 0.078±0.020 d, and it more than doubled the removal efficiency and the average removal rate. All parameters were statistically different between the suspended and the immobilized cultures (p<0.001, p=0.043, p<0.001 and p<0.001, respectively). From the analysis of Figure VI.2 (Annex VI) it can be assessed that the final concentration of ciprofloxacin of the suspended culture was similar to the one obtained in the second trial. However, as the immobilized culture in this trial had a higher removal of ciprofloxacin, its final concentration was of (1.03±0.01)x10^3 μg L^{-1}.

In order to assess if the sodium alginate had any influence on the removal kinetic parameters of phosphorous, nitrogen and ciprofloxacin, it was also monitored the evolution of the phosphorous, nitrogen and ciprofloxacin concentrations in culture mediums with a C:N:P ratio of 1.8:9:1 with sodium alginate beads but in the absence of *C. vulgaris*. As expected, there was no significant change in the concentration

of the three substances. Thus, it can be concluded that the nutrient removal was only due to the microalga and it was not influenced by any action with the sodium alginate beads.

4.4. Trial 4: C:N:P ratio variation and *C. vulgaris* immobilization

4.4.1. Microalgal growth

In order to further access the carbon effect on immobilizes microalgal growth, its concentration was increased again to a ratio of 20:9:1. On the other hand, as the previous trial (Trial 3) presented interesting results on phosphorous removal, the ratio was changed to 1.8:9:10. As for the concentration of ciprofloxacin it was doubled to 5.00×10^3 µg L^{-1}, in order to access there was algal growth inhibition from the increase in concentration. Table 4.12 and Figure VII.4 (in Annex VII) present the average biomass productivities and specific growth rates for each C:N:P ratio and ciprofloxacin concentration. Once more, the control of the trial was of the suspended culture with a C:N:P ratio of 1.8:9:1 without ciprofloxacin.

Table 4.12 – Average biomass productivities (in mg DW L^{-1} d^{-1}) and specific growth rates (in d^{-1}) for each C:N:P ratio.

C:N:P ratio	[Ciprofloxacin] ($\times10^3$ µg L^{-1})	P (g DW L^{-1} d^{-1})	µ (d^{-1})
1.8:9:1	0.00	0.061±0.002	0.237±0.002
20:9:1	0.00	0.040±0.001	0.201±0.002
20:9:1	5.00	0.039±0.001	0.195±0.003
20:9:1*	0.00	0.063±0.007	0.412±0.004
20:9:1*	5.00	0.060±0.008	0.405±0.003
1.8:9:10	0.00	0.038±0.001	0.195±0.002
1.8:9:10	5.00	0.037±0.002	0.194±0.002
1.8:9:10*	0.00	0.086±0.004	0.457±0.013
1.8:9:10*	5.00	0.051±0.006	0.420±0.006

Values are presented as mean ± standard deviation of two independent experiments.
* *C. vulgaris* immobilized in sodium alginate beads.

When comparing between the controls from this trial (Trial 4) and Trials 3, there was not a statistically difference for the values of the average biomass productivities (p=0.237) and for the specific growth rates (p=0.118). The values for the average biomass productivity showed a minimum of 0.037±0.002 g DW L^{-1} d^{-1} for the C:N:P ratio of 1.8:9:10 with the suspended biomass in the presence of ciprofloxacin, which was statistically different from the control (p=0.049). The maximum of 0.086±0.004 g DW L^{-1} d^{-1} was obtained using C:N:P ratio of 1.8:9:10 with the immobilized biomass and in the absence of ciprofloxacin, which was also statistically different from the control (p=0.040).

The increase of the carbon concentration using C:N:P ratio of 1.8:9:1 to 20:9:1 produced a significant decrease on the productivities on the suspended cultures in the absence of ciprofloxacin, 0.061±0.002 and 0.040±0.001 g DW L^{-1} d^{-1} respectively, as they were statistically different (p=0.004). The increase in phosphorous concentration also resulted in a decrease of the average biomass productivity when comparing to the control (0.038±0.001 g DW L^{-1} d^{-1}). Both values were statistically different (p=0.049). Liang et al. (2013) reported an increase in algal biomass production for phosphorous concentrations between 18 and 80 mM and a decrease for phosphorous concentrations higher than 80 mM. Thus, suggesting that high concentrations of phosphorous inhibits the algal metabolism, e.g. the phosphorous concentration of 450 mg L^{-1} used for the C:N:P ratio of 1.8:9:10. As for the immobilization of the microalga, it caused an increase of the average biomass productivity in both C:N:P ratios and in the presence and absence of ciprofloxacin. For instance, the immobilization of the microalgae for the C:N:P ratio of 20:9:1 in the absence of ciprofloxacin increased the productivity from 0.040±0.001 to 0.063±0.007 g DW L^{-1} d^{-1}. These values were statistically different (p=0.044). While, the immobilization of the culture using C:N:P ratio of 1.8:9:10 in the absence of ciprofloxacin increased the productivity from 0.038±0.001 to 0.086±0.004 g DW L^{-1} d^{-1}. Both values were also statistically different (p<0.01). The addition of ciprofloxacin caused a decreased in productivity for the cultures of 1.8:9:10 ratio, which was also observed in the previous trials (Trial 2 and 3). However, for the cultures of 20:9:1 ratio the addition of antibiotic did not showed a significant effect on the parameter.

The increase of the concentration of ciprofloxacin in this trial did not cause a significant difference in the kinetic parameters, e.g. when comparing the productivities from the suspended culture of a 20:9:1 ratio with a ciprofloxacin concentration of 5.00×10^3 µg L^{-1} for this trial (Trial 4), 0.039±0.001 g DW L^{-1} d^{-1} to the suspended culture of the same C:N:P ratio but with a ciprofloxacin concentration of 2.50×10^3 µg L^{-1} from Trial 2, 0.041±0.001 g DW L^{-1} d^{-1}, there was no statistically difference (p=0.042).

Relatively to the values of the specific growth rate, it presented a minimum of 0.194±0.002 d^{-1}, for the suspended culture of a 1.8:9:1 ratio in the absence of ciprofloxacin, which was statistically different from the control (p=0.004). While, the maximum of 0.457±0.013 d^{-1} was obtained for C:N:P ratio of 1.8:9:10 with the immobilized biomass and in the absence of ciprofloxacin, which was also statistically different from the control (p=0.040). Likewise to the average biomass productivity, the specific growth rate of the suspended culture with the 20:9:1 ratio in the absence of ciprofloxacin was lower than the control, 0.201±0.002 d^{-1} and 0.237±0.002 d^{-1}, respectively. Both values were statistically different (p=0.011). As for the increase in the phosphorous concentration, it also resulted in a decrease of the specific growth rate, 0.195±0.002 d^{-1}. The value was statistically different from the control (p=0.004). The immobilized cultures without ciprofloxacin showed a significant increase between their rates and the control, 0.412±0.004 d^{-1} for the 20:9:1 ratio and 0.457±0.013 d^{-1} for the 1.8:9:10 ratio. Both samples were statistically different from the control (p=0.0011 and p=0.004, respectively). The addition of ciprofloxacin provoked a decrease of the

rate in both ratios, as observed in the previous trials. However, the increase in concentration did not cause a significant difference in the specific growth rate. The suspended culture of 20:9:1 ratio with ciprofloxacin concentration from this trial (Trial 4) presented a rate of 0.195±0.003 d^{-1}, while the suspended culture of the same C:N:P ratio with ciprofloxacin from Trial 2 presented a 0.235±0.006 d^{-1}. Both values were not statistically different (p=0.005).

In overall, the increase in the phosphorus concentration seem to have inhibited the growth of *C. vulgaris*, as when comparing to the control there was a decrease in both kinetic growth parameters. Martínez et al. (1999) also reported that an increase in the concentration of phosphorous caused an increase in the inhibitory effect for the microalga *Scenedesmus obliquus*.

On the other hand, as the ciprofloxacin concentration was incremented it was also applied the paper-disc agar method for a ciprofloxacin concentration of 5.00x10^3 µg L^{-1}. The paper-disc agar is presented in Figure XIV.2 (Annex XIV). From its analysis it can be assessed that the increase in concentration did not caused any inhibition effect on *C. vulgaris*.

4.4.2. Nutrients removal

Identically to the previous trials, the uptake rate, lag time, removal efficiency and average removal rate was estimated for nitrogen and are presented in Table 4.13 and in Figure VIII.4 (Annex VIII).

Table 4.13 – Uptake rates (k, in d^{-1}), lag time (λ, in d), nutrient removal efficiency (R, in %) and average removal rate (Rs, in mg N L^{-1} d^{-1}) of nitrogen for each C:N:P ratio.

C:N:P ratio	[Ciprofloxacin] (×10^3 µg L^{-1})	k (d^{-1})	λ (d)	R (%)	Rs (mg N L^{-1} d^{-1})
1.8:9:1	0.00	0.309±0.002	0.968±0.021	41.60±0.10	8.614±0.028
20:9:1	0.00	0.316±0.002	0.959±0.056	39.67±0.19	8.472±0.058
20:9:1	5.00	0.435±0.008	3.411±0.112	42.51±0.08	9.012±0.032
20:9:1*	0.00	0.329±0.025	0.576±0.178	36.94±0.12	7.866±0.032
20:9:1*	5.00	0.307±0.002	0.461±0.053	35.78±0.21	7.392±0.056
1.8:9:10	0.00	0.345±0.011	2.340±0.322	15.26±0.20	3.228±0.037
1.8:9:10	5.00	0.282±0.002	1.366±0.037	28.86±0.06	6.114±0.024
1.8:9:10*	0.00	0.345±0.002	0.904±0.063	25.66±0.15	5.232±0.034
1.8:9:10*	5.00	0.744±0.012	2.454±0.026	50.63±0.25	10.714±0.042

Values are presented as mean ± standard deviation of two independent experiments.
* *C. vulgaris* immobilized in sodium alginate beads.

The addition of ciprofloxacin to the culture medium caused contradictory effects on the uptake rate of nitrogen, e.g. it caused an increase on the suspended cultures with a 20:9:1 ratio, from 0.316±0.002 to 0.435±0.008 d^{-1}, but it provoked a decrease when added to the suspended culture of a C:N:P ratio of

1.8:9:10, from 0.345±0.011 to 0.282±0.002 d^{-1}. As for the immobilization of the microalga, in overall it did not cause a significant difference on the uptake rate. However, the immobilization of the culture with the 1.8:9:10 ratio in the presence of ciprofloxacin, there was a substantial increase in the uptake rates, from 0.282±0.002 to 0.744±0.012 d^{-1}. Both values were statistically different (p=0.037).

The value of lag time, in general, was lowered by the immobilization of the microalga. As such the minimum was of 0.461±0.053 d for the immobilized culture with a 20:9:1 ratio in the presence of ciprofloxacin, which was statistically different from the control (p=0.043). Similarly to the uptake rate, the addition of ciprofloxacin to the medium did not show a consistent effect on the lag time. However, there was a value decrease for the suspended cultures with a 1.9:9:10 ratio and the immobilized cultures with a 20:9:1 ratio, as it was expected.

The increase in the phosphorous concentration caused an inhibitory effect on the algal growth and in the removal of nitrogen, as there was a decrease in the removal efficiency and average removal rate when compared to the control. However, the immobilization of the microalga and the addition of ciprofloxacin counteracted this effect. As such, the immobilized culture with a 1.8:9:10 ratio in the presence of ciprofloxacin presented the maximum value for the removal efficiency (50.63±0.25%) and for the removal rate (10.714±0.042 mg N L^{-1} d^{-1}). Both values were statistically different from the control (p<0.001 and p=0.001, respectively).

On the other hand, as maximum value of the removal rate was lower than 70%, none of the cultures achieved the EU legislation minimum. From the analysis of Figure IV.5 (Annex IV), it can be also inferred that none of the cultures had a final concentration lower than the minimum required by the EU legislation.

From examination of nitrogen parameters, the best results were obtained by the immobilized culture with an increased concentration of phosphorous in the presence of ciprofloxacin, as it had the highest uptake rate, removal efficiency and average removal rate.

In Table 4.14 and in Figure IX.4 (Annex IX) the kinetic removal parameters relatively to phosphorous are presented. The uptake rate presented a maximum 3.660±0.179 d^{-1} for the immobilized culture with a 20:9:1 ratio in the presence of ciprofloxacin, which was statistically different from the control (p=0.004). It appears that the effects from the immobilization of *C. vulgaris* and from the increase of carbon concentration combined and as a result it was achieved the highest uptake rate value for phosphorous from all the cultures.

It was expected that the immobilized culture with a 20:9:1 ratio in the presence of ciprofloxacin would have the minimum value for the lag time, as its uptake rate was so elevated. However, this did not happen as the minimum value (0.902±0.025 d) was presented by the immobilized culture with a 1.8:9:10 ratio in the absence of ciprofloxacin, which was statistically different from the control (p=0.043). As for

the maximum value, it was of 2.667±2.074 d for the suspended culture with a 1.8:9:10 ratio in the absence of ciprofloxacin, which was also statistically different from the control (p=0.002).

Table 4.14 – Uptake rates (k, in d^{-1}), lag time (λ, in d), nutrient removal efficiency (R, in %) and average removal rate (Rs, in mg P L^{-1} d^{-1}) of phosphorous for each C:N:P ratio.

C:N:P ratio	[Ciprofloxacin] ($\times 10^3$ µg L^{-1})	k (d^{-1})	λ (d)	R (%)	Rs (mg P L^{-1} d^{-1})
1.8:9:1	0.00	0.317±0.008	2.186±0.154	88.12±0.35	3.244±0.007
20:9:1	0.00	0.418±0.037	2.451±0.249	72.54±2.21	2.604±0.104
20:9:1	5.00	0.352±0.024	2.479±0.491	70.62±0.19	2.706±0.037
20:9:1*	0.00	3.070±0.962	1.301±0.540	98.49±0.40	3.731±0.081
20:9:1*	5.00	3.660±0.179	1.716±0.023	98.04±0.08	3.810±0.062
1.8:9:10	0.00	0.554±0.353	2.667±2.074	15.71±2.92	6.421±1.267
1.8:9:10	5.00	0.334±0.031	1.894±0.505	15.14±0.61	5.733±0.247
1.8:9:10*	0.00	0.422±0.027	0.902±0.025	54.08±1.52	22.445±0.880
1.8:9:10*	5.00	1.133±1.037	2.442±0.746	18.80±1.79	7.826±0.733

Values are presented as mean ± standard deviation of two independent experiments.
* *C. vulgaris* immobilized in sodium alginate beads.

In Figure V.6 and V.7 (Annex V) the time-course evolution of phosphorous concentration is presented. It can be assessed that only the immobilized cultures with a C:N:P ratio of 20:9:1 achieved final phosphorous concentration below the minimum required by the EU regulation. On the other hand, both cultures also achieved a removal efficiency higher than 80%. The maximum value was 98.49±0.40% for the immobilized culture with a 20:9:1 ratio in the absence of ciprofloxacin, which was statistically different from the control (p<0.001).

