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MICROALGAE FOR THE TERTIARY TREATMENT OF URBAN EFFLUENTS IN A PILOT-SCALE SYSTEM

Master thesis in Molecular and Microbial Biology

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Declaração de autoria de trabalho

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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"If a person has no dreams, they no longer have any reason to live. Dreaming is necessary, although in the dream reality should be glimpsed. For me this is a principle of life."

Ayrton Senna

Resumo

Este estudo relata o primeiro teste piloto dos fotobiorreatores GreenDune. No tratamento terciário de águas residuais urbanas, *blooms* naturais de microalgas foram em duas condições sazonais diferentes: outono e inverno. Foram realizados o isolamento e identificação de estirpes de microalgas, avaliação da eficiência de remoção de nutrientes pelas microalgas e avaliação das melhores condições operacionais e composição bioquímica da biomassa produzida. Na fase de caracterização do *bloom*, as principais classes que compuseram o consórcio foram: *Cyanophyceae*, *Chlorophyceae* e *Bacillariophyceae*. Para uma aprofundada identificação, as microalgas foram isoladas. *Desmodesmus abundans* GTM11, *Scenedesmus* sp. GTM2, *Chlorella* sp. GTM4 e *Chlorella* sp. GTM 5 foram as estirpes identificadas. A maior concentração celular registada ocorreu na experiência de inverno, W1.12 com uma média de 1,64 ± 2,23 gL^{-1}. Relativamente às condições de cultivo, a temperatura registada no Algarve (Portugal), que oscilou entre os 15,2 e 30,5 ° C durante os estudos no outono e no inverno, revelou ter condições adequadas e favoráveis ao crescimento mesmo em estação mais fria. Em todas as experiências, o piloto foi eficiente na remoção de N-NH4 e a concentração de nitratos permaneceu abaixo dos limites legais estipulados pela legislação Portuguesa. Nitratos totais e fósfuros totais foram encontrado em altas concentrações como sendo reflexo da presença de sólidos suspenso nas amostras tratadas, provavelmente constituídas por células microalgais. Estes resultados de TN e TP poderão ter sido causa de baixa eficiência de sedimentação. Porém, de uma forma geral, todos os limites definidos pela legislação foram cumpridos com sucesso. A biomassa obtida apresentou elevados níveis de proteínas (27,99-31,98%) e carbohidratos (31,62-37,50%), o que é indicativo de possíveis aplicações promissoras para a produção de biogás, biometano ou bioetanol. O conteudo lipídico, neste caso, foram os mais baixos (6,61-8,12%), pelo que a produção de biodiesel não deverá ser uma opção. Relativo à composição mineral presente nas cinzas (25,63-32,61%), esta mostrou ser rica em macro- e micronutrientes, que são fundamentais e de enorme interesse para aplicações na agricultura como biofertilizantes. Neste estudo, é de destacar o potencial dos fotobioreactores piloto GreenDune, que foram capazes de operar em 2 dias ou menos com uma capacidade de trabalho de 450L por 1 m^2 (relação volume / área elevada). Dessa forma, menos espaço será necessário, a pegada ambiental reduzida e a eficiência econômica aumentada.

Palavras-chave: fotobiorreator piloto; remoção eficaz; sustentabilidade; produtos de valor agregado;

Abstract

This study reports the first pilot trial of the GDPBRs in urban wastewater tertiary treatment using a natural bloom of microalgae in two different seasonal conditions: autumn and winter. Isolation and identification of microalgae strains, assessment of nutrient removal efficiencies by the microalgal as well as the assessment of both the best operational conditions and biochemical composition of the produced biomass were executed. The main classes that composed the consortium were *Cyanophyceae*, *Chlorophyceae*, *Bacillariophyceae*. Upon isolation, four strains were identified, namely *Desmodesmus abundans* GTM11, *Scenedesmus* sp. GTM2, *Chlorella* sp. GTM4 and *Chlorella* sp. GTM 5. The highest registered cellular concentration was in W1.12 experiment (average 1.64 ± 2.23 gL^{-1}). The temperature registered in Algarve (Portugal) that ranged between 15.2 and 30.5°C during studies in autumn and winter, proved to have proper cultivation conditions even in the coldest season. The pilot was efficient in the removal of $N\text{-}NH_4$ and nitrates concentration remained below legal limits in all experiments. High TN and TP concentrations were a reflection of the presence of solids, probably constituted by microalgal cells, caused by poor sedimentation efficiency. Nevertheless, all the limits defined by the Portuguese legislation were fulfilled. The biochemical composition of the microalgal biomass showed elevated proteins (27.99-31.98 %) and carbohydrates (31.62-37.50 %) with promising applications for the production of biogas, biomethane or bioethanol. Due to lower lipids (6.61-8.12 %), the production of biodiesel should not be an option. The mineral composition present in the ashes (25.63-32.61 %) proved to be a rich source of macro and micronutrients of interest for agriculture applications as biofertilizers. The highlights of this study were the potential of the pilot GreenDune-PBR that was able to operate under 2 days or less with a working capacity of 450L per 1 m^2 (high volume/area ratio). That way, less land is required, the environmental footprint reduced, and the economic efficiency augmented.

Keywords: pilot photobioreactor; removal efficiency; sustainability; value-added products;

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Abbreviations

EC_{50} – Effective concentration in 50% of tested population

GDPBR – GreenDune Photobioreactors

HRAP – High Rate Algal Ponds

HRT – Hydraulic Retention Time

PBR - Photobioreactor

PPCP – Pharmaceutical and Personal Care Products

SRT – Solid Retention Time

WWTP – Wastewater Treatment Plant

A2 – Autumn with hydraulic retention time of 2d

W2 – Winter with hydraulic retention time of 2d

W1.12 – Winter with hydraulic retention time of 1.12d

RE – Removal Efficiency

E – inflow wastewater

T – outflow or treated wastewater

1. Introduction

1.1. General Principles of Wastewater Treatment

During the 20th century, the accelerated population growth along with agriculture, urbanization, and industrialization became key factors to increase the consumption and limit the access of safe water sources (Naidoo & Olaniran, 2014). Consequently, the anthropogenic activities enhanced the amount of water residues. The development of treatment systems was necessary for proper sanitation to protect the environment and large populations from diseases (Naidoo & Olaniran, 2014). Otherwise, untreated waters may constitute a source of pollution in the environment. For example, the eutrophication phenomenon, that is often referred as a consequence of an excessive input of nutrients, mainly nitrates and phosphates, into the water column leading to the development of algal blooms, accelerated growth of aquatic plants, oxygen depletion and, thereby, resulting in the degradation of freshwater, and/or seawater, and loss of key species (Gonçalves et al., 2017; EU Directive 98/15/EC, 1998). Also, contaminated water increases the risk of illness of communities due to different bacteria, viruses, and protozoan pathogens which may be a source of various infectious diseases either by direct body contact or ingestion (Naidoo & Olaniran, 2014).

The water bodies whose original composition changed are termed wastewaters (WW). Its composition varies according to anthropogenic interference at different locations (Acién et al., 2016). The sources can be agriculture, industrial, and/or urban that may have significant organic matter contents, oil and grease, heavy metals and toxic chemicals leading to the contamination and/or pollution of water bodies if not properly treated (Acién et al., 2016; Udaiyappan et al., 2017). The main sources from agro-industrial wastewater are dairy, olive oil, winery industries and animal manure (Cuellar-Bermudez et al., 2017; Markou et al., 2018). Urban wastewater includes runoff waters, domestic, commercial, industrial and hospital effluents (Deblonde et al., 2015; Teijon et al., 2010).