As for the values obtained for the average removal rate, the minimum was of 2.604±0.104 mg P L^{-1} d^{-1} for the suspended culture with a 20:9:1 ratio in the absence of ciprofloxacin, which was statistically different from the control (p=0.001). While, the maximum value was 22.445±0.880 mg P L^{-1} d^{-1} for the immobilized culture with a 1.8:9:10 ratio in the absence of ciprofloxacin, which was statistically different to the control (p=0.001). This value was superior to the rate of both immobilized cultures of a 20:9:1 ratio, as the initial phosphorous concentration of the 1.8:9:10 culture was ten times greater than the former. As such, this culture presented a greater removal rate and a smaller efficiency.

Contrary to the nitrogen removal kinetic parameters, the kinetic parameters for phosphorous removal were best for the immobilized culture with an increased concentration of carbon and in the presence of ciprofloxacin, as it had the highest uptake rate and average removal rate and the second highest removal efficiency.

In other respects, in Table 4.15 and in Figure X.3 (Annex X) the kinetic removal parameters relatively to ciprofloxacin are presented.

Table 4.15 – Uptake rates (k, in d^{-1}), lag time (λ, in d), nutrient removal efficiency (R, in %) and average removal rate (Rs, in mg L^{-1} d^{-1}) of ciprofloxacin for each C:N:P ratio.

C:N:P ratio	k (d^{-1})	λ (d)	R (%)	Rs (mg L^{-1} d^{-1})
20:9:1	(1)	(1)	14.21±0.27	0.057±0.001
20:9:1*	0.447±0.001	0.096±0.002	48.67±0.20	0.198±0.002
1.8:9:10	(1)	(1)	13.25±0.11	0.056±0.001
1.8:9:10*	0.305±0.001	0.098±0.001	35.10±0.39	0.142±0.003

Values are presented as mean ± standard deviation of two independent experiments.
* *C. vulgaris* immobilized in sodium alginate beads.
(1) It was not possible to calculate the parameter as the software could not fit the experimental values to the Gompertz model since the nutrient removal was too low.

As the removal of ciprofloxacin was too low for both the suspended cultures, its uptake rates and lag times were not estimated. However, the increase in phosphorous and in carbon provoked an increase in the uptake rate of the immobilized cultures relatively to the previous trial (Trial 3). As such, the maximum uptake rate value was of 0.447±0.001 d^{-1} for the 20:9:1 ratio. The estimated values of the lag time of the different C:N:P ratios were not statistically different from each other (p=0.728). On the other hand, when comparing to the immobilized culture from the previous (Trial 3), there was an increase on the lag time of ciprofloxacin.

Similarly, to the phosphorous removal efficiency, the highest value was obtained from the immobilized culture with a 20:9:1 ratio (48.67±0.20%), which was statistically different from the control (p<0.001). Githinji et al. (2011) reported, for a domestic wastewater, considerable lower removal efficiencies, ranging between 10 and 16% under different pH values. As such, it can be inferred that the immobilization of the microalga provoked a positive effect on the bioremediation of ciprofloxacin and on the removal of nutrients.

5. Conclusion

The aim of this work was to evaluated the effect of C:N:P ratio on nutrients removal by *C. vulgaris*, in the absence and presence of an emergent contaminant, ciprofloxacin. As a first approach the C:N:P ratio of the cultures were varied in order to assess its effect on the removal of nutrients (phosphorous and nitrogen). Then, the emergent contaminant ciprofloxacin was added to the medium to evaluate its removal from the culture and its effects on the removal of nutrients. Lastly, *C. vulgaris* was immobilized in sodium alginate beads in order to assess the role of immobilization on nutrient removal.

The C:N:P ratio affected the growth of *C. vulgaris* and the nutrients removal efficiency. The best results were obtained by the culture with a ratio of 1.8:9:1, which was the ratio of the original composition of the OECD medium, as it obtained an average biomass productivity of 0.061±0.002 g DW L^{-1} d^{-1}. The variations of C:N:P with the best results were 1:10:10 and 20:9:1, with a maximum average biomass productivity of 0.046±0.005 g DW L^{-1} d^{-1} and 0.040±0.001 g DW L^{-1} d^{-1}, respectively.

The microalgal immobilization greatly improved the average biomass productivity, nitrogen, phosphorus and ciprofloxacin removal efficiencies.

From the addition of ciprofloxacin to the algal cultures there was no observation of growth inhibition and it was achieved a satisfying removal rates of the antibiotic and nutrients, for instance it was obtained a removal efficiency of ciprofloxacin of 48.67±0.20% and an average removal rate of 0.198±0.002 mg L^{-1} d^{-1}.

In conclusion, the microalgal cultures of *C. vulgaris* present an effective alternative to the conventional methods for wastewater treatment, particularly for wastewater polishing. Removal rates and minimum concentrations imposed by the EU regulation were achieved, helping to reduce current environmental problems from insufficiently treated wastewaters.

6. Perspectives for future work

This thesis has demonstrated the huge potential of *C. vulgaris* on the removal of nutrients and of an emerging contaminant, ciprofloxacin. It was noted that different tests and more information is needed in this topic but due to time limitations and material resources many of these tests were not possible to perform.

Firstly, in order to confirm the validity of the findings it is needed to increase the model scale. It would be interesting to assess if the high removal rates achieved in this work would stand or if they had a significant decrease, as the nutrient removal rates usually decreases with the increase of scale of the reactor.

Biofilms represent the scaled-up version of the microalgal immobilization performed in this work with sodium alginate beads. As such, the cultivation and environmental conditions between the two types of scales varies dramatically. As it was obtained such favorable results from the algal immobilization, it should be assessed the scale-up effects on the algal growth and removal of nutrients.

It would be also interesting the use of real wastewaters from different sources, e.g. dairy, textile, municipal. In that way it would be tested different C:N:P ratios and also its effects on the algal growth. On the other hand, as the microalgal metabolism is shared by different species, the same culture conditions could be used for other species.

As new contaminants constantly appear in wastewaters, its effects on the microalgal growth and in nutrient removal kinetics need further studies. On the other hand, as ciprofloxacin did not show any inhibition effect on *C. vulgaris* it would be interesting to test different microalgal species against ciprofloxacin or to test new emerging contaminants against *C. vulgaris*.

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Annexes

Annex I: Calibration Curves

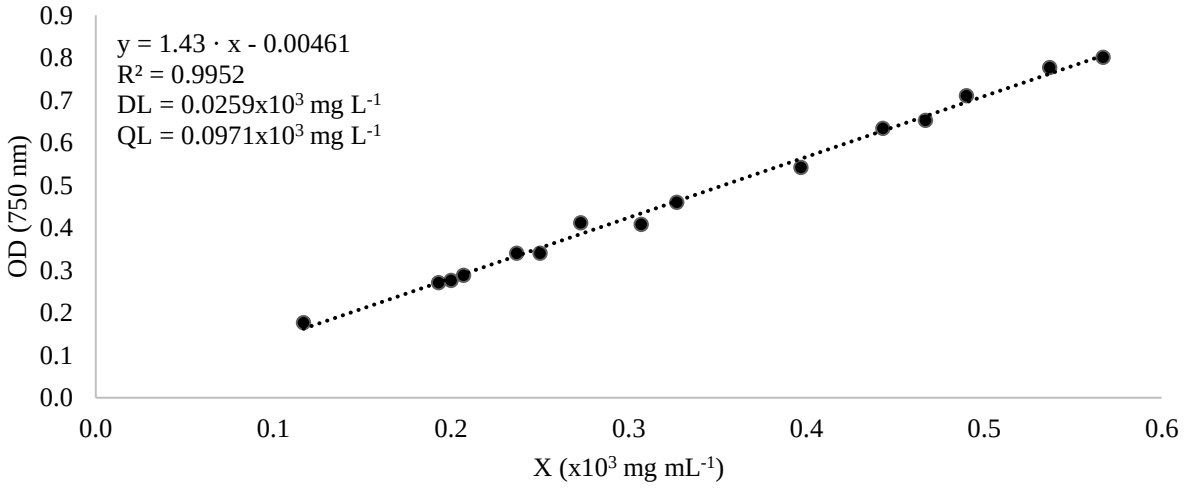

Figure I.1 - Linear regression between the OD measured at 750 nm and biomass concentration (X, mg DW L^{-1}).

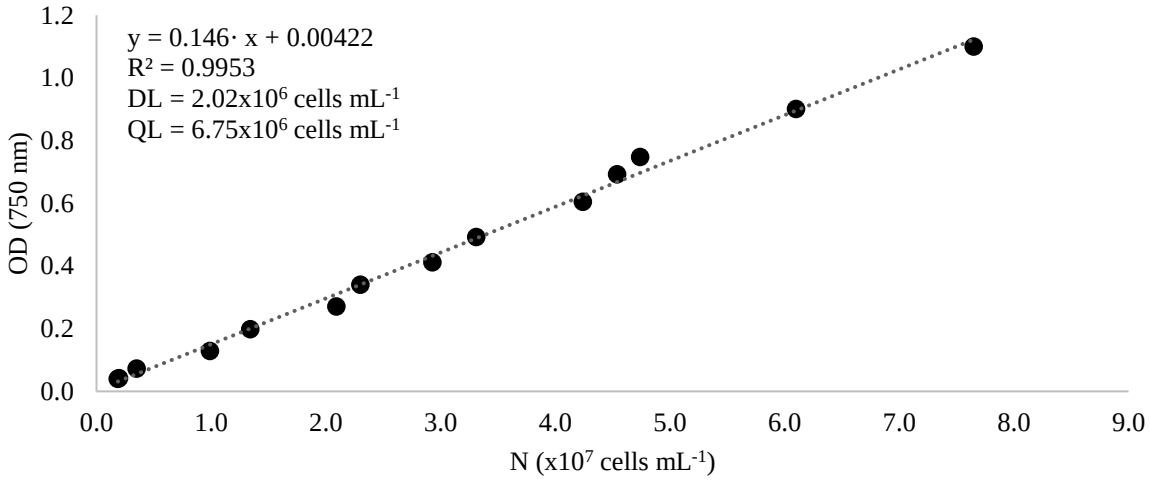

Figure I.2 - Linear regression between the OD measured at 750 nm and cell concentration (N, cells mL^{-1}).

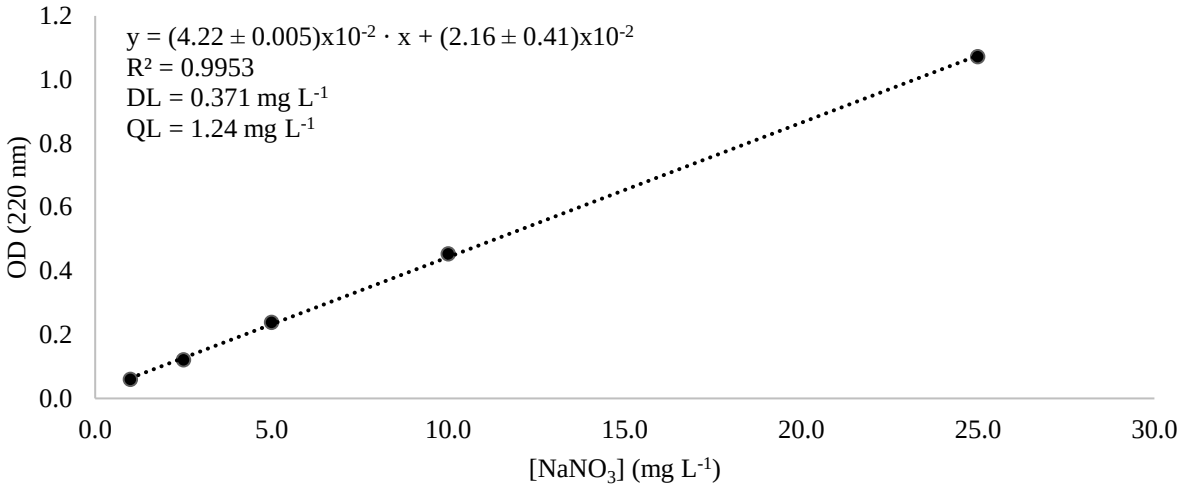

Figure I.3 - Linear regression between the OD measured at 220 nm and $NaNO_3$ concentration (in mg L^{-1}).

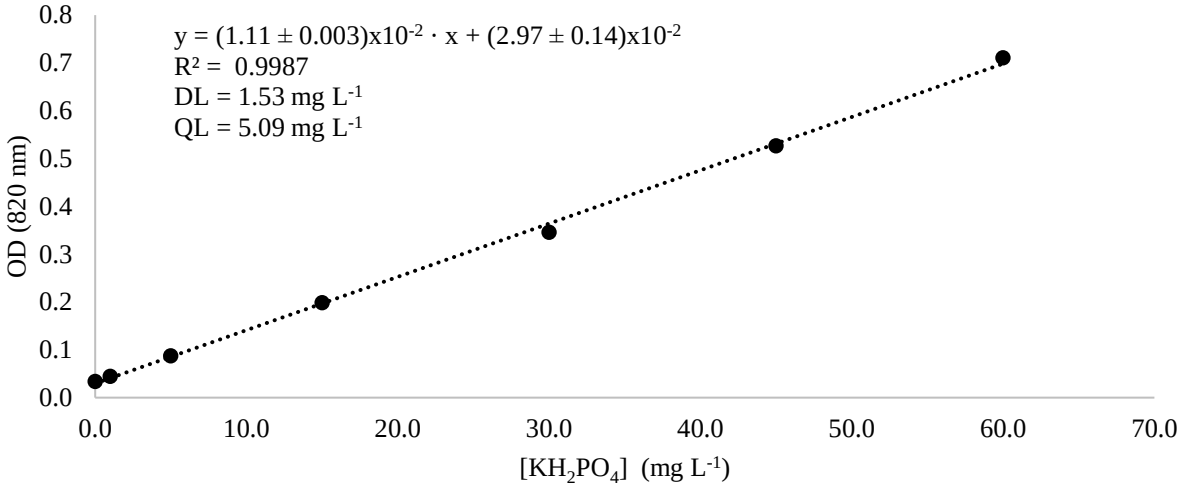

Figure I.4 - Linear regression between the OD measured at 820 nm and KH_2PO_4 concentration (in mg L^{-1}).

Annex II: Ciprofloxacin Quantification – Method Validation

Figure II.1 - Emission spectrum of a ciprofloxacin sample with a concentration of 1.00×10^3 μg L^{-1}.

Figure II.2 - Emission spectrum of a ciprofloxacin sample with a concentration of 10.00×10^3 μg L^{-1}.

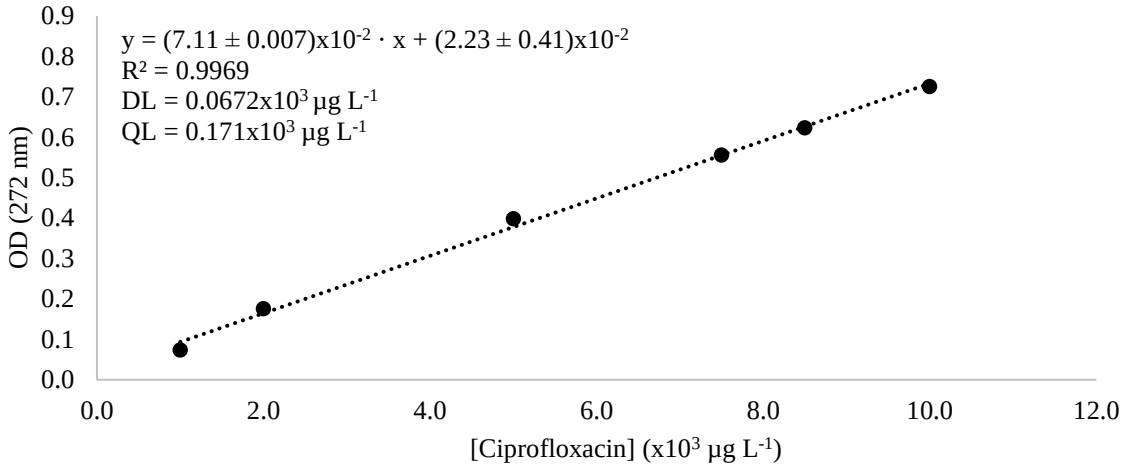

Figure II.3 - Linear regression between the OD measured at 272 nm and ciprofloxacin concentration (in µg L^{-1}).

Table II.1 - Coefficient of variation and repeatability of the method.

	OD (272 nm)		
[Ciprofloxacin] (x10³ µg L^{-1})	1.00	7.50	10.0
Coefficient of variation (%)	0.969	0.284	0.150
Repeatability	0.002	0.004	0.003

Table II.2 - Detection and quantification limits and robustness of the method.

Detection Limit (x10³ µg L^{-1})	0.0672
Quantification Limit (x10³ µg L^{-1})	0.171
Robustness (%)	99.8

Annex III: Growth curves

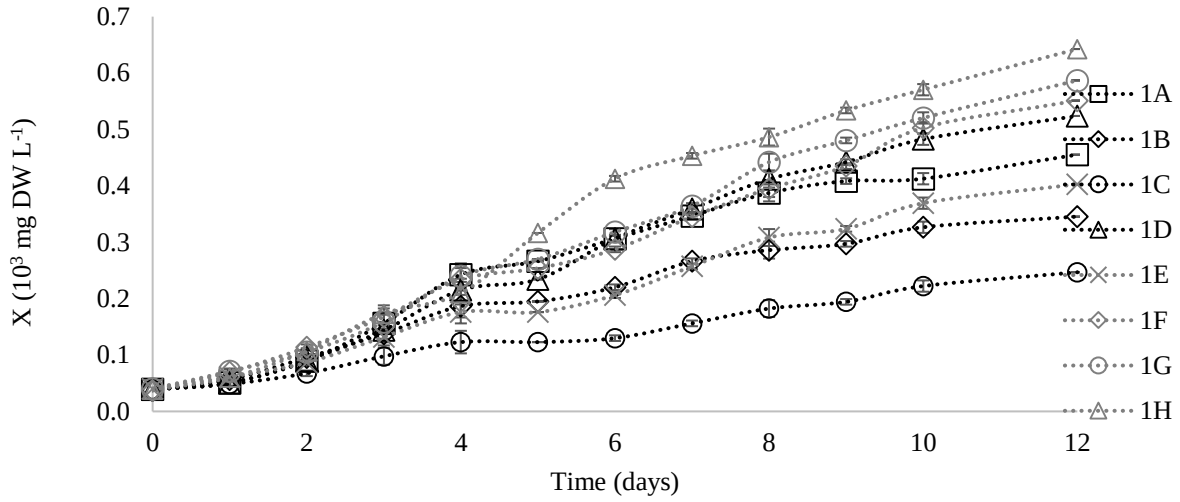

Figure III.1 - Growth curves obtained from each culture of Trial 1. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

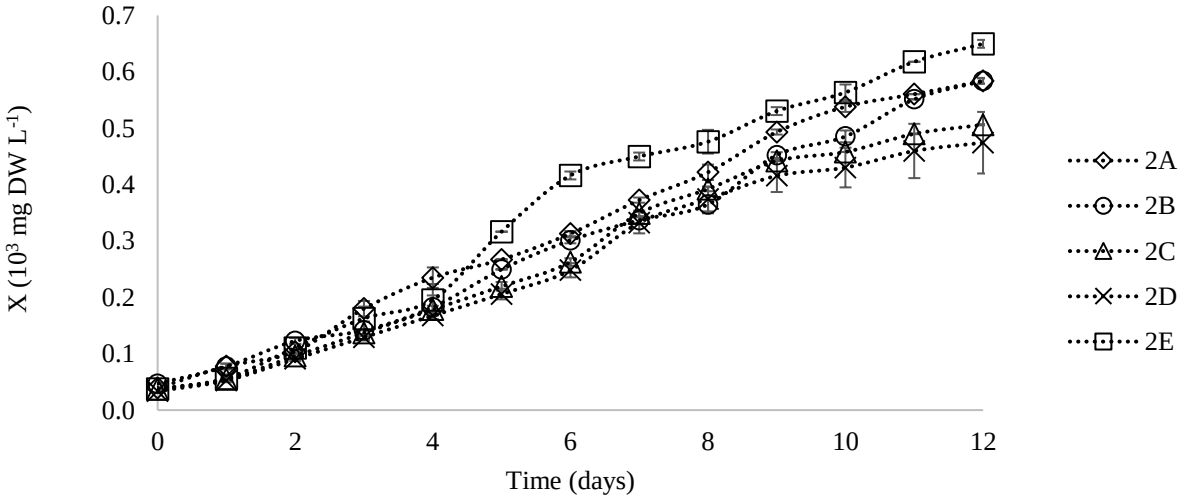

Figure III.2 - Growth curves obtained from each culture of Trial 2. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

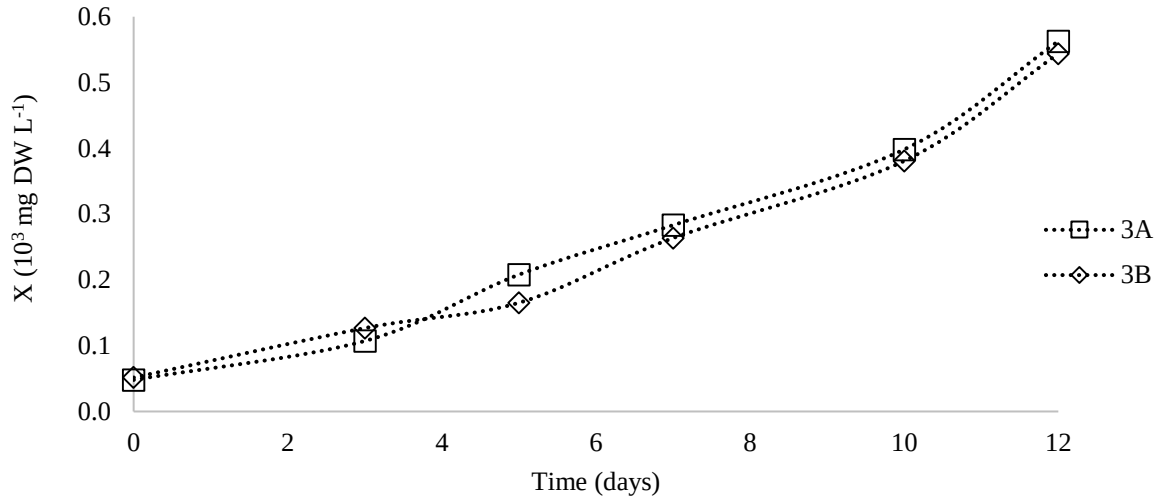

Figure III.3 - Growth curves obtained from each suspended culture of Trial 3. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

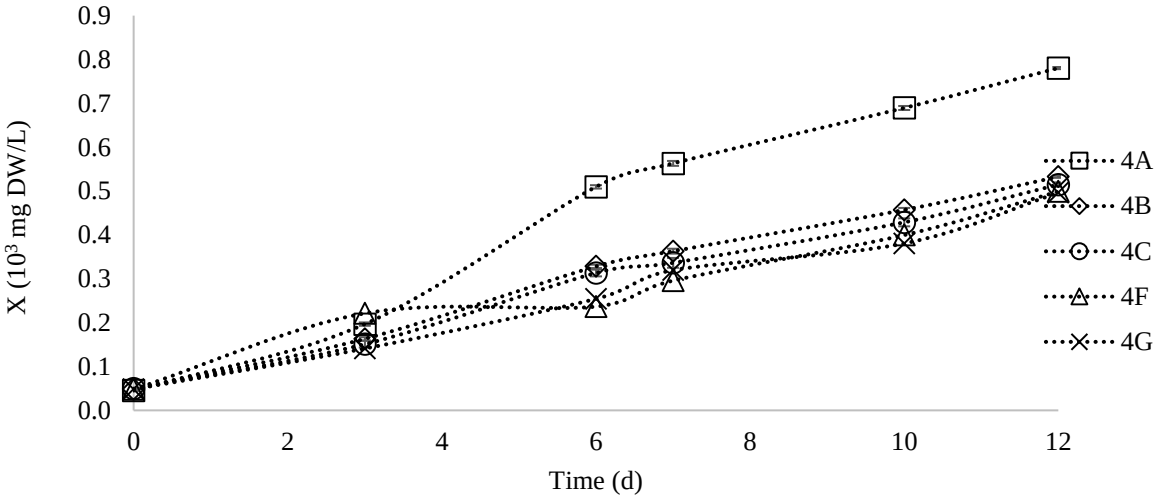

Figure III.4 - Growth curves obtained from each suspended culture of Trial 4. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

Annex IV: Nitrogen evolution

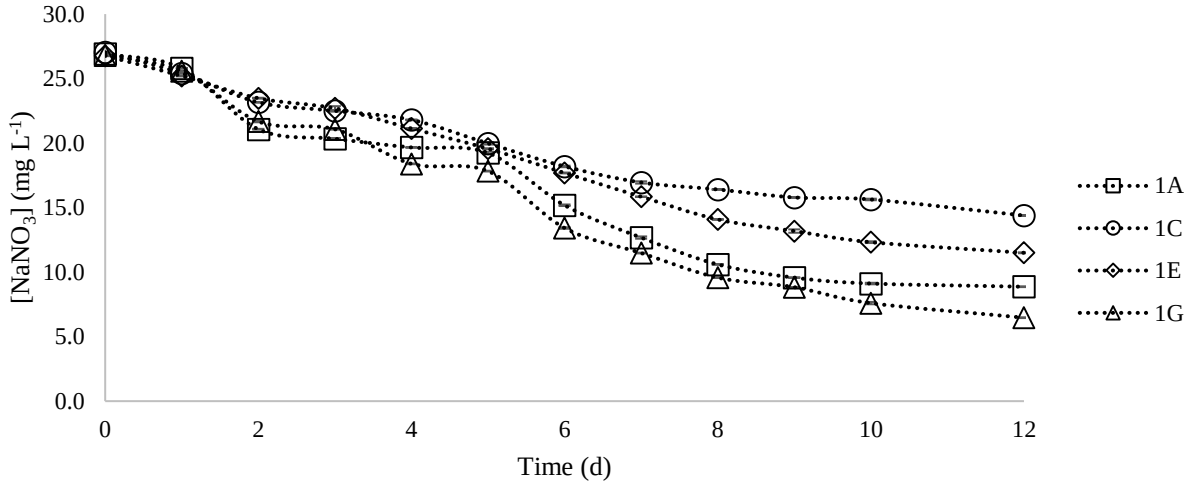

Figure IV.1 - Time-course evolution of $NaNO_3$ concentration (in mg L^{-1}) from each culture of Trial 1. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

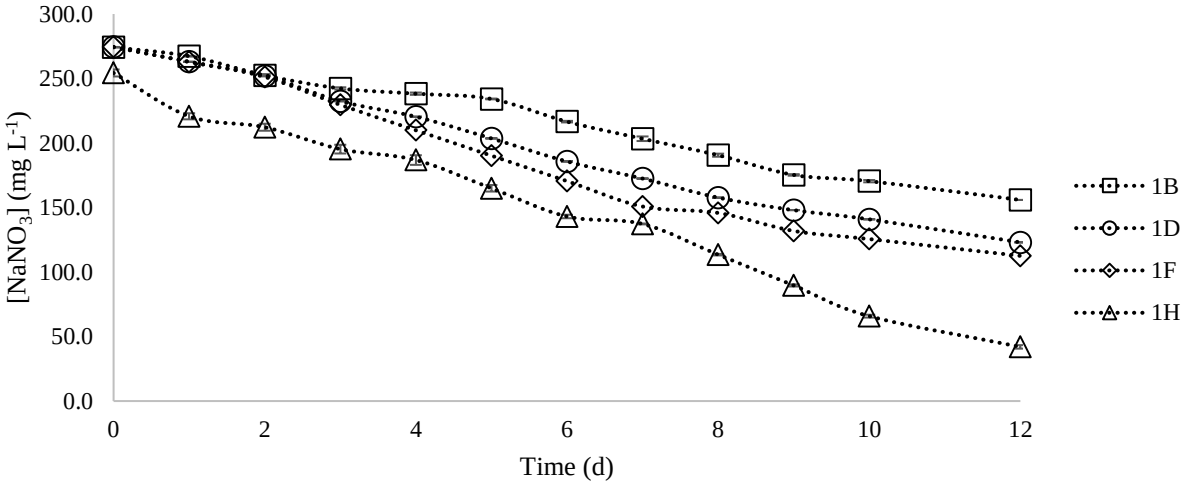

Figure IV.2 - Time-course evolution of $NaNO_3$ concentration (in mg L^{-1}) from each culture of Trial 1. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

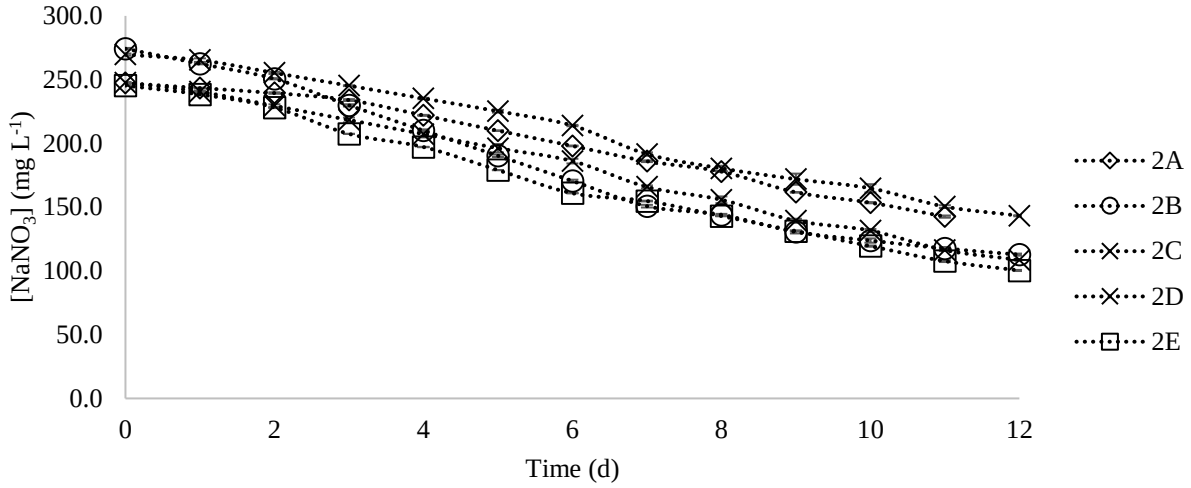

Figure IV.3 - Time-course evolution of $NaNO_3$ concentration (in mg L^{-1}) from each culture of Trial 2. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