The conventional methods for effluents treatment at wastewater treatment plants (WWTPs) are commonly divided into preliminary, primary, secondary, and tertiary. The preliminary stage involves the elimination of large solid materials to prevent the obstruction of the flow or equipment damaging. Then, a physical or primary process is responsible for the removal of organic and inorganic solids by sedimentation and flotation, reducing the chemical oxygen demand (COD) by 25-50%, the total suspended solids (TSS) by 50-70%, oil and grease by 65% (Sonune & Ghate, 2004). The secondary stage consists of a biological treatment with bacteria that reduces biochemical oxygen demand (BOD) by at least 90% that is accomplished by aeration under high-rate air conditions to achieve a certain effluent quality. Then, wastewater goes through a settling that results in the formation of sludge, often designated as activated sludge (Sonune & Ghate, 2004). Therefore, a complementary or tertiary step using biological agents could be necessary if remnant organic micropollutants such as nitrogen, phosphorus or toxic materials persist (Sonune & Ghate, 2004). The nitrification step performed by bacteria converts ammonia (NH_4^+) to nitrite (NO_2^-), and nitrite to nitrate (NO_3^-) under oxygen-rich conditions (Lewkowska et al., 2016). Subsequently, denitrifying bacteria reduces nitrogen under anoxic conditions, where nitrate is transformed into molecular nitrogen, which is stripped out as gas by aeration (Schulze et al., 2017). However, external carbon sources (e.g. methanol) are often required by denitrifying bacteria (Schulze et al., 2017).

The described conventional methods are efficient to treat wastewaters in a short time. Despite the benefits of the treatment, mechanical aeration in secondary and/or tertiary phases increases up to 50% the energy consumption of the wastewater treatment plant, resulting also in increased greenhouse gases emission, as well as having other environment impacts (Yoshida et al., 2014; Zou et al., 2018). Also, the supplementation with flocculants to allow the precipitation of phosphorus could possibly contaminate the sludge with toxic, metal-containing flocculants (Christenson & Sims, 2011). However, wastewater treatment in developed countries are mainly influenced by direct capital and operational costs. In addition, most of the treatments does not include tertiary treatment or advanced sludge processing. Life-cycle assessment in environmental impacts and costs are rarely considered, as long as discharged or reused waters meet the standards defined by EU Directive 98/15/CE (Awad et al., 2019). Hence, to minimize negative impacts in environment and benefit WWTPs it is necessary to study sustainable alternatives.

1.2. Wastewater Treatment Using Microalgae

Taxonomically, microalgae belong to different kingdoms such as Plantae, Bacteria, Protozoa and Chromista (Di Caprio, 2020). They are photosynthetic microorganisms that use sunlight as a source of energy, thus producing oxygen and consuming inorganic compounds such as carbon (C), nitrogen (N) and phosphorus (P) that are essential nutrients for their cell multiplication and maintenance (Delgadillo-Mirquez et al., 2016;

Posadas et al., 2015). Wastewaters have been considered as a great source of C, N and P, that could work as a microalgal cultivation medium, recycling these compounds. For that reason, microalgae have been proposed by Oswald in 1960, as an alternative to effluents treatment. The availability of nutrients strongly depends on several conditions, such as wastewater composition that changes over time, thus being necessary its characterization. Also, the molar ratio C/N/P is important to ensure molecular and metabolic processes for proper microalgae functioning (Acién et al., 2016).

Microalgae are considered a great alternative to the treatment of effluents helping in CO_2 mitigation, reducing greenhouse gas emissions removing and recycling nutrients, and producing biomass that could be considered a cost-effective feedstock for value-added compounds. Biofertilizers, bioplastics, animal feed, biofuels, and bioactive compounds could be obtained as valuable products and could reduce the cost of effluents treatment through microalgae culture (Arias et al., 2018; Batista et al., 2015). **Table 1** shows the removal percentages of COD, total nitrogen (TN) and total phosphorus (TP) by different microalgae species. Also, the treatment of wastewater varied according to its source, such as cattle farm, piggery, aquaculture, or urban effluents, as well as the time needed (**Table 1**).

Table 1 – Removal of nutrients by different microalgae species.

Specie	**WW**	**Time required (days)**	**TN removed (%)**	**TP removed (%)**	**COD removed (%)**	**References**
Chlorella vulgaris	Cattle Farm	3 to 5	81.16	85.29	62.3	(Lv et al., 2018)
Desmodesmus sp.	Piggery		90	70		(Chen et al., 2020)
Chlorella vulgaris and Scenedesmus obliquus	Aquaculture	1	86.1	82.7		(Gao et al., 2016)
Chlorella sp., *Acutodesmus obliquus* and *Oscillatoria* sp.	Piggery	27	82-85	90-92		(García et al., 2018)
Mixed microalgae culture	Municipal	8	Complete removal	Complete removal	70% within 8 days	(Arias et al., 2018)

Three microalgae and isolated consortium	Urban	2 to 6	84-98%	95-100%	36-64%	(Gouveia et al., 2016)
Natural consortium	Municipal	4.5	91-93		62-65	(Arashiro et al., 2019)

The most common forms of inorganic carbon are carbon dioxide (CO_2), carbonate (CO_3^{2-}), and bicarbonate (HCO_3^-). In autotrophic microalgae, CO_2 uptake occurs by diffusion and HCO_3^- is incorporated into the cells by active transport through carbonic anhydrase activity (Sayre, 2010). Then, HCO_3^- is converted to CO_2 and fixed by RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase), producing two molecules of 3-phosphoglycerate (Sayre, 2010). In some cases, certain microalgae are heterotrophic, using the respiratory metabolism to integrate organic carbon such as acetate, glucose, glycerol and ethanol as a carbon source (Gonçalves et al., 2017). Others are mixotrophic by combining both the autotrophic and heterotrophic mechanisms by assimilating atmospheric CO_2 and organic compounds as a carbon source (Gonçalves et al., 2017).

Regarding inorganic nitrogen, the fixation and assimilation by microalgae are important steps. Prokaryotic microalgae (e.g. cyanobacteria) can fix molecular nitrogen (N_2) and convert into ammonia-nitrogen (NH_3-N). Eukaryotic microalgae (e.g. green algae, red algae, glaucophytes) need to assimilate reduced nitrogen forms that have previously been fixed by nitrifying bacteria. However, WW already contains abundant amounts of inorganic nitrogen in the form of ammonium, nitrite, and nitrate that could be incorporated by microalgal cells through ammonium and nitrate/nitrite transporter proteins (Gupta et al., 2019b). Inside the cell, the reduction of nitrate into nitrite is catalyzed by nitrate reductase, and nitrite is reduced into ammonia by nitrite reductase. After that, ammonia can be converted into amino acids via the glutamine-glutamate synthase pathway catalyzed by glutamine synthase or released to the environment (Gonçalves et al., 2017). Concerning phosphorus, the uptake occurs in the forms of orthophosphate ions (e.g. $H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-}) through active transport, which is important for the synthesis of nucleic acids and energy transfer processes (Gonçalves et al., 2017). The mechanism of transport involves phosphorylation at the substrate level, oxidative phosphorylation, and photophosphorylation (Gonçalves et al., 2017).

1.3. Cultivation Conditions

1.3.1. Effect of Nutrients

The molar ratio 100/14/2 (C/N/P) found in primary effluent is considered ideal for microalgae growth because of the presence of higher amounts of organic carbon (Acién et al., 2016). When secondary effluent is used, microalgae could be carbon limited because of the lack of organic load (Acién et al., 2016). An alternative that improved microalgae productivity was CO_2 injection, either used as pure, flue or residual gases (Guo et al., 2017; Posadas et al., 2015).

The effect of CO_2 supplementation with sewage provided as a nutrient source had significant impacts on lipid and biomass productivity, and total chlorophyll and protein contents of the microalgae consortia (Sharma et al., 2020). A study demonstrated that the presence of carbon exhibited a linear correlation with biomass productivity shown by *Chlorella pyrenoidosa*, while treating primary piggery wastewater efficiently, removing over 90% of ammonium (Wang et al., 2012). Posadas et al. (2015) showed that when CO_2 from flue gas was applied, the highest removal rates for COD, total organic carbon (TOC), and TP, as well as higher biomass productivity were registered using inoculated *Scenedesmus* sp. with activated sludge to treat primary domestic wastewater.

Nitrogen is an essential nutrient for microalgae growth and cultivation. The limitation of N supply to microalgae could result in a higher energy dissipation by decreased photosynthetic activity (PSII) and consequently a gradual decrease in biomass growth as well as chlorophyll content (Markou et al., 2017). Caporgno et al. (2015) showed that N-starvation in *Chlorella kessleri* and *Chlorella vulgaris* led to increased lipid production. However, WW provides a limitless source of N mainly in the forms of nitrate (NO_3^-) or ammoniacal nitrogen (NH_4^+/NH_3). It is often assumed that the uptake of ammoniacal nitrogen is preferred over other forms of N (Gutierrez et al., 2016). That way, NH_4^+/NH_3 could be recovered as microalgal biomass byproduct (Gutierrez et al., 2016). However, NH_3 is dominant at elevated pH which could be toxic at a certain concentration with a direct impact on photosynthetic apparatus (Markou & Muylaert, 2016). Ammonia can damage the oxygen evolving complex (OEC) of the photosystem II by displacing a water ligand and acting as an uncoupler of the Mn cluster of the OEC or, ammonia could diffuse through membranes and accumulate, disrupting the ΔpH component of the thylakoid proton gradient (Gutierrez et al., 2016; Markou & Muylaert, 2016; Rossi et al., 2020). Experiments with cyanobacteria monocultures of three different species of

Synechococcus sp., *Synechocystis* sp., and *Leptolyngbya* sp., and cyanobacteria-bacteria consortia mainly dominated by *Synechoccus* sp. and *Synechocystis* sp. presented an average half maximal effective concentration for ammonia ($EC_{50,NH3}$) of 14.1 mg NH_3 L^{-1} and 26.2 mg NH_3 L^{-1}, respectively (Rossi et al., 2020). Then, it was concluded that cyanobacteria were more sensitive to ammonia comparing to the results for a microalgae monoculture tested with four different strains *Chlorella vulgaris* SAG211-11j ($EC_{50,NH3}$= 60.9 mg NH_3 L^{-1}), *Chlorella sorokiniana* SAG211-8k ($EC_{50,NH3}$= 96.3 mg NH_3 L^{-1}), *Scenedesmus* spp. SAG276-4d ($EC_{50,NH3}$= 77.7 mg NH_3 L^{-1}), and *Scenedesmus obliquus* ($EC_{50,NH3}$= 52.6 mg NH_3 L^{-1}) (Rossi et al., 2020). Cyanobacteria was also more sensitive than microalgae-bacteria consortia dominated by *Chlorella* sp. and *Scenedesmus* sp., that presented an $EC_{50,NH3}$= 88.4 mg NH_3 L^{-1} (Rossi et al., 2020). Although, including other variables such as the impact of pH and temperature on ammonia, phototrophs-bacteria consortia showed to be more robust when compared to monocultures (Rossi et al., 2020).

Phosphorus is a finite, non-renewable resource that is important to global food and feed production by modern agriculture (Solovchenko et al., 2016). P is another essential nutrient to microalgae cells, and plays an important role in nucleic acids synthesis, structural phospholipids, proteins, sugar phosphates, and other metabolites (Solovchenko et al., 2019). Microalgae can take up inorganic P in the forms of orthophosphate ions (e.g. $H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-}) through active transport (Solovhenko et al., 2019). Phosphorus could be bioavailable and recovered from wastewaters into microalgal biomass, which could be converted into feed additives and biofertilizers (Solovchenko et al., 2016). Also, the removal efficiency of N from waste effluents could depend on their P-content (Choi & Lee, 2015; Xin et al., 2010).

The biomass productivity is highly depend on the N/P ratios. Choi & Lee (2015) showed that biomass productivity increases within ratios up to 10 (2.97 g L^{-1} d^{-1}), where the maximum TN removal occurred at N/P of 11-15. After, a biomass productivity decrease, until a constant value, was obtained with a ratio of 30 (0.40–0.78 g L^{-1} d^{-1}), which means that higher P-content favors more the biomass productivity than higher N-content (Choi & Lee, 2015). The effect of adding phosphate as a supplement in *Chlamydomonas* sp. led to a 90.7% removal of ammonia in comparison to a removal of 51.7% if without supplementation (Paskuliakova et al., 2016). Micronutrients such as magnesium (Mg), calcium (Ca), manganese (Mn), molybdenum (Mo), copper (Cu), zinc (Zn) and vitamins also play an important role in microalgal growth (Kumar et al., 2010).

1.3.2. Effect of Light, Temperature and pH

Photosynthesis determines the uptake of nutrients, its removal efficiency, and biomass productivity of microalgae. The combined effects of light, temperature, or pH are crucial factors that have impacts on photosynthesis. The photosynthetic activity is proportional to light intensity until the saturation point (Gonçalves et al., 2017). The effect of increased irradiance enhances microalgae photosynthetic activity, thus increasing CO_2 consumption and consequently increasing the pH of the culture (Foladori et al., 2018). In dark conditions, the absence of photosynthesis leads to cellular respiration, CO_2 production, and therefore, the pH decreases (Foladori et al., 2018).

Light intensity and temperature between 100 and 800 μmol photon m^{-2} s^{-1} and 20–35 °C, respectively, are often considered optimal conditions for microalgae cultivation(Gupta et al., 2019). In outdoor conditions, critical parameters such as light availability and temperature, present fluctuations during the day (i.e. lower photon flux density occurs at the morning and evening, and higher at noon) and also depends on the season and location of the treatment plant (i.e. different latitudes and photoperiods, self-shading of microalgal cells) (Holdmann et al., 2019).

A study in Germany showed that the amount and distribution of photons during the day had impact on productivity. Also, it showed that longer photoperiods yielded higher productivity (Holdmann et al., 2019). In Spain, an outdoor study with different regimes of light intensity (0, 150 and 300 μmol m^{-2} s^{-1}), light:dark cycles (12:12 h and 24:0 h) and photoperiods (on/off: 1.5:1.5 h, 0.75:0.75 h and 1:2 h) showed no differences in performance of microalgae mainly composed by *Chlorella* sp. and *Scenedesmus* sp. (González-Camejo et al., 2019). Therefore, the authors concluded that microalgae performance probably depends on the net photon flux (Gonzále-Camejo et al., 2019).

Beyond optimal culture conditions, excessive light and temperature may induce photodamage to the culture that could be avoided by shading, which limits the incident light in cells (Martínez et al., 2017). As demonstrated by Hindersin et al. (2013), both photoinhibition and temperature could be reduced and regulated by controlling irradiance and/or shading (Hindersin et al., 2013).

Microalgae-based wastewater treatments uses well adapted and resistance strains including *Chlorella* and *Scenedesmus* that were found to grow in a wide range of temperatures (15-30 °C and 10 to 30 °C, respectively)(González-Camejo et al., 2019; Xin et al., 2011). Delgadillo-Mirquez et al. (2016) showed that native microalgae-bacteria consortium growth was absent at lower temperatures (5 °C), while at 25 °C it was registered the highest productivity.

Microalgae mainly consumes soluble inorganic nutrients from the effluents in the forms of inorganic carbon (CO_3^{2-}, HCO_3^- and CO_2), inorganic nitrogen (NH_4^+, NO_3^-) and inorganic phosphorus ($H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-}). Microalgae are susceptible to pH changes and is growth can also be assessed by the pH of the culture. The optimal pH condition ranges between 7 and 9 (Posadas et al., 2015). The higher photosynthetic activity by microalgae, that is dependent in other factors such as light irradiance and temperature, increases the inorganic carbon consumption leading to higher culture pH (González-Camejo et al., 2020).

The solubility of nutrients is pH dependent. At higher pH, the dominant form of carbon is bicarbonate (Binnal & Babu, 2017). Its consumption also increases pH (Foladori et al., 2018). At pH higher than 8, ionized forms such as NH_4^+ are dissolved into nonionized forms, such as NH_3 that are extremely toxic to microalgae (Binnal & Babu, 2017), although, at increased pH and temperature significant amounts of ammonia can be volatilized, which in turn may lead to N limitation (Cai et al., 2013). The pH could be counterbalanced by nitrifying bacteria that could oxidize NH_4^+ into NO_3^-, which is less toxic (Foladori et al., 2018). The removal of orthophosphate, similarly to nitrogen, is not only removed by the uptake into microalgal cells, but also by the effect of pH. At pH values between 9 and 11, phosphorus will precipitate and deposit (Cai et al., 2013).