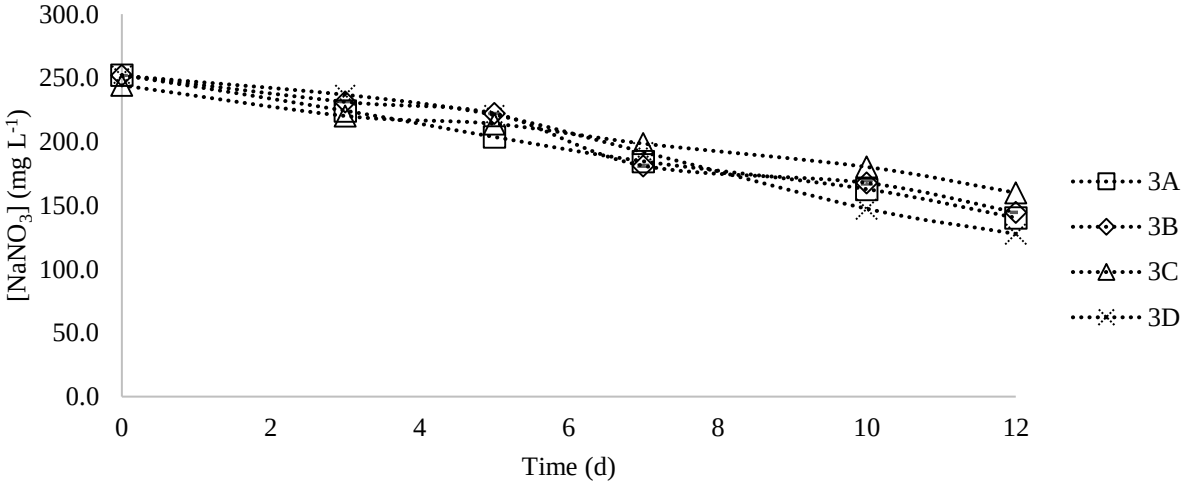

Figure IV.4 - Time-course evolution of $NaNO_3$ concentration (in mg L^{-1}) from each culture of Trial 3. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

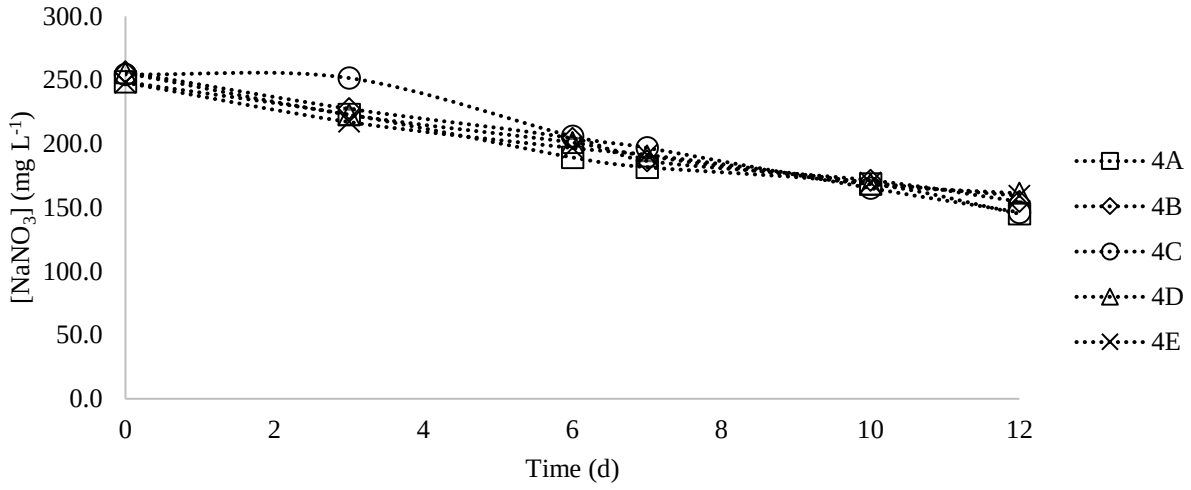

Figure IV.5 - Time-course evolution of $NaNO_3$ concentration (in mg L^{-1}) from each culture of Trial 4. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

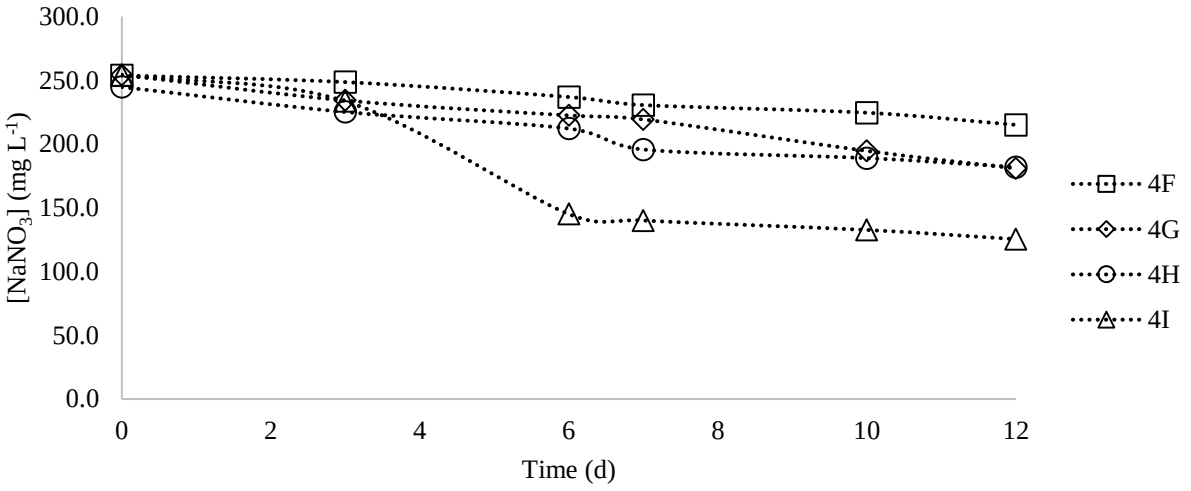

Figure IV.6 - Time-course evolution of $NaNO_3$ concentration (in mg L^{-1}) from each culture of Trial 4. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

Annex V: Phosphorous evolution

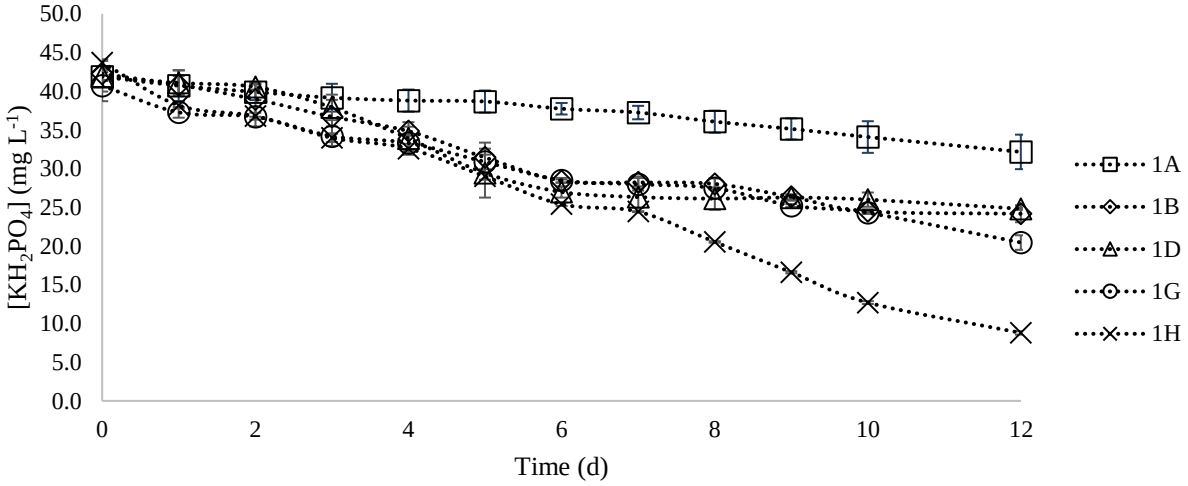

Figure V.1 - Time-course evolution of KH_2PO_4 concentration (in mg L^{-1}) from each culture of Trial 1. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

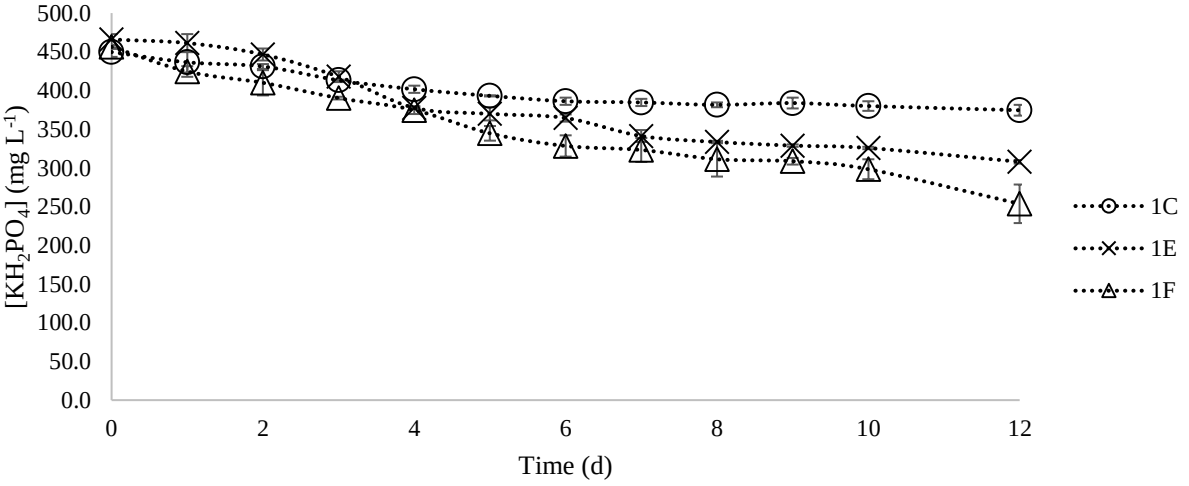

Figure V.2 - Time-course evolution of KH_2PO_4 concentration (in mg L^{-1}) from each culture of Trial 1. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

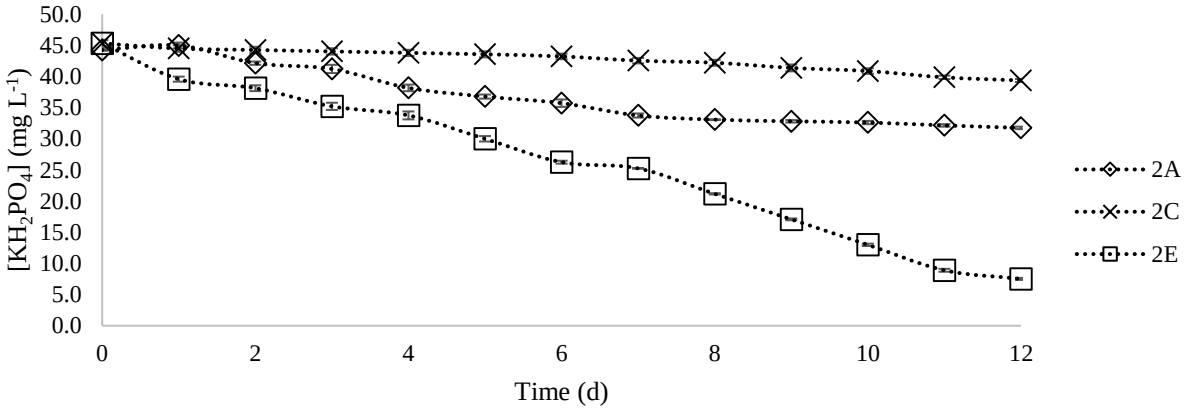

Figure V.3 - Time-course evolution of KH_2PO_4 concentration (in mg L^{-1}) from each culture of Trial 2. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

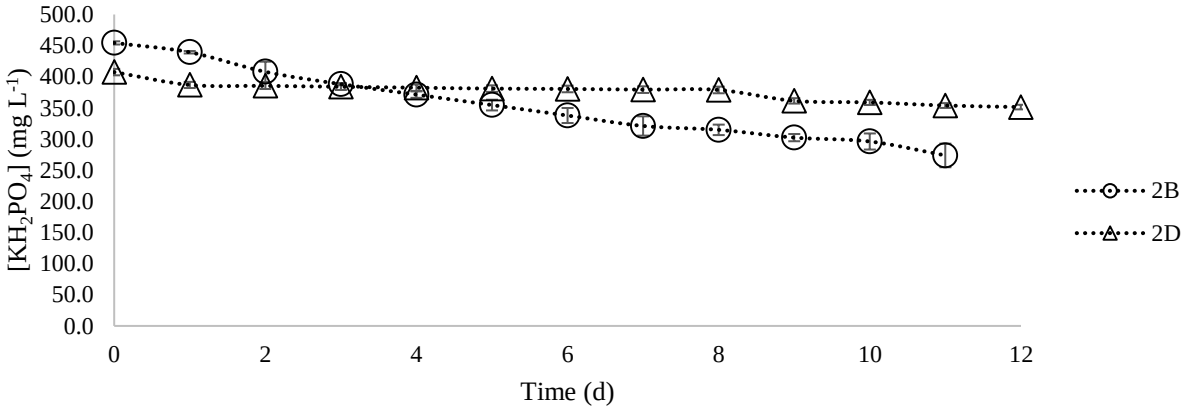

Figure V.4 - Time-course evolution of KH_2PO_4 concentration (in mg L^{-1}) from each culture of Trial 2. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

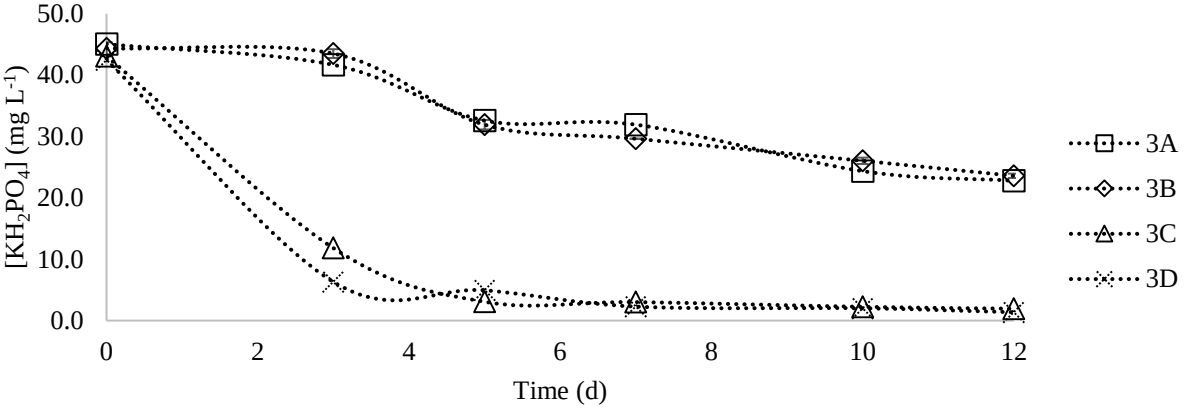

Figure V.5 - Time-course evolution of KH_2PO_4 concentration (in mg L^{-1}) from each culture of Trial 3. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