1.3.3. Operational Conditions

The cultivation conditions could be performed under batch, semi-continuous or continuous modes. In a batch process, all the nutrients are initially provided without further addition. Here, nutrients are limited and rapidly consumed until its exhaustion marking the end of the process. A semi-continuous process or fed-batch technique provides the necessary nutrients to the culture making possible the manipulation of the substrate or productivity of the culture and duration of the treatment. In the continuous mode, constant fresh medium is added to the culture and nutrients are steadily removed during stable and long-term periods of cultivation. Mostly, all the processes provide different nutrient and energy sources that significantly affect microalgae growth and biomass composition (Chojnacka & Marquez-Rocha, 2004). Those cultivations could be under photoautotrophic, heterotrophic, mixotrophic, photoheterotrophic modes well reviewed and detailed in (Chew et al., 2018).

The efficiency of wastewater treatment using microalgae also depends on operational conditions such as gas transfer, mixing, solids residence time (SRT), and hydraulic retention time (HRT) (Gonçalves, Pires, & Simões, 2017). The HRT controls some parameters such as nutrient loading, biomass productivity, and biochemical composition, while SRT consists in the solid fraction of a suspended system (Bradley et al., 2019). Bradley et al. (2019) demonstrated that short SRT promoted higher eukaryotic diversity, increased functional stability, and better TN removal, while longer SRT promoted stable bacterial nitrification. Valigore et al. (2012) showed that biomass from native microalgal-bacterial consortia with longer SRT enhanced settleability while shorter HRT enhanced productivity. Takabe et al. (2016) suggested an HRT of 2-3 d to obtain maximum biomass yield from indigenous microorganisms in secondary effluent treatment.

Gas transfer conditions are also important to ensure proper aeration, gases supply and dissolution (e.g. CO_2), sufficient light exposure, nutrients distribution, and internal mixing among microalgal cells (Kumar et al., 2010). Mixing is key to ensure that biomass is prevented from settling, avoiding stratification zones (Gonçalves et al., 2017; Sutherland et al., 2014). Stratification leads to stagnant zones that could create anaerobic conditions, reduce nutrient availability, biomass productivity, and even accumulate toxic compounds (Kumar et al., 2010).

1.3.4. Heavy Metals, Pharmaceuticals and Personal Care Products in Wastewaters

The environment is prone to pollution and/or contamination by chemical substances either released to the air, land, or water. Heavy metals such as arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), lead (Pb), manganese, (Mn), mercury (Hg), silver (Ag), zinc (Zn), and others, are commonly originated from industrial activities, including mining, metal plating, refineries, battery, dye and pigment manufacturing, fertilizers, and others (Leong & Chang, 2020). Heavy metals pose serious threats to environmental and human health, since some can be highly toxic, carcinogenic, mutagenic and teratogenic (Leong & Chang, 2020). To remove toxic substances from wastewater, conventional methods use technics such as chemical precipitation, ion exchange, membrane filtration, flotation, coagulation-flocculation and electrochemical methods (e.g. electrodeposition, electroflotation and electrocoagulation) (Fu & Wang, 2011).

Regarding urban effluents, they are also a rich source of pharmaceuticals and personal care products (PPCPs). These group contains antibiotics, analgesics, anti-inflammatory and cardiovascular drugs, tranquilizers, stimulants, steroid and hormones, lipid regulators, skin care products, soaps, and other agents (Muñoz et al., 2008). These substances are widely found in wastewaters (Santos et al., 2013). PPCPs have bioactive properties used to treat human and animal diseases, and for that reason they are widely used, thus threatening the environment health and that of aquatic organisms (Santos et al., 2013). Antibiotics either from human, veterinary, and agriculture purposes might be potentially suitable for the development of resistant bacteria (e.g. enterococci and coliforms) in environment (Rizzo et al., 2013; Varela et al., 2013).

The removal of PPCPs involves advanced technologies, such as active oxidation (O_3, H_2O_2 and Fenton-type oxidations), photodegradation and photocatalytic degradation processes, membrane filtration, or activated carbon adsorption (Yinghui Wang et al., 2017). These advanced technologies add costs associated to their operation and maintenance, as well as secondary pollution because of the formation of toxic sludges (Leong & Chang, 2020; Yinghui Wang et al., 2017). Alternatively, the removal of contaminants associated with microalgae treatment includes both abiotic and biotic factors such as photolysis, hydrolysis, oxidation/reduction, and bioadsorption, bioaccumulation, and biodegradation, respectively (Yinghui Wang et al., 2017; Xiong et al., 2016).

In a textile effluent, chromogenic substances such as Al, Cu, V, Pb and Se were removed by green microalgae between 47% to 70% (Oyebamiji et al., 2019). The study of Ajayan et al. (2015), showed that not only *Scenedesmus* sp. was able to reduce pollution load by the heavy metals Cr (96%), Cu (98%), Pb (98%) and Zn (98%), but also remove the nutrients NO_3 (>44.3%) and PO_4 (>95%) from tannery wastewater.

In another study, *Chlorella minutissima* could efficiently remove Cd, Cu, Mn and Zn under heterotrophic conditions without growth inhibition, while manifesting increased lipid content by 21% and 94% with Cd and Cu addition, respectively (Yang et al., 2015). Alam et al. (2015) showed that the self-flocculating *Chlorella vulgaris* JSC-7 removed more than 80% of Zn and 60% of Cd within 3 days, presenting much higher results than the non-flocculating *Chlorella vulgaris* CNW11.

Regarding PPCPs removal, *Scenedesmus obliquus* presented a removal efficiency of diclofenac above 79%, 2.6 and 3.7 times higher than *Chlorella sorokiniana* and *Chlorella vulgaris*, respectively (Escapa et al., 2016). The removal of salicylic acid and paracetamol by *Chlorella sorokiniana* in batch culture was 73% and 67%, respectively, and in semi-continuous culture was 42% and 93%, respectively (Escapa et al., 2015). Matamoros et al. (2015) tested 26 organic pollutants (such as caffeine, ibuprofen, carbamazepine, diclofenac, triclosan, bisphenol A, etc.), and depending on the compound the removal efficiencies ranged from negligible to more than 90%.

The addition of an anaerobic step showed to improve higher removal rates to ibuprofen, naproxen, salicylic acid, triclosan and propylparaben was respectively, 94 ±1%, 52 ± 43%, 98 ± 2%, 100 ± 0%, 100 ± 0% (López-serna et al., 2019). Eventually, the combination and testing of different conventional technics with microalgae-based technologies are needed to achieve an efficient wastewater treatment.

1.4. Microalgae Monocultures *versus* Microalgae-bacteria Consortia for Wastewater Treatment

Microalgae-based technology for wastewater treatment can use monocultures of isolated species or microbial consortia. Consortia could be shaped by different species of microalgae and bacteria, which occur naturally (natural microalgal bloom) or by selection and/or artificial engineering (Gonçalves et al., 2017). The most widely employed microalgae species are from the genera *Chlorella*, *Scenedesmus*, and some cyanobacteria due to their high growth rate, environmental tolerance, and lipid and starch accumulation (Li et al., 2019; Zhou et al., 2012). Common groups of the bacteria used belong to the genera *Proteobacteria*, *Bacteroides*, *Flavobacteria*, *Sphingobacteria* and *Actinobacteria* (Lakaniemi et al., 2012; Yue Wang et al., 2016).

A key advantage of natural occurring species in wastewater treatment is that they are resistant to environmental changes (Sharma et al., 2020). For that reason, the consortium could be less prone to microbial contamination by competitors, predators, and parasites when compared to monocultures (Luo et al., 2020). That is possible because complex interactions (e.g. cooperation and competition) are able to maintain a stable community (**Fig. 1**) (Liu et al., 2017). However, such interactions depend on the communication between microorganisms through the exchange of metabolites and molecular signals that lead to diversified pathways for nutrient consumption (Liu et al., 2017). These pathways are shaped by photoautotrophic, mixotrophic or heterotrophic cultures reviewed in Nirmalakhandan et al. (2019).