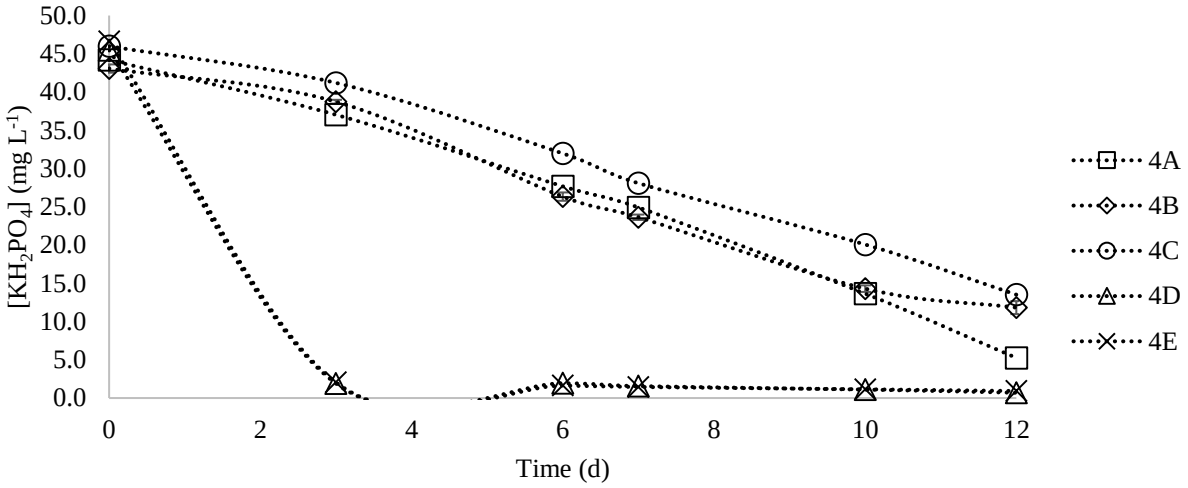

Figure V.6 - Time-course evolution of KH_2PO_4 concentration (in mg L^{-1}) from each culture of Trial 4. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

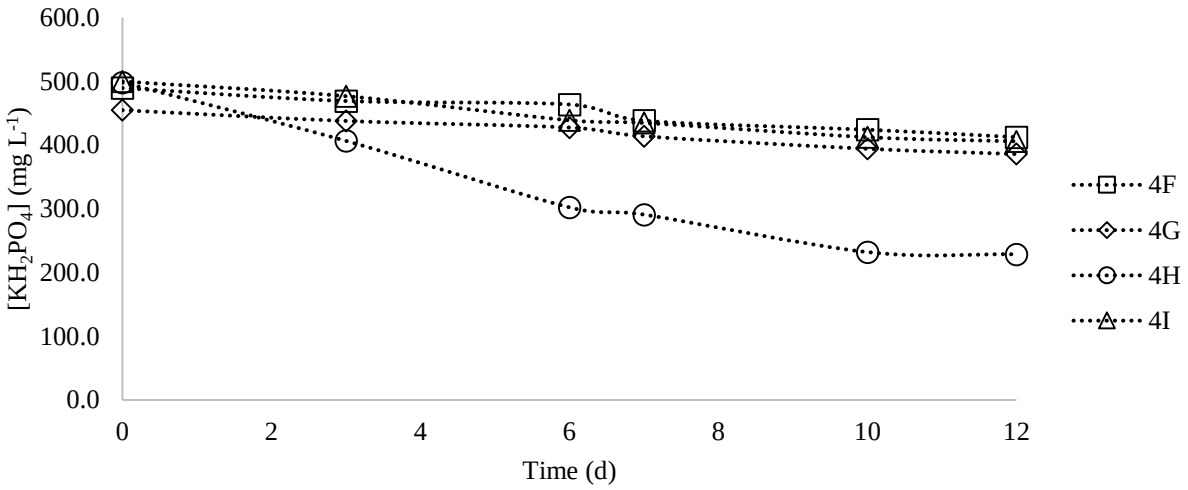

Figure V.7 - Time-course evolution of KH_2PO_4 concentration (in mg L^{-1}) from each culture of Trial 4. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

Annex VI: Ciprofloxacin evolution

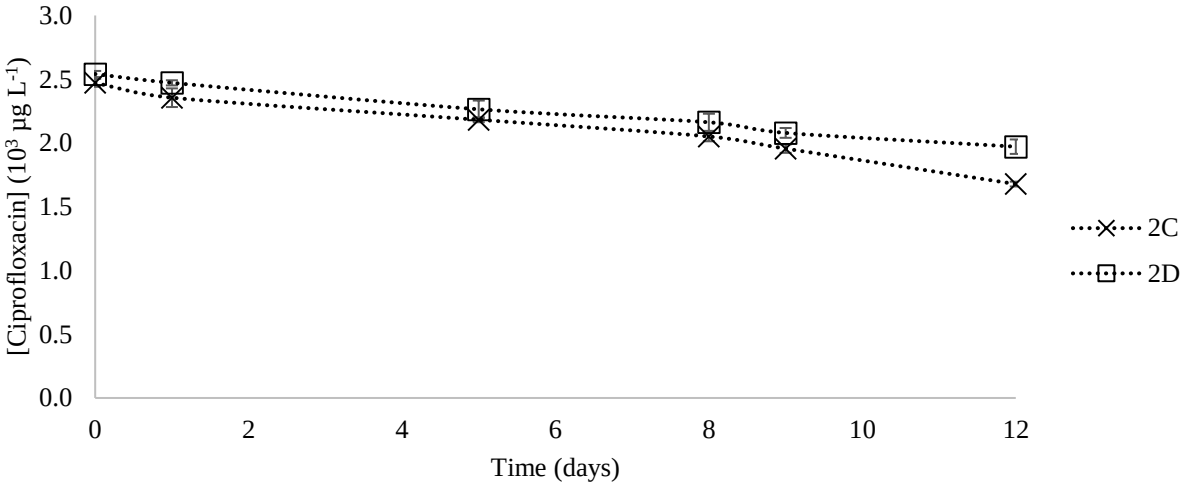

Figure VI.1 - Time-course evolution of ciprofloxacin concentration (in µg L^{-1}) from each culture of Trial 2. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

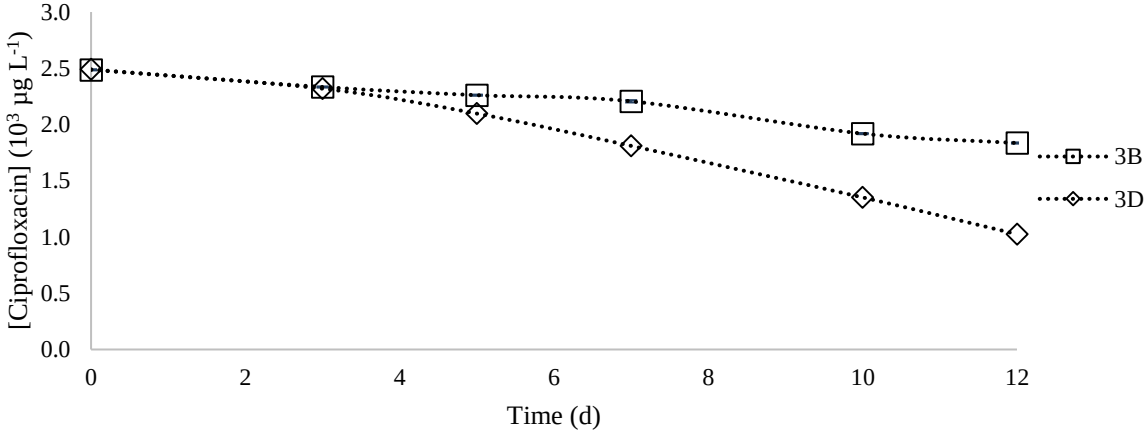

Figure VI.2 - Time-course evolution of ciprofloxacin concentration (in µg L^{-1}) from each culture of Trial 3. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

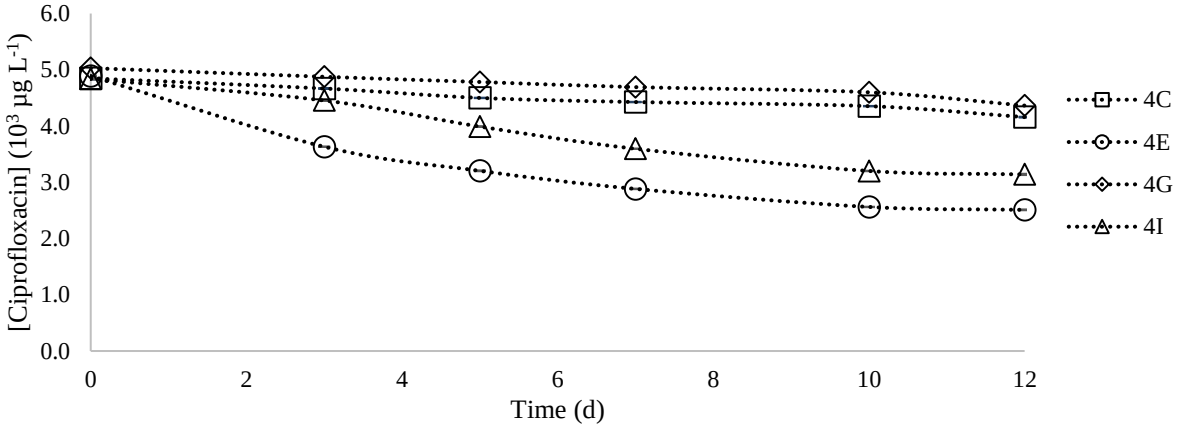

Figure VI.3 - Time-course evolution of ciprofloxacin concentration (in µg L^{-1}) from each culture of Trial 4. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

Annex VII: Kinetic growth parameters

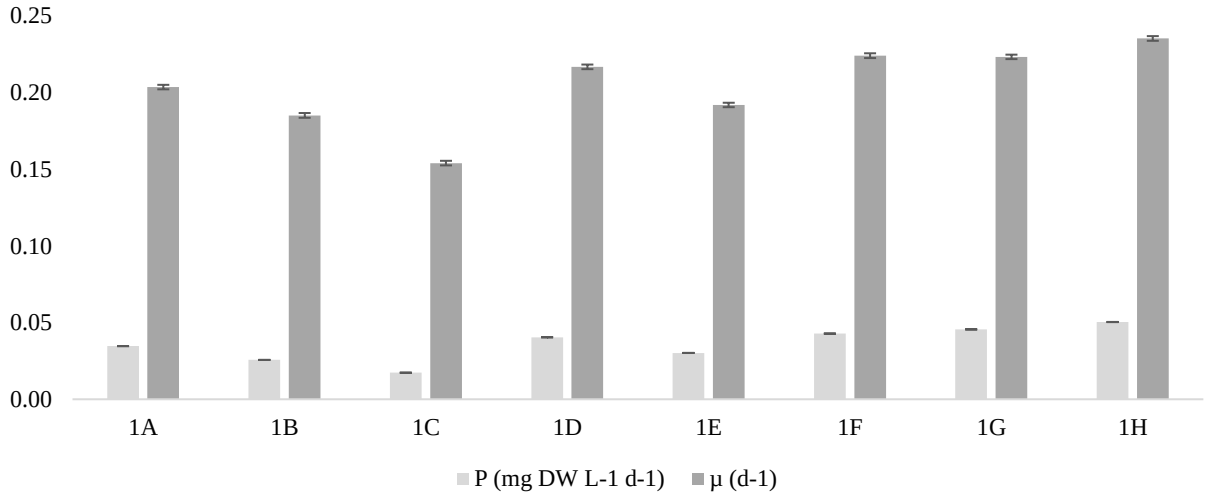

Figure VII.1 - Average biomass productivities (in mg DW L^{-1} d^{-1}) and specific growth rate (in d^{-1}) from each culture of Trial 1. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

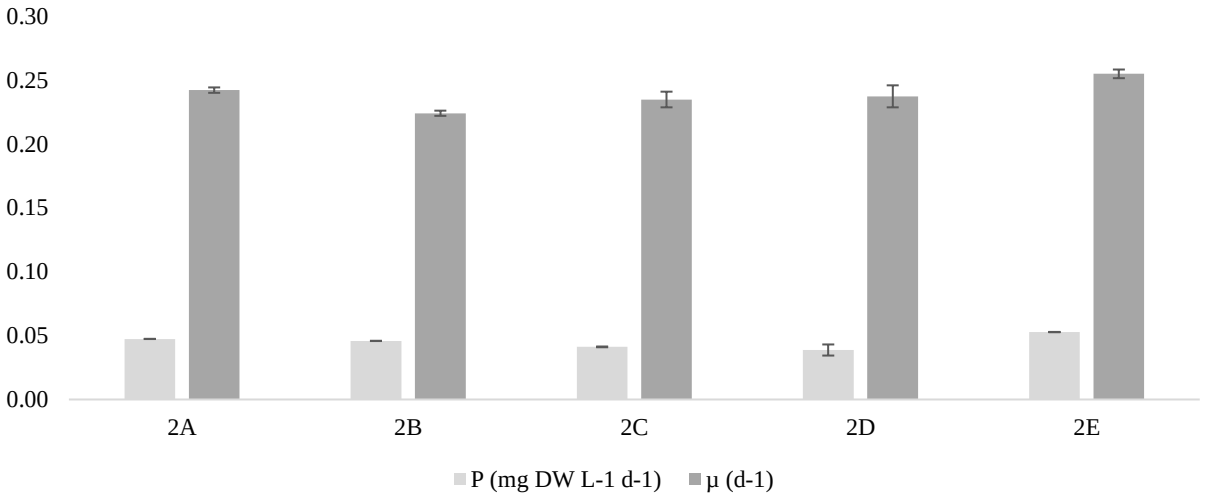

Figure VII.2 - Average biomass productivities (in mg DW L^{-1} d^{-1}) and specific growth rate (in d^{-1}) from each culture of Trial 2. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

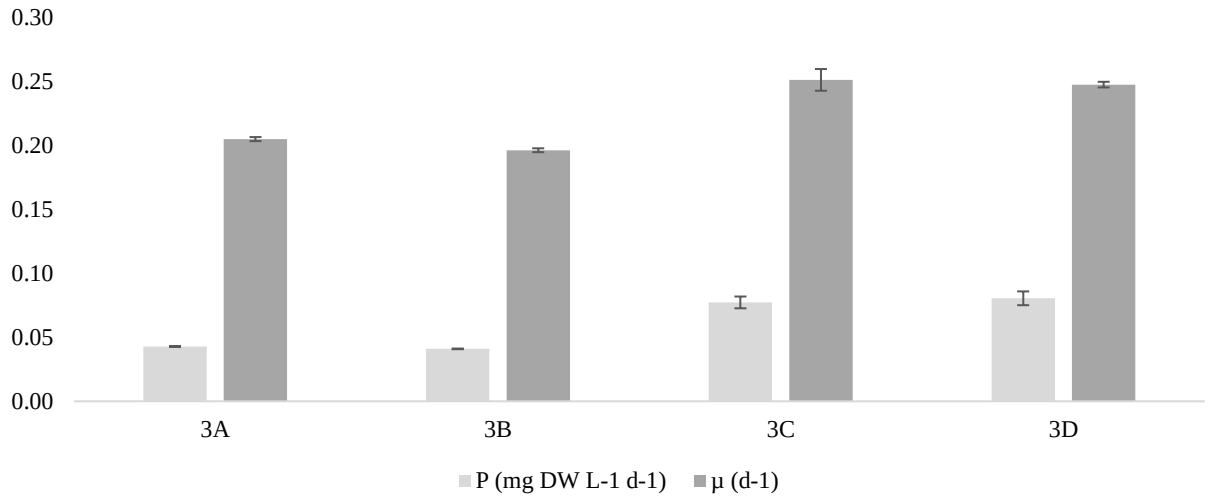

Figure VII.3 Average biomass productivities (in mg DW L^{-1} d^{-1}) and specific growth rate (in d^{-1}) from each culture of Trial 3. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