In WWT systems microalgae-bacteria consortia dominate under mixotrophic conditions, which combines both photoautotrophic and heterotrophic processes. That way, mixotrophic culture combines inorganic and organic carbon sources in different light regimes (Nirmalakhandan et al., 2019). For example, Rashid et al. (2019) demonstrated that co-cultivation in mixotrophic conditions yielded higher biomass productivity and stabler composition. Zhang et al. (2013) showed that mixotrophic microalgae (*Scenedesmus* sp. and *Chlorella* sp.) isolated from domestic effluent could be also operated under heterotrophic conditions.

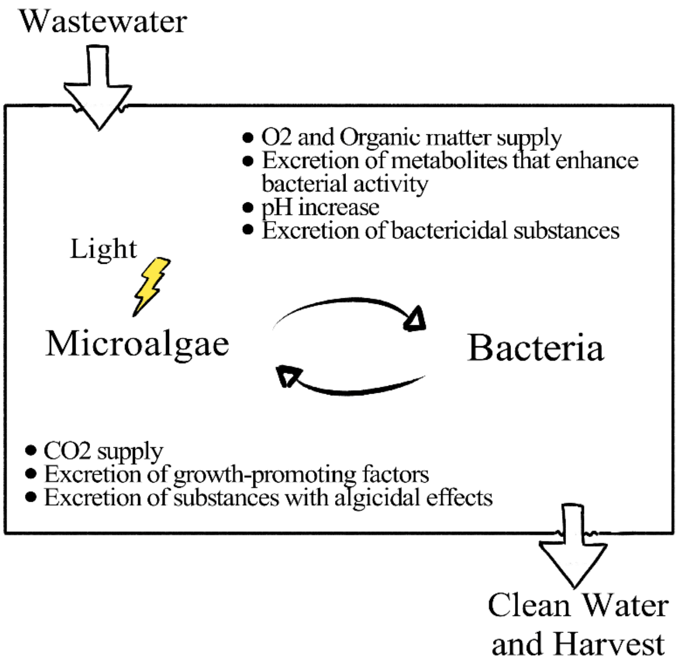

Figure 1 - Microbial interactions using microalgae-based technology for wastewater treatment (adapted from Gonçalves et al., 2017).

1.5. Microalgal Biomass Composition and Valorization

Microalgal biomass is comprised by 50-70% of proteins, 10-50% of lipids, and about 50% of carbohydrates contents (Chew et al., 2017; Costa et al., 2020). The composition may vary according to microalgae genera and species, as well as the culture conditions (Becker, 2013).

Protein products are important for human or animal nutrition (Chew et al., 2017). Although, previous analysis must be performed to assess application of proteins as end-products (Chew et al., 2017). Regarding lipids, they are classified into three major categories according to the number of double bonds in the side chains, i) saturated fatty acids (SFAs, e.g. palmitic and stearic acid), ii) monounsaturated fatty acids (MUFAs, e.g. oleic acid) and iii) polyunsaturated fatty acids (PUFAs) (Saini & Keum, 2018). Among the fatty acids, microalgae are a rich source of PUFAs, such as omega-3 fatty acids (e.g. linolenic, ALA; eicosapentaenoic, EPA, docosapentaenoic, DPA and docosahexaenoic acids, DHA) and omega-6 fatty acids (e.g. linoleic acid, LA and arachidonic acid, AA) that are considered high value bioproducts in food and health industries (Kumar et al., 2019; Saini & Keum, 2018). Also, microalgae oils could be an attractive lipid-based energy source as a raw material for biodiesel production (Chisti, 2007). Carbohydrates are accumulated in the plastid as a reserve material, like glucose and starch, or as a component of cell walls, such as cellulose, pectin and sulfated polysaccharides, being suitable for bioethanol, biobutanol, biohydrogen and biomethane production (Chen et al., 2013). Ashes content are the total amount of minerals present in the biomass that determines the quality of the biomass to produce animal feed, biofertilizers, human food or biofuel (Liu, 2019). High ash contents in microalgae diminishes their inclusion levels for feed, food and fertilizer applications, causes concerns regarding heavy metals composition, and also poses major operational problems for energy conversion using microalgal biomass in combustion systems (Austic et al., 2013; Liu, 2017).

The produced biomass from microalgae cultured in wastewater, usually, presents high-protein (>30% DW), low-lipid (<10% DW), and high-ash (>25% DW) contents (Huang et al., 2016). Biomass productivities, proteins, lipids, carbohydrates, and ash contents from different strains, effluents and culture conditions are presented in **Table 2.**

Despite the costs involved in the harvesting processes, the association between microorganisms from cultures were found to be beneficial for settleability through bioflocculation and natural aggregation (Luo et al., 2020; Pires et al., 2013). Furthermore, the quality of the biomass produced using wastewater as substrate must be checked because potential contaminations with pathogens (bacteria, viruses, parasites, etc.), metals, and xenobiotics (hormones, antibiotics, parasiticides, etc.) could occur (Markou et al., 2018; Schulze et al., 2017).

Regarding costs, effluent treatment using microalgae must achieve similar levels of cost as the conventional (0.2€/m^3) (Gouveia et al., 2016). To reduce capital and scalability costs, existing infrastructures of WWTPs could be utilized (Christenson and Sims, 2011). Therefore, the process of combining wastewater treatment and the production of useful microalgal biomass could enhance environmental sustainability and provide economic benefits (Batista et al., 2015; Christenson and Sims, 2011).

The production of bioenergy could be spontaneously implemented without major restrictions when compared to other bioproducts, thus being energetically feasible while removing nutrients, mitigating CO_2, and generating heat from combustion (Pires et al., 2013). Additionally, techno-economic assessment for wastewater-based algal biofuel production, such as bioethanol, biobutanol, biohydrogen, biomethane, and biodiesel, became an attractive option for the desired fuel market while practicing circular economy (Batista et al., 2015; C. Xin et al., 2018; Ranganathan and Savithri, 2019).

Table 2 - Chemical composition of microalgal biomass cultured in different wastewaters.

Species	**Culture Medium**	**Culture Condition**	**Biomass Productivity (g L^{-1} d^{-1})**	**Proteins (% DW)**	**Lipids (% DW)**	**Carbohydrates (% DW)**	**Ashes (% DW)**	**Ref**
C. vulgaris, *S. obliquus* and *Natural Consortium*	Urban	Closed system	0.05, 0.22, and 0.14, respectively	56.4, 32.7, and 51.6, respectively	10, 8.1, and 13.6, respectively	27.7, 11.7, and 20.6, respectively		(Gouveia et al., 2016)
Co-cultivation *Chlorella* sp./*Ettlia* sp.	BG-11	Closed system. Autotrophy and mixotrophy	0.7 ± 20 and 0.74 ± 60, respectively	41 and 30, respectively	11 and 18%, respectively	33 and 20%, respectively		(Rashid et al., 2019)
C. sorokiniana and consortium (*C. sorokiniana* and *M. capsulatus*)	Industrial	Closed system. Bubbbed gas (60% CH_4, 40% CO_2)		45 and 28, respectively	30 and 34, respectively	23 and 32, respectively		(Rasouli et al., 2018)
Natural Consortium	Municipal	Continuous flow stirred open system.			14		29	(Roberts et al., 2013)
Chlorella spp.	Swine	Open system		50.3	1.3	41		(Michelon et al., 2019)
S. obliquus	Municipal	Mixotrophic cultivation with different			18.4 to 22.9%, 18.4 23.3%, and	20.5 to 26.5%, 23.8 to 25.5%, and		(Ji et al., 2015)

		dilutions of FW and 5%, 10% and 14.1% flue gas CO_2			19.7 to 22.5%, respectively	25.5 to 28.8%, respectively		
Chlorella fusca LEB111	Industrial	Addition of CO_2, SO_2, NO and ash	0.100 to 0.14	50.2	15.5	19.7		(Duarte et al., 2016)
Different set of *D. communis* and a consortium	Urban (synthetic, primary or secondary effluent)	Different conditions of light (low and high) and air-CO_2 mixture	0.018 to 0.227	9.5 to 39.2	1.4 to 9.3	7.1 to 39.3	1.9 to 13.3	(Samorì et al., 2013)
S. obliquus, C. vulgaris and *Consortium C*	Urban	Closed system		32.7, 56.4 and 51.6, respectively	8.1, 10 and 13.6, respectively	11.7, 27.7 and 20.6, respectively		(Batista et al., 2015)
S. obliquus, C. vulgaris and *Consortium C*	Urban	Closed system. Nutritional stress.		35.7, 34, 45.3, respectively	25.2, 12.8 and 12.8, respectively	42.6, 21.9, 28.6, respectively		(Batista et al., 2015)