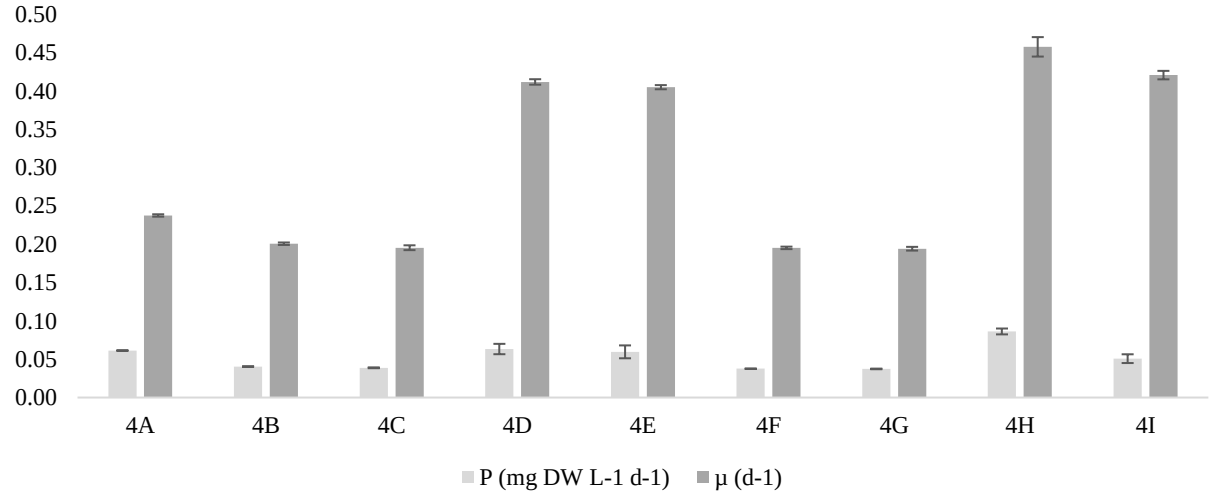

Figure VII.4 - Average biomass productivities (in mg DW L^{-1} d^{-1}) and specific growth rate (in d^{-1}) from each culture of Trial 4. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

Annex VIII: Nitrogen kinetic parameters

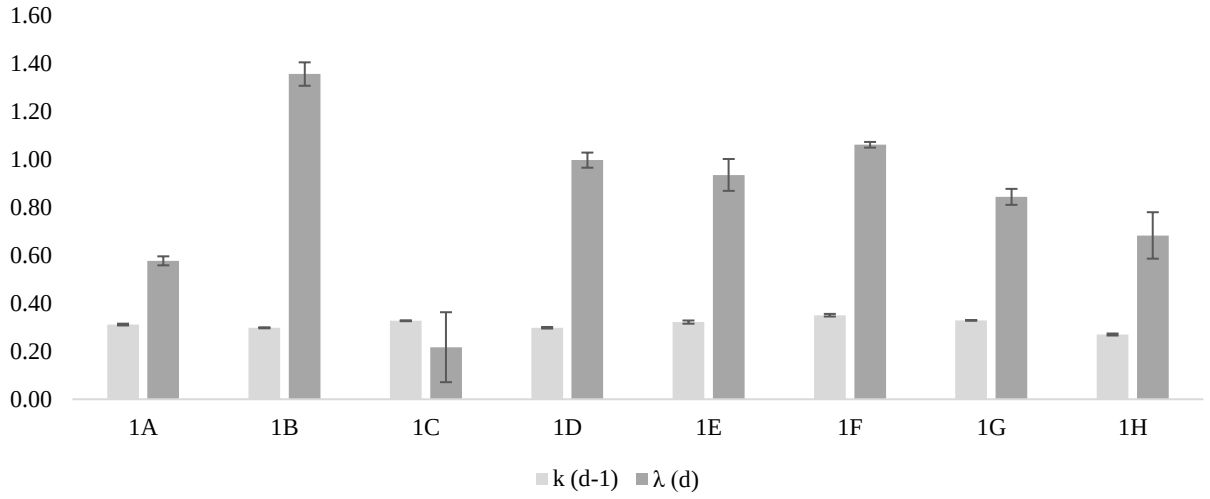

Figure VIII.1 - Uptake rates (k, in d^{-1}) and lag time (λ, in d) for nitrogen from each culture of Trial 1. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

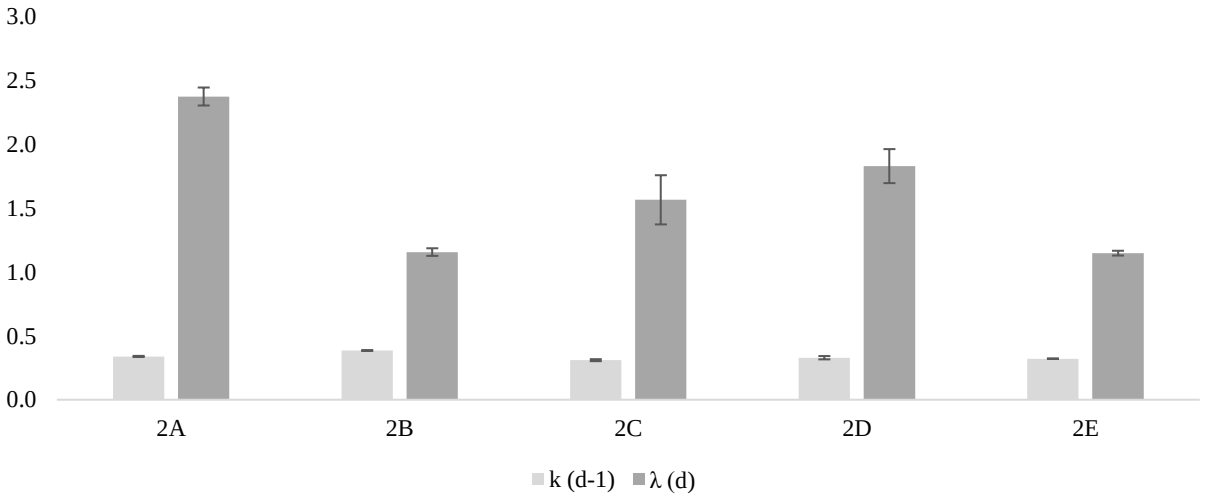

Figure VIII.2 - Uptake rates (k, in d^{-1}) and lag time (λ, in d) for nitrogen from each culture of Trial 2. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

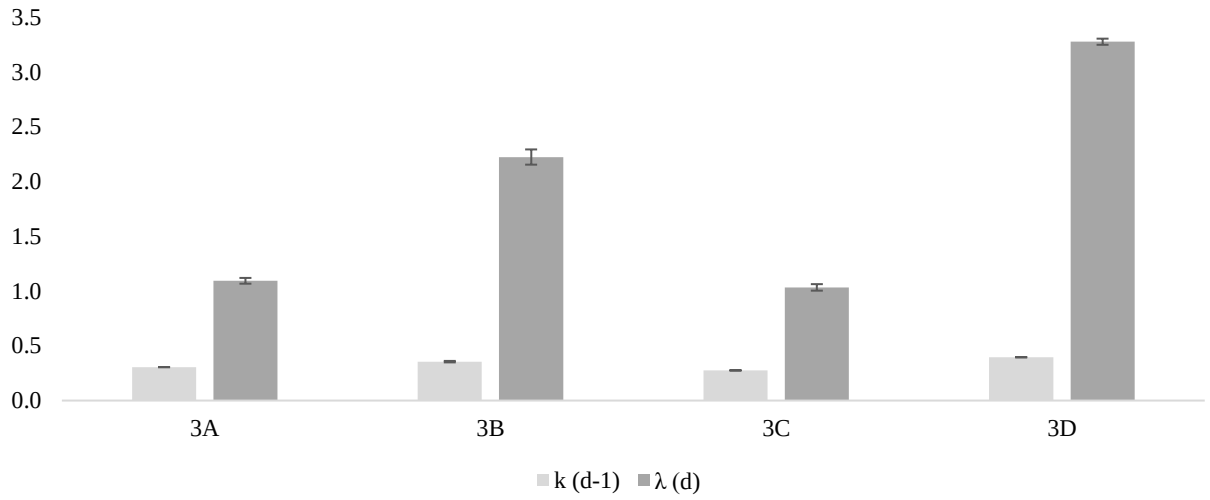

Figure VIII.3 - Uptake rates (k, in d^{-1}) and lag time (λ, in d) for nitrogen from each culture of Trial 3. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

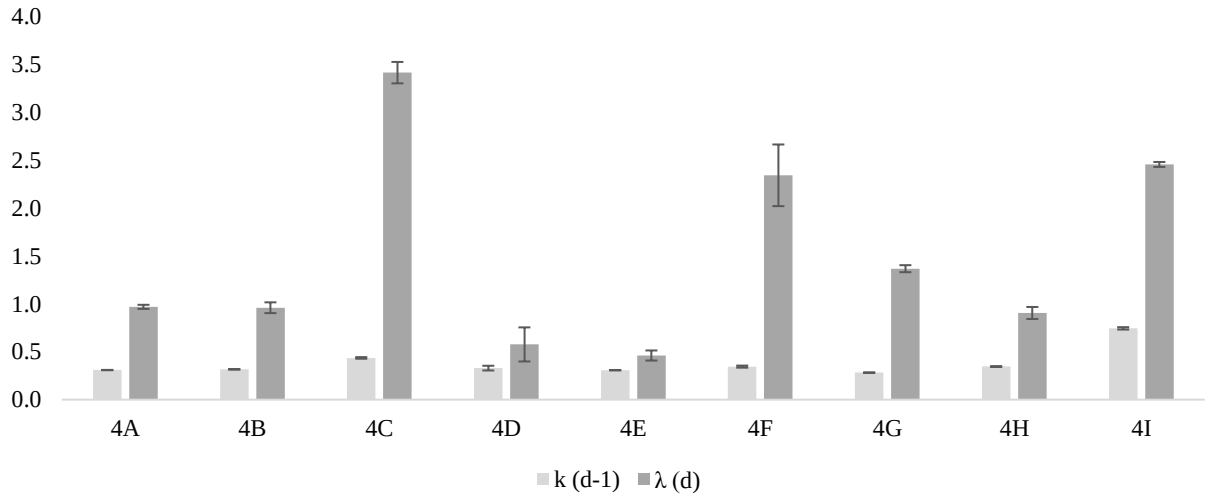

Figure VIII.4 - Uptake rates (k, in d^{-1}) and lag time (λ, in d) for nitrogen from each culture of Trial 4. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

Annex IX: Phosphorous kinetic parameters

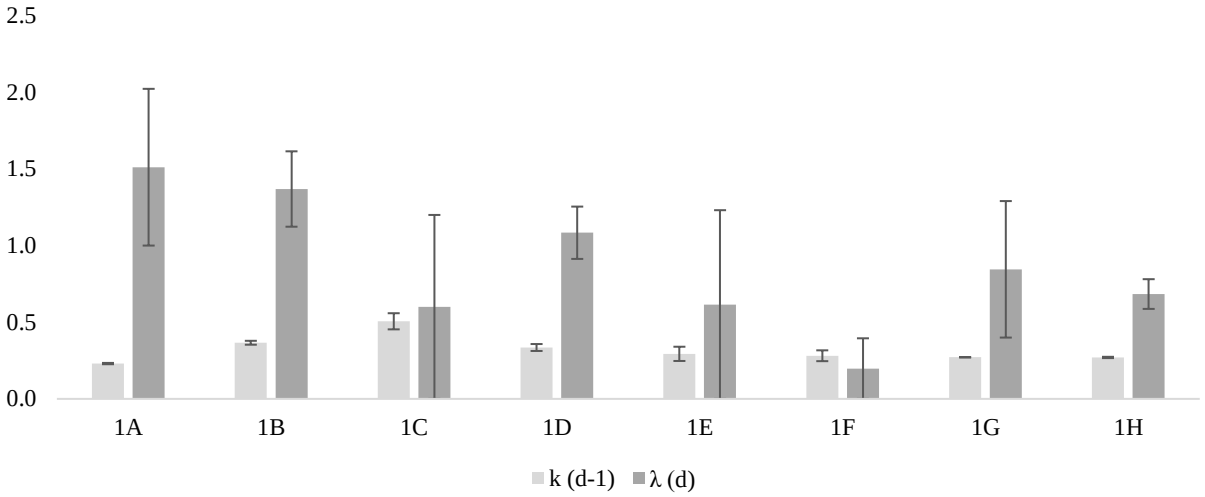

Figure IX.1 - Uptake rates (k, in d^{-1}) and lag time (λ, in d) for phosphorous from each culture of Trial 1. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

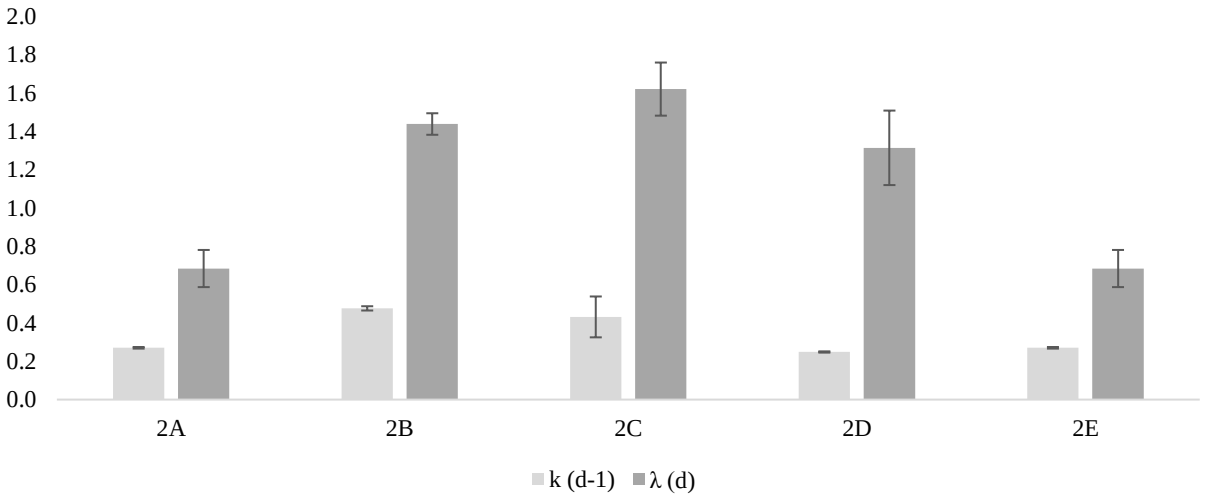

Figure IX.2 - Uptake rates (k, in d^{-1}) and lag time (λ, in d) for phosphorous from each culture of Trial 2. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

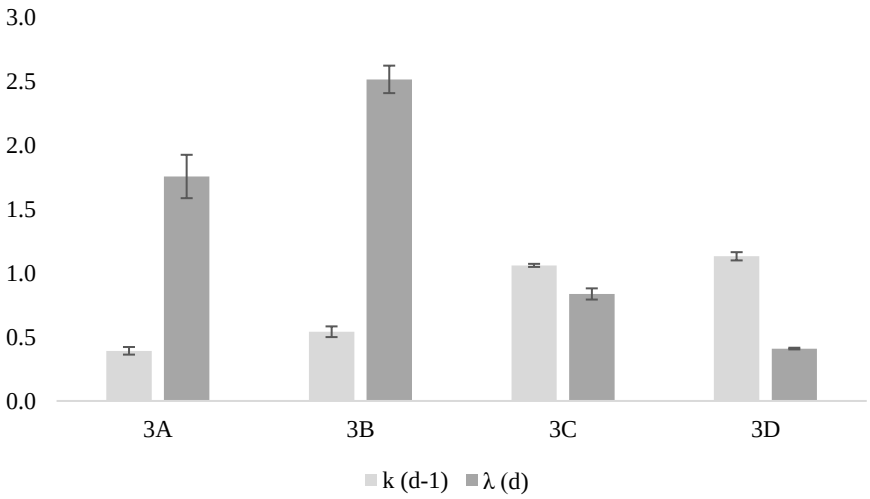

Figure IX.3 - Uptake rates (k, in d^{-1}) and lag time (λ, in d) for phosphorous from each culture of Trial 3. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

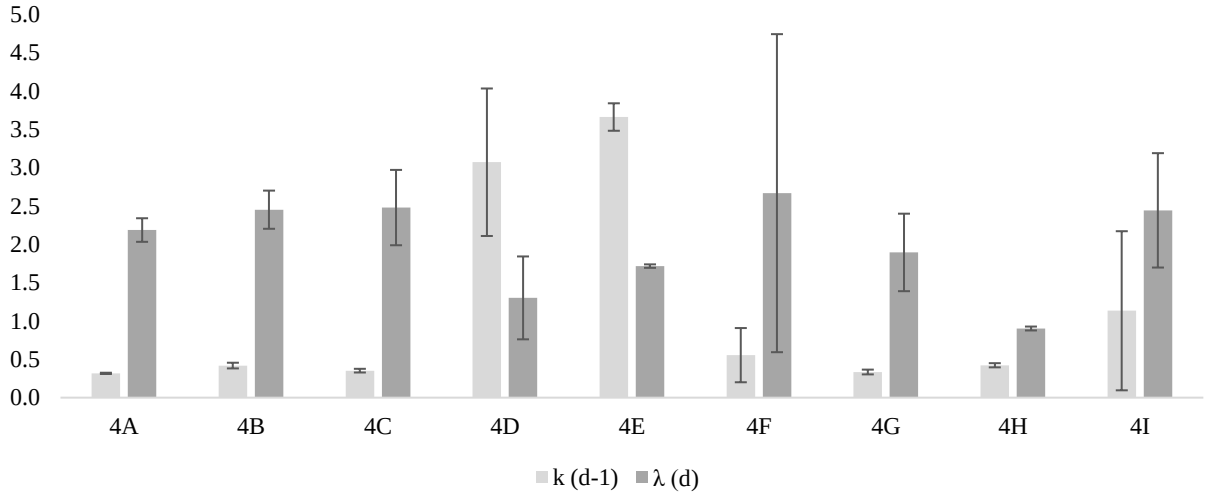

Figure IX.4 - Uptake rates (k, in d^{-1}) and lag time (λ, in d) for phosphorous from each culture of Trial 4. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

Annex X: Ciprofloxacin kinetic parameters

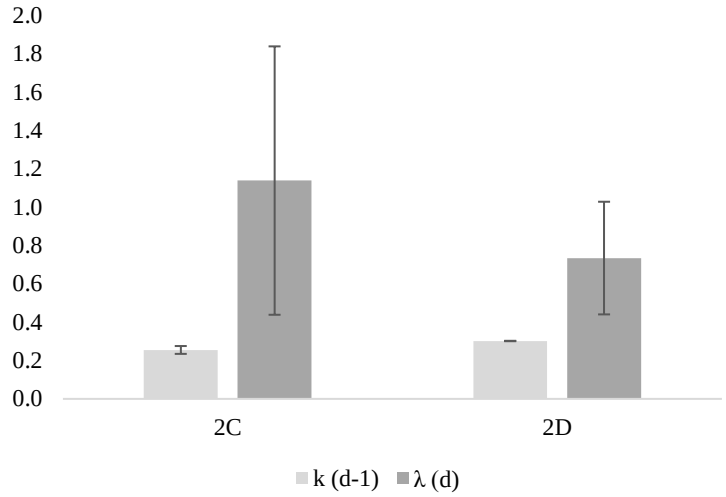

Figure X.1 - Uptake rates (k, in d^{-1}) and lag time (λ, in d) for ciprofloxacin from each culture of Trial 2. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

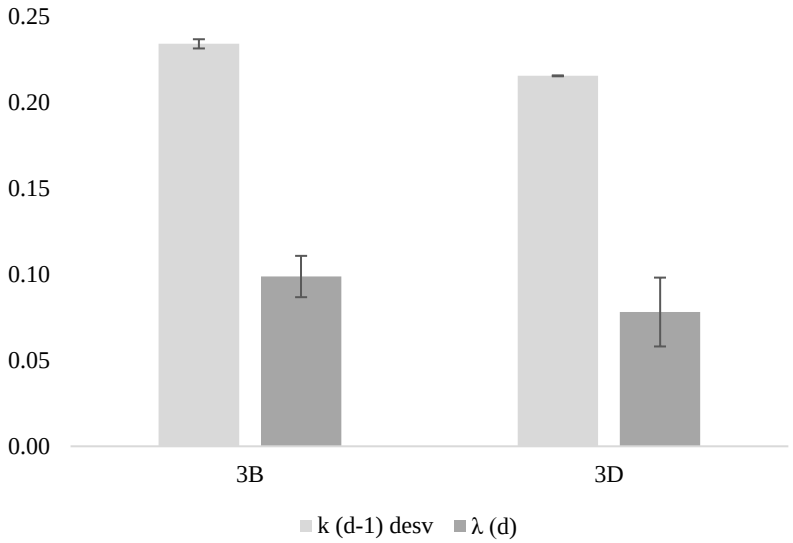

Figure X.2 - Uptake rates (k, in d^{-1}) and lag time (λ, in d) for ciprofloxacin from each culture of Trial 3. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

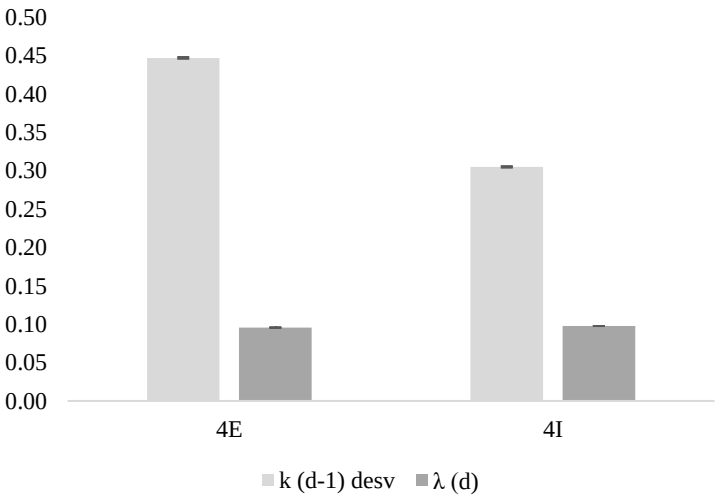

Figure X.3 - Uptake rates (k, in d^{-1}) and lag time (λ, in d) for ciprofloxacin from each culture of Trial 4. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

Annex XI: Removal efficiencies

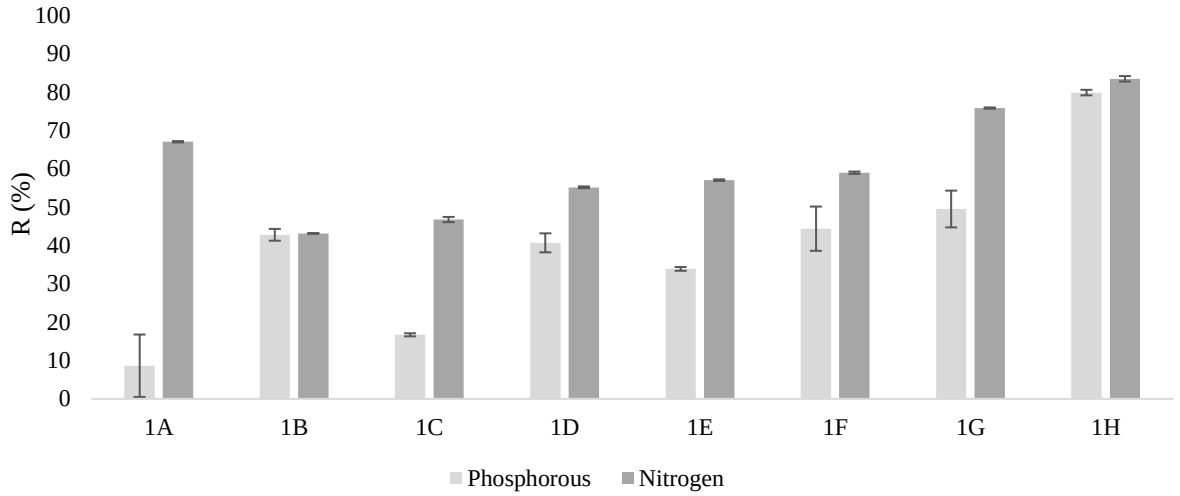

Figure XI.1 – Removal efficiency (R, in %) for phosphorous and nitrogen from each culture of Trial 1. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

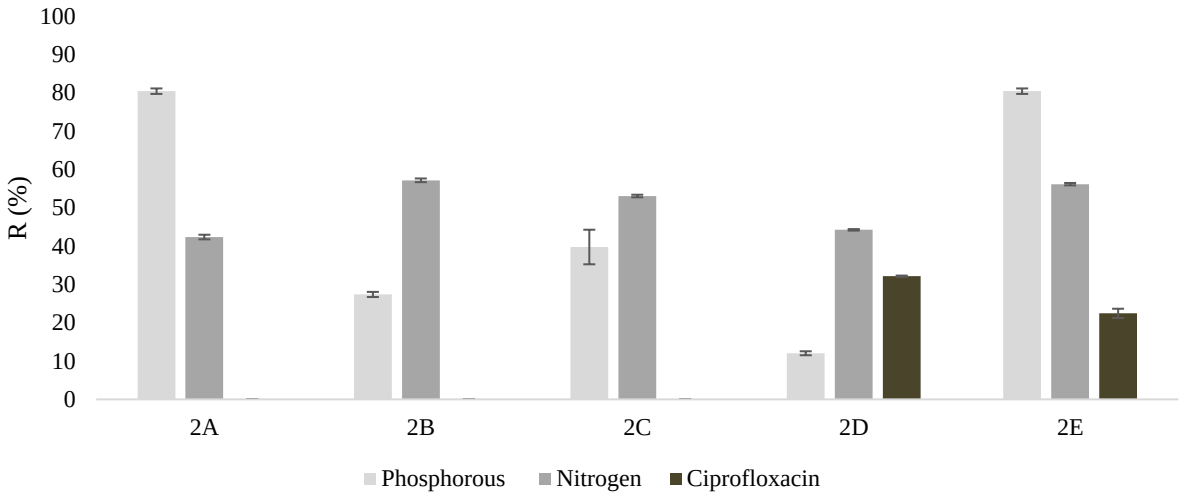

Figure XI.2 – Removal efficiency (R, in %) for phosphorous, nitrogen and ciprofloxacin from each culture of Trial 2. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

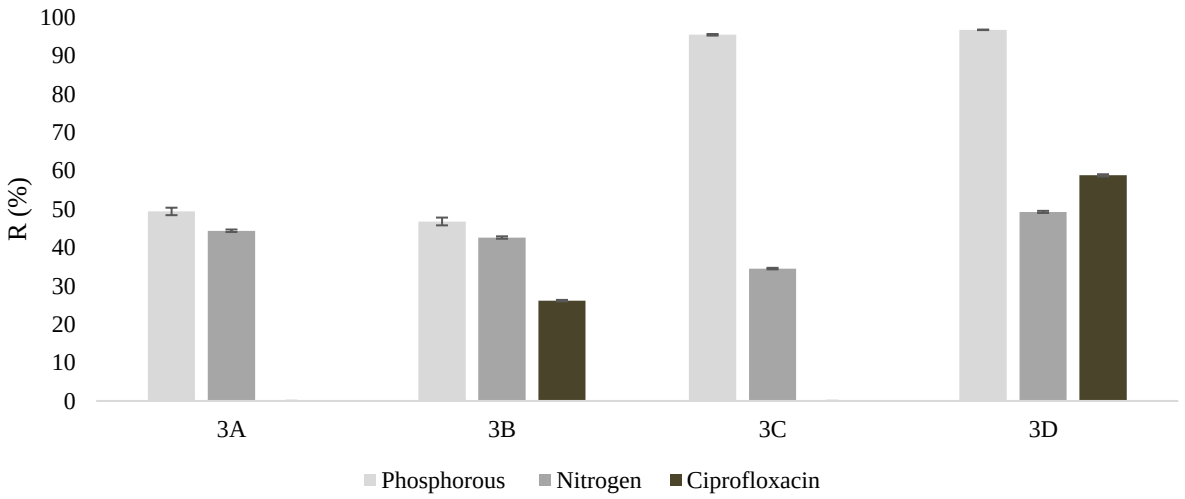

Figure XI.3 – Removal efficiency (R, in %) for phosphorous, nitrogen and ciprofloxacin from each culture of Trial 3. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

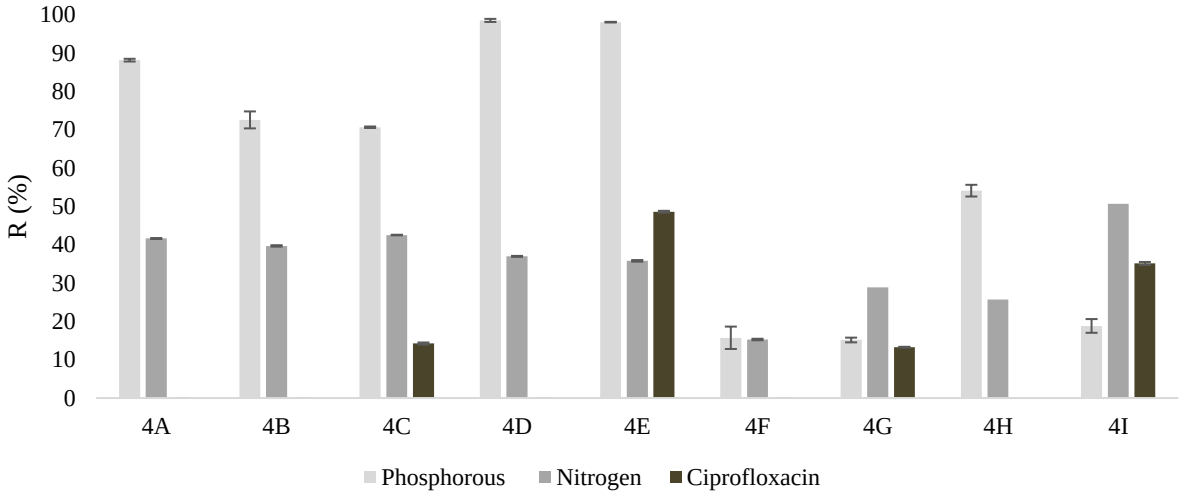

Figure XI.4 – Removal efficiency (R, in %) for phosphorous, nitrogen and ciprofloxacin from each culture of Trial 4. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