1.6. Types of Photobioreactors for Microalgae Cultivation

The most common technology used for microalgae cultivation in wastewater treatment are suspended cultures in either open or closed bioreactors (Gonçalves et al., 2017). Open systems include raceways or high rate algal ponds (HRAPs) that are typically about 0.3 m deep occupying large areas (**Fig. 2a**) (Chisti, 2007). In closed system, the most common are tubular or column photobioreactor (PBRs). These reactors are generally 0.1 m or less in diameter (Chisti, 2007). Compared to HRAPs, uses less area and gives better control over pH, temperature, and contaminations in cultures (**Fig. 2b**) (Christenson and Sims, 2011). Often, tubular PBRs suffer from overheating, accumulation of toxicity by oxygen, and high material and maintenance costs (Christenson and Sims, 2011). Both raceways and column PBRs are limited by harvesting processes. An alternative could be immobilized -cell bioreactors that uses a matrix of biofilm, but bacteria in wastewaters are likely to benefit, and high operation costs are associated (Christenson and Sims, 2011).

In terms of construction, scalability and maintenance, open systems are less expensive (Gonçalves et al., 2017). Although, for large scale wastewater treatment using microalgae a major challenge other than harvesting involves land requirements (Christenson and Sims, 2011).

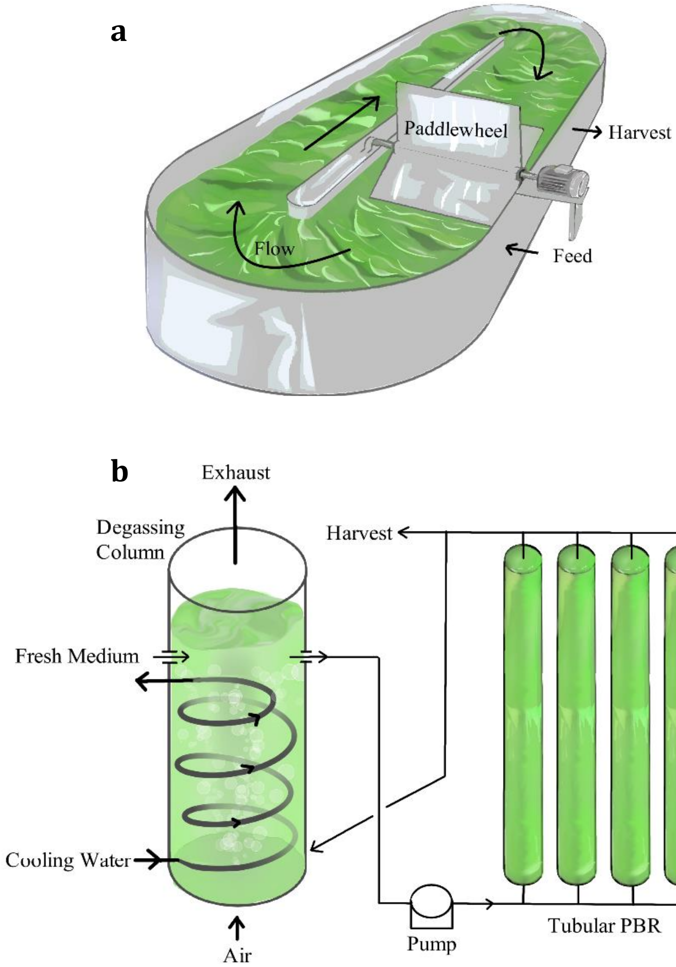

Figure 2 - Representation of a raceway pond (**a**), and tubular photobioreactors (**b**).

1.7. Current Framework of the Study

Since 2005, numerous photobioreactor configurations have been designed and the microalgal biomass market expanded until current days (Kirnev et al., 2020). The present experiment is part of the GreenTreat project from the MarBiotech Group (CCMar, UAlga).

The GreenTreat Project aims to develop an integrated and sustainable process for the tertiary treatment of urban wastewater using microalgae in the Algarve region using innovative PBRs designed and patented by Bluemater – eco-efficient solutions, Lda.. The project is performed by a consortium that includes CCMAR, the University of Algarve, REQUIMTE (Rede Química e Tecnologia), and Bluemater, with the support of Águas do Algarve.

The system used specifically for wastewater treatment are designated by GreenDune PBR (GDPBR) (**Fig. 3**). GDPBR have prismatic shape by 1 m x 1 m x 0.8 m in dimensions with a maximum volume of 490 L and a working capacity of 450L. They are an open system, where the contact between the culture and the atmosphere is established at the top. The gas transfer occurs at the bottom by air bubbling, providing mixing and preventing sedimentation. This system can be displaced sequentially, increasing the volume/area ratio and capacity of the treatment. Comparing the GDPBR to other reactors, the raceway ponds, for example, in a 450 L raceway with 2.5 m long, a channel width of 0.3 m and average depth of 0.1 m, the working volume is less than 150 L. In a tubular PBR with 0.2 m in diameter and 1 m height, the volume capacity is approximately 125 L. Overall, GDPBR presents higher working volume per area capacities and for that reason the higher land requirement of reactors construction could be avoided, as well as being cheap to be produced and maintained.

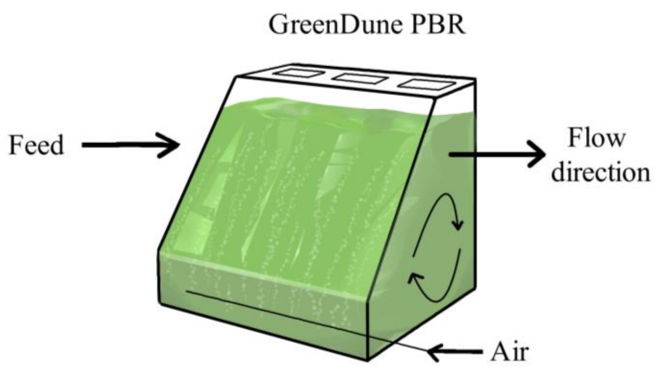

Figure 3 – Pilot GreenDune PBRs at WWTP of Quinta do Lago, Algarve, Portugal.

2. <u>Objectives</u>

The aim of this thesis was to study the removal efficiency of nutrients from urban wastewater (after secondary treatment) using pilot-scale photobioreactors for microalgae culture. The treatment of the effluent was conducted by natural bloom-forming microalgae within different seasons.

Specifically, to achieve a more effective and viable system that minimizes both space and economic costs, the objectives included:

i. Isolation and identification of microalgae strains appropriate for tertiary wastewater treatment;
ii. Assessment of nutrient removal efficiencies by microalgae in outdoor conditions in Autumn and Winter;
iii. Assessment of the best operational conditions (e.g., hydraulic retention time) for optimum nutrient removal in each season;
iv. Assessment of the biochemical composition of the microalgal biomass produced and its applicability for added-value applications;

3. Materials and Methods

3.3. Experimental Set-up

The experiments were conducted in GreenDune photobioreactors (GD-PBR) placed at the WWTP of Quinta do Lago, Algarve, Portugal (37°02'15.9"N 8°00'32.0"W) as presented in **Fig. 4**. GDPBR have prismatic shape by 1 m x 1 m x 0.8 m in dimensions with a maximum capacity of 490 L. Also, they can be connected increasing the total volume of the set-up. In this study, three GDPBR were connected setting up a treatment volume of 3x490L (triplicates) designated by treatment line (T), and three different lines, further designated T1, T2 and T3, were used. The air stream was bubbled from the bottom to prevent sedimentation, provide mixed conditions, and avoid the accumulation of biofilms in the photic zone. All experimentation was performed in outdoor conditions with ambient light and air temperature.

Before starting the experiments in autumn and winter, one or two weeks of acclimation were performed at outdoor conditions and using the chosen hydraulic retention time. Therefore, a natural microalgae-bacteria consortium was formed. The system was operated in a continuous mode and fed with secondary effluent from WWTP of Quinta do Lago. In the Autumn, GD-PBRs were operated for 4 weeks at 2 d of HRT (A2: 30th of September, 2019 to 30th of October, 2019). In the Winter, the GD-PBRs were operated for 4 weeks at an HRT of 2 d (W2: 27th of January, 2020 to 17th of February, 2020), and another 4 weeks at 1.12 d (W1.12: 17th of February, 2020 to 4th of March, 2020).

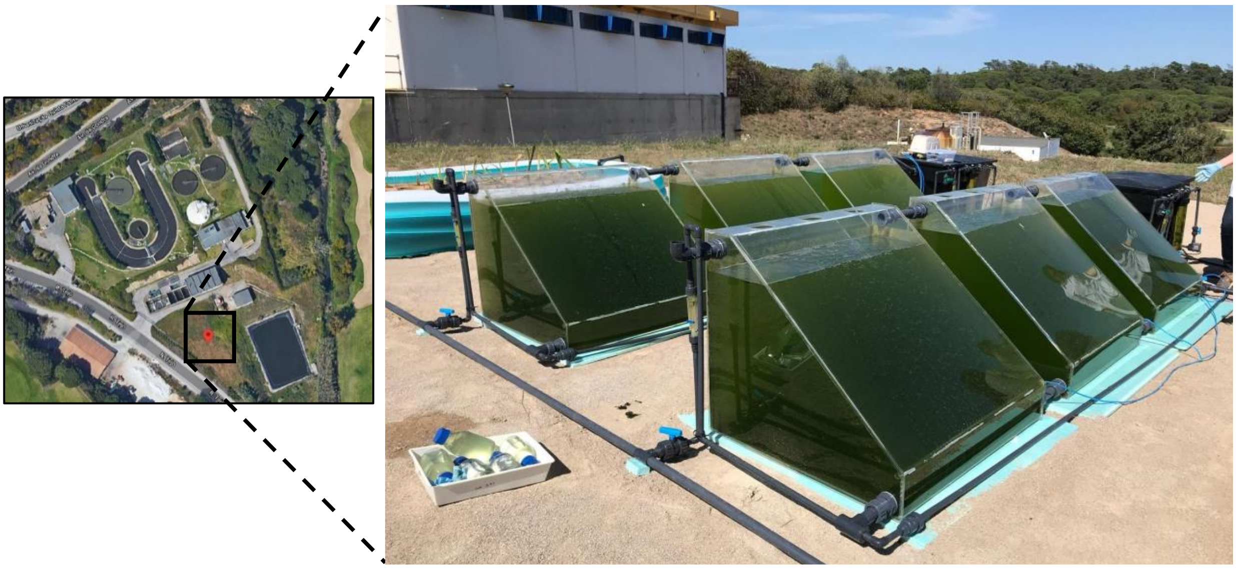

Figure 4 – GreenDune PBRs placed at Quinta do Lago, Algarve, Portugal (37°02'15.9"N 8°00'32.0"W).

3.4. Analytical Methods

Water temperature and pH were measured in situ weekly using a pH/mV/°C meter (HI 83141 Hanna Instruments, Italy). Microalgal biomass concentration (g L^{-1}) was determined weekly by measuring the optical density of the cell culture in a 96-well plate spectrophotometer (Biotek Synergy 4, USA) at 750 nm. The cellular concentration was obtained using a standard curve ($y= 2.0167x – 0.1351$, $r^2= 0.9972$). Both pH, temperature and biomass concentration parameters were expressed as mean ± SD.

The following chemical parameters were analyzed in the inflow wastewater (E) and in the outflow (T) from the GD-PBR according to standard methods (APHA, 2012.). The water was analyzed for TSS, TN, NH_4^+-N, NO_3^-, TP, and PO_4^{3-}-P using commercial kits (Hach, USA).

For biochemical characterization, the biomass was collected into falcon tubes and centrifuged at 2,500 x g for 10 min. Then, it was stored at -20 °C prior to lyophilization. All analyses were carried out in freeze-dried biomass except for moisture content which was done in fresh biomass.

3.5. Biochemical Analysis of the biomass

Protein content was obtained by elemental analysis of carbon, hydrogen and nitrogen assessed using and elemental analyzer (Vario E III, Elementar). Nitrogen content was multiplied by a factor of 5.13 to determine total protein content (Lourenço et al., 2002), as shown in equation (1):

$$(1)\ \text{Protein}\ (\%) = 5.13\ \times \text{N content}\ (\%)$$

Total lipids were determined gravimetrically using the Bligh and Dyer (1959) method with few modifications as described in Pereira et al. (2011). Briefly, dried biomass was homogenized with an Ultra-Turrax disperser (IKA-Werke GmbH, Germany) and extracted with a mixture of chloroform, methanol, and water (2:2:1). Then, separation phase was achieved by centrifugation, and a known volume of chloroform was pipetted to pre-weighted tubes and evaporated in a dry bath at 60 °C overnight. The resulting dried residue was weighed, and total lipids calculated using the following equation (2):

$$(2)\ \text{Total Lipids}\ (\%) = \frac{\dfrac{(\text{Final weight} - \text{Initial weight}) \times \text{Volume total chloroform}}{\text{Volume evaporated chloroform}}}{\text{Weight dried sample}} \times 100$$

Ash content was determined by incinerating the dried biomass placed in crucibles for 8 hours in a muffle furnace at 525 °C. After incineration, the resulting residue was weighed, and the ash content calculated using the formula (3):

$$(3)\ \text{Ashes}\ (\%) = \frac{\text{Weight capsule with ashes} - \text{Weight capsule}}{\text{Weight dried biomass}} \times 100$$

Carbohydrates were determined by subtraction following the equation (4):

$$(4)\ \text{Carbohydrates}\ (\%) = 100\ \% - (\text{proteins} + \text{total lipids} + \text{ashes})$$

The mineral profile was determined by microwave-induced plasma atomic emission spectrometry (Agilent Technologies 4200 MP-AES, USA) after acid digestion using an automated microwave digestor (Discover SP-D 80, CEM). Briefly, an aliquot of dried biomass was placed in quartz tubes followed by addition of 68% nitric acid and mili-Q water to a final volume of 15 mL. After, the biomass was digested under high temperature that increased for 4 min until Tmax at 200 °C for 3 min, and high constant pressure of 145 psi. The conditions for the elemental analysis were set as follows: construction of calibration curves with coefficient limit of 0.8 per each analyzed element using standard stock solutions, pump speed 15 rpm, 15 s stabilization time, 55 s samples uptake time, rinse time of 50 s, and 4 replicates. The intensity of the following elements were determined and plotted with respective calibration curves: Ag, Al, As, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, Pb, Sb, Se, Sn, Tl, V and Zn.

3.6. Microscopy

Microscopic images were acquired in a Zeiss AXIOMAGER Z2 microscope, with a coollSNApHQ2 camera and AxioVision software version 4.8 (Carl Zeiss MicroImaging GmbH, Göttingen, Germany), using the 100 × lens. Optical microscopic (Motic BA310, Hong Kong) was used for observations with 40 x lens. All images were not treated.

3.7. Microalgae Isolation

Culture dominance was assessed by flow cytometry in a Becton Dickinson FACS Aria II (BD Biosciences, Belgium) using FACS Diva software (version 6.1.3). The side scatter (SSC) and forward scatter (FSC) were used to measure inner cell complexity and relative cell size, respectively. The chlorophyll fluorescence signal was recorded in the PerCP-Cy5-5-A and APC-A channel (695/40) after excitation with the blue (488 nm) laser. The sorted cells were divided into 96-well plates containing Algal culture medium without sugar or salt sources to select photosynthetic freshwater microalgae. The principles of fluorescent activated cell sorting (FACS) are well documented in (Pereira et al., 2018) (**Fig. 5**).