Annex XII: Average removal rates

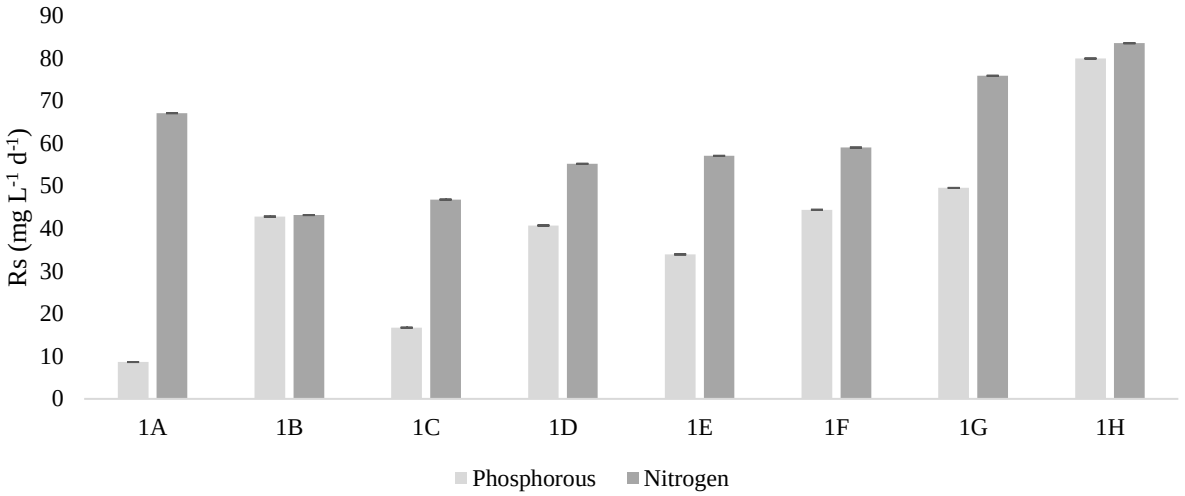

Figure XII.1 – Average removal rate (Rs, in mg L^{-1} d^{-1}) for phosphorous and nitrogen from each culture of Trial 1. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

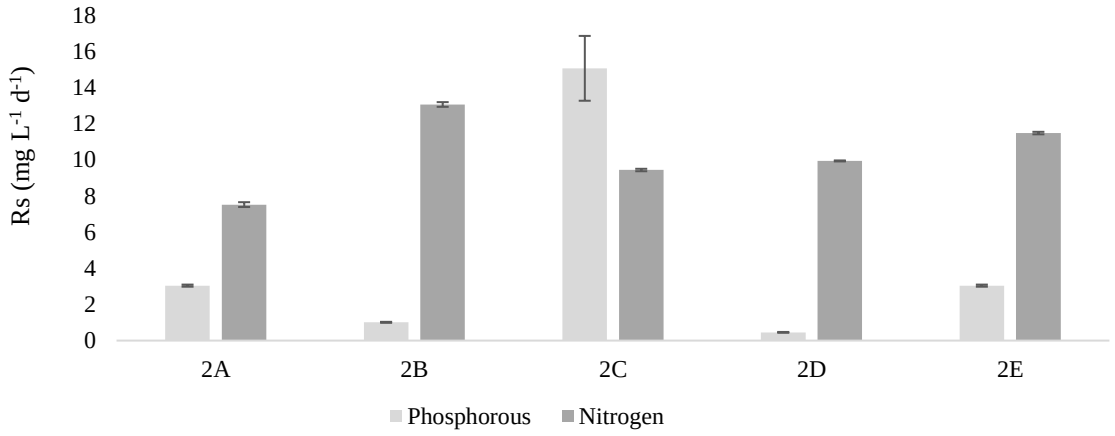

Figure XII.2 – Average removal rate (Rs, in mg L^{-1} d^{-1}) for phosphorous and nitrogen from each culture of Trial 2. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

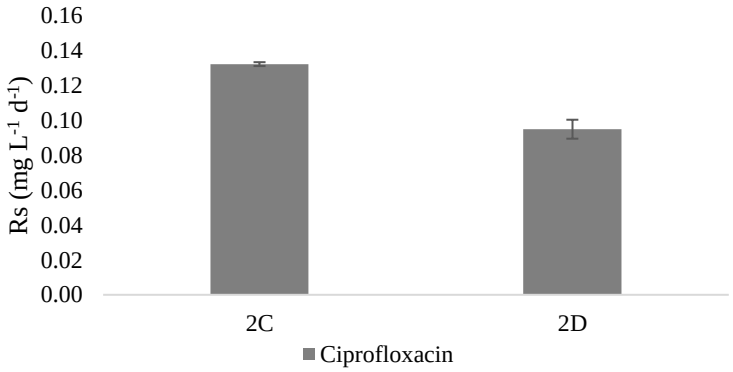

Figure XII.3 – Average removal rate (Rs, in mg L^{-1} d^{-1}) for ciprofloxacin from each culture of Trial 2. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

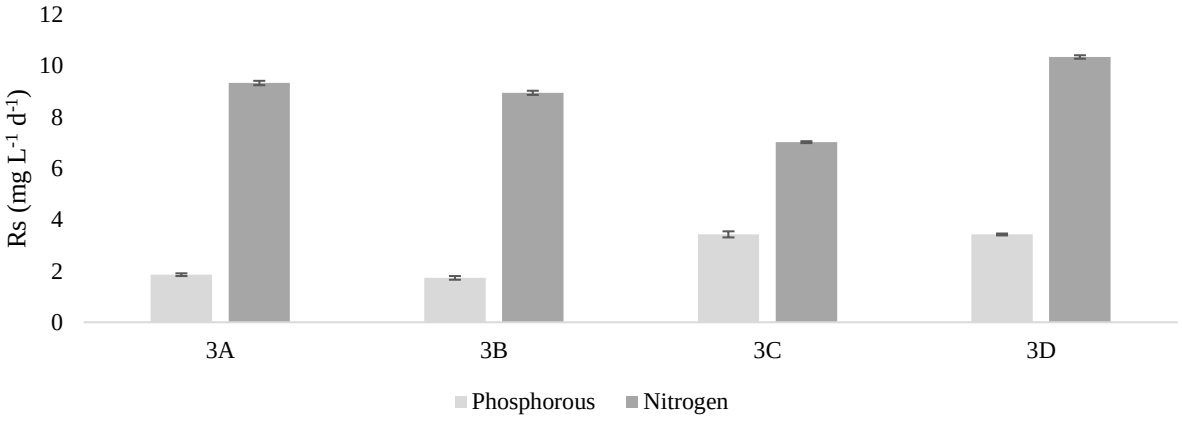

Figure XII.4 – Average removal rate (Rs, in mg L^{-1} d^{-1}) for phosphorous and nitrogen from each culture of Trial 3. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

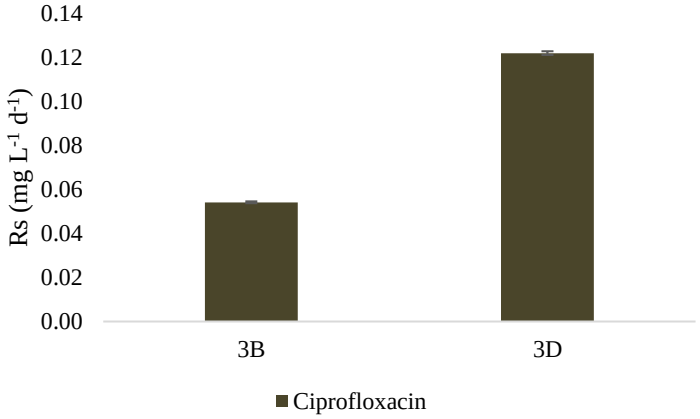

Figure XII.5 – Average removal rate (Rs, in mg L^{-1} d^{-1}) for ciprofloxacin from each culture of Trial 3. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

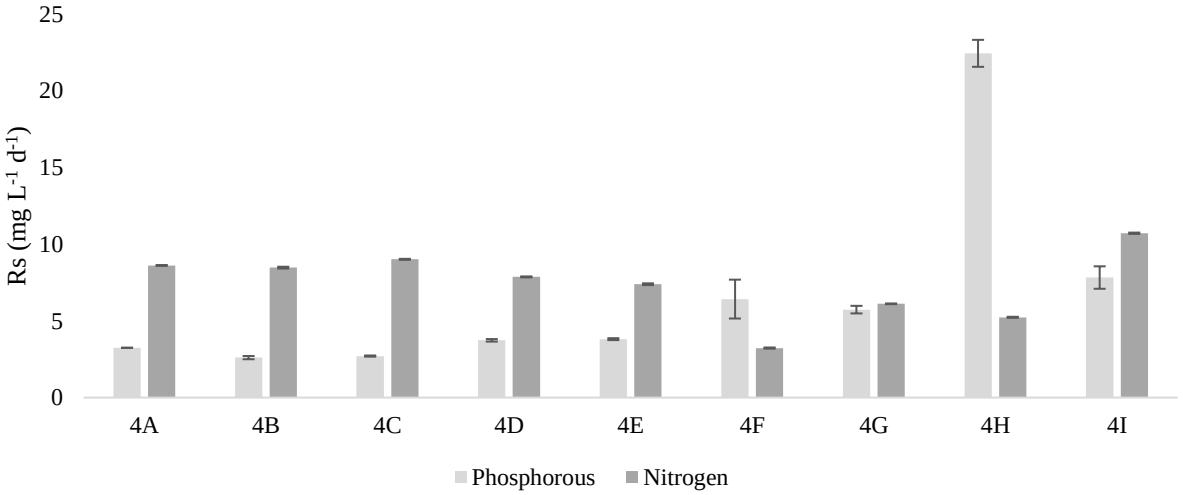

Figure XII.6 – Average removal rate (Rs, in mg L^{-1} d^{-1}) for phosphorous and nitrogen from each culture of Trial 4. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

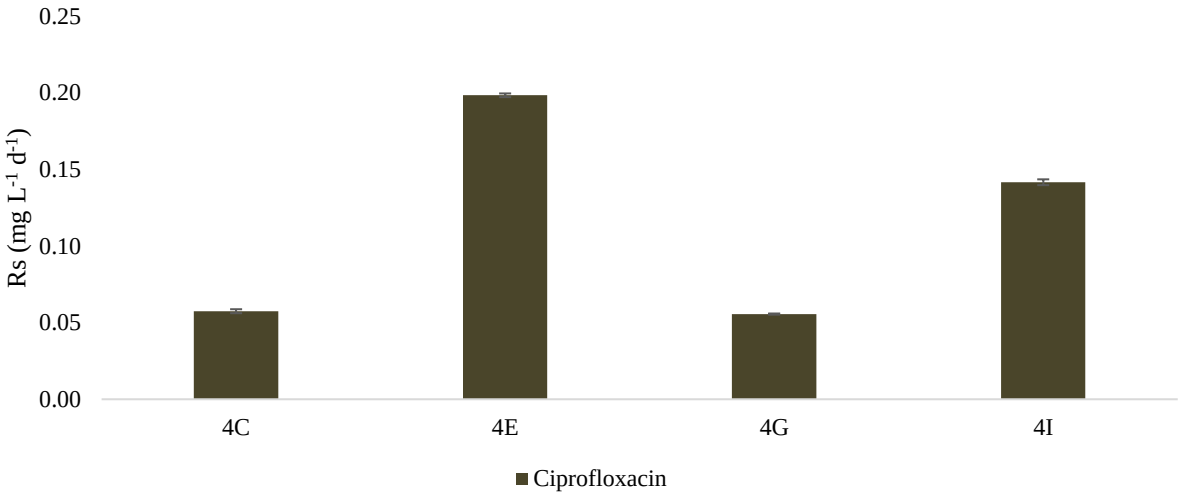

Figure XII.7 – Average removal rate (Rs, in mg L^{-1} d^{-1}) for ciprofloxacin from each culture of Trial 4. Error bars correspond to the standard deviation of the mean determined for two independent experiments.

Annex XIII: Growth inhibition

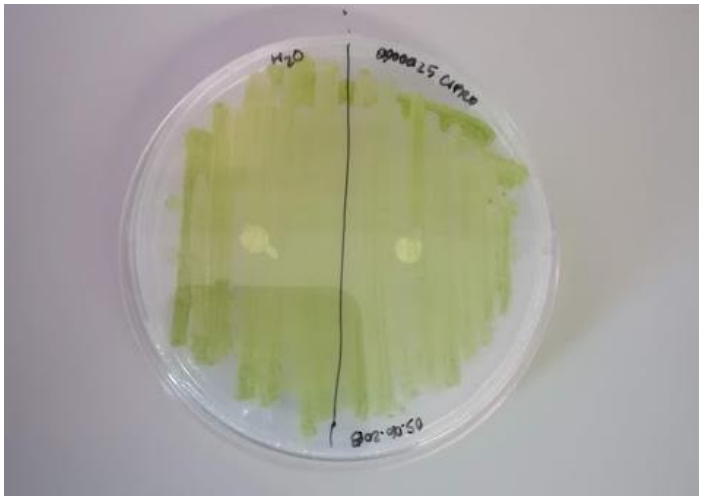

Figure XIII.1 – Paper-disc agar method with a ciprofloxacin concentration of 2.50 x 10^3 µg L^{-1} and water as blank.

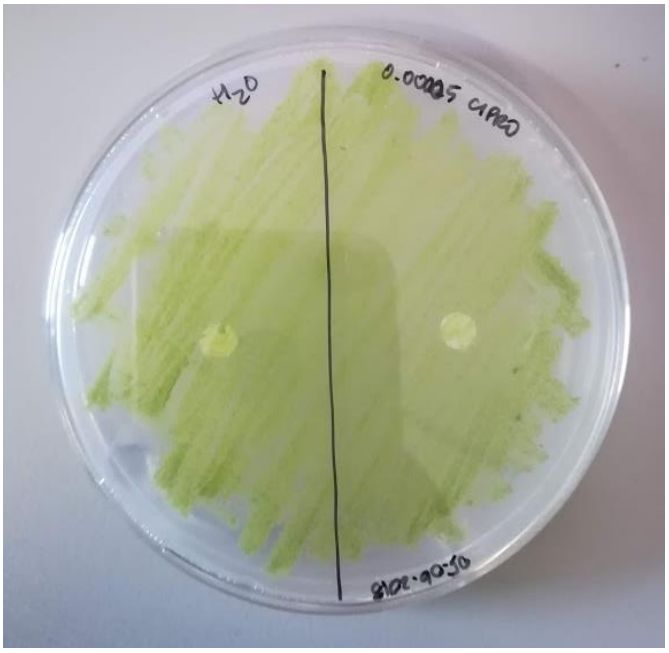

Figure XIII.2 – Paper-disc agar method with a ciprofloxacin concentration of 5.00 x 10^3 µg L^{-1} and water as blank.