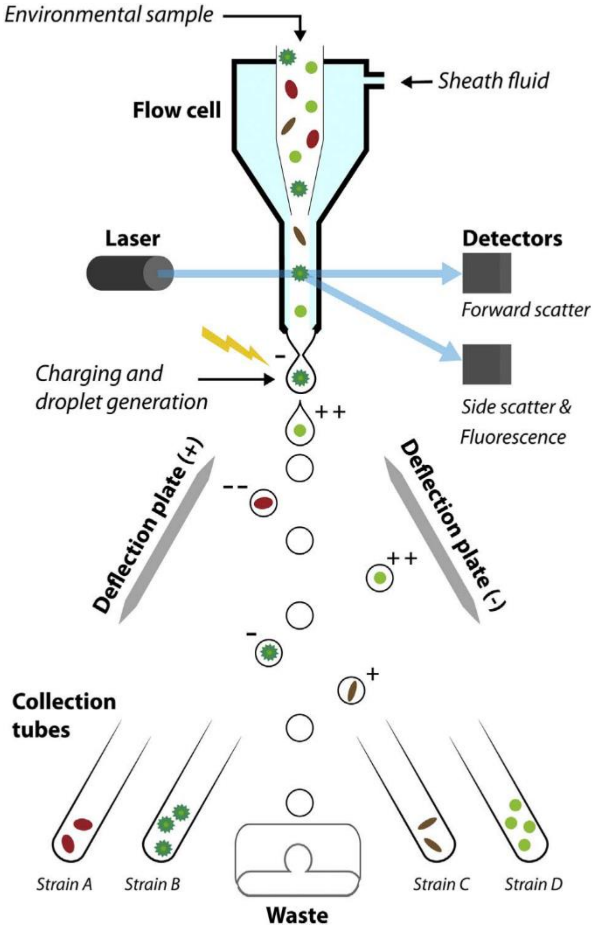

Figure 5 – Fluorescent activated cell sorting scheme (Source: Pereira et al., 2018). Microalgae cells from samples passes through the flow cell in a narrow liquid stream that is divided into droplets that are charged positively or negatively. The cells are directed to different wells in the microplate by deflection according to the electrical charges of droplets.

3.8. Taxonomic Identification

Microalgae strains were identified by means of 18S rDNA sequencing. DNA extraction was performed with the ZYMO Plant Kit (Zymo Research, USA) according to the manufacturer's guidelines. The obtained DNA was amplified by PCR (initial denaturation 5 min at 94 °C; 35 cycles repeating 30s denaturation at 95 °C, 30s annealing at 55 °C and 1 min elongation at 72 °C; final elongation during 10 min at 72 °C and hold step at 10 °C) with the primers 18SUnivFor (5′-ACCTGGTTGATCCTGCCAGT-3′) and 18SUnivRev (5′-TCAGCCTTGCGACCATAC-3′) as described in Pereira et al. (2016). Each sequence was compared with GenBank database using BLASTn available at NCBI (https://blast.ncbi.nlm.nih.gov). The obtained sequences were aligned and visualized using the editor CLC Sequence Viewer (v. 7.6.1, Quiagen). The curation of the alignment using Gblocks was performed using standard setups. Phylogeneti analysis was performed using Maximum-likelihood (ML) assuming GTR + I + G substitution model. Probabilities were determined by Likelihood-Ratio Test (aLRT) using PhyMLv .3.0 (http://www.atgc-montpellier.fr/). The final tree was drawn with FigTree v.1.3.1 software (http://tree.bio.ed.ac.uk/software/figtree).

3.9. Statistical Analysis

Statistical analyses were performed using SPSS statistical package for Windows (release 15.0, SPSS Inc.). The parametricity of the data set was assessed by the analysis of normality and homogeneity of variances. Statistical differences were determined through students *t*-Test or Mann-Whitney U Test (removal efficiency of nutrients, $p < 0.05$) and one-way ANOVA followed by Dunnett's multiple comparison test or Kruskal-Wallis Test (biomass composition, $p < 0.05$). Correlations between pH, temperature, cellular concentration or removal efficiency were assessed via two-tailored Pearson's test (r). The data was analyzed individually for each season.

4. Results and Discussion

4.1. Microalgae Isolation and Identification

Microscopical observation of water samples collected from the GDPBRs allowed for the identification of spontaneously growing microalgae belonging mainly to the classes *Cyanophyceae*, *Chlorophyceae*, *Bacillariophyceae*, as well as some predators (**Fig. 6**).

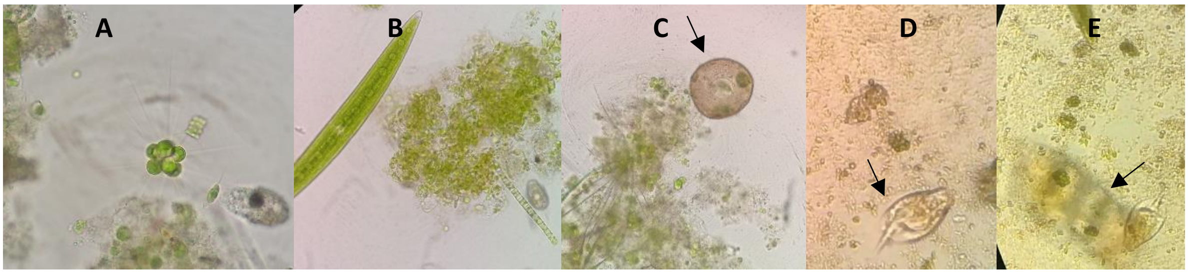

Figure 6 - Optical microscope visualization (40x) of microalgae spontaneous growing in the GDPBRs (**A** and **B**). The arrows highlight predators (**C**, **D** and **E**).

Focusing on photosynthetic organisms, it was possible to isolate some of these strains by flow cytometry coupled with fluorescence activated cell sorting. For this, a first combination of the side scatter (indicating the level of cellular inner complexity) and fluorescence at 695 nm signals (resulting from the presence of chlorophyll pigments) was used to differentiate non-photosynthetic microorganisms and debris from photosynthetic cells (**Fig. 7**).

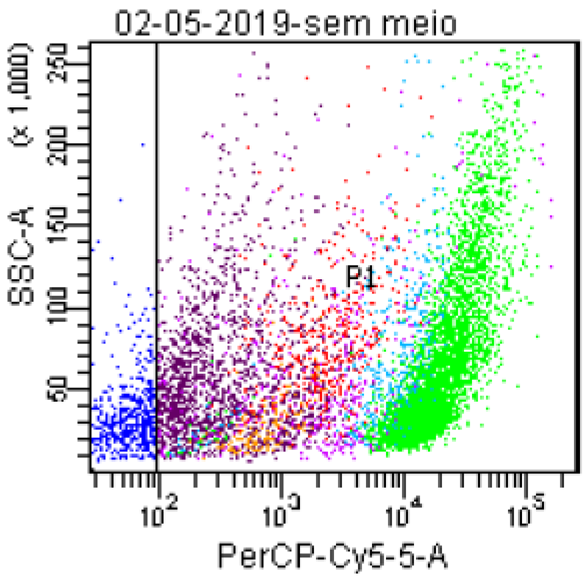

Figure 7 – Side scattering (SSC) combined with fluorescence emission by chlorophyll pigments in a two-dimensional dot plot. The first sorting corresponds to P1 gate defined with more than 100 arbitrary units of chlorophyll autofluorescence to isolate photosynthetic cells.

After the initial gating (P1), several signal combinations were tested to see which would help to better differentiate between populations of autotrophic microorganisms. The best combination was the PE-A channel, related with phycoerythrin-derived autofluorescence of cells, and the APC-A channel associated with phycocyanin-derived autofluorescence (Pereira et al., 2011). That way, five gates (P2, P3, P4, P5 and P6) were set in the two-dimensional plot which could correspond to five different populations (**Fig. 8**).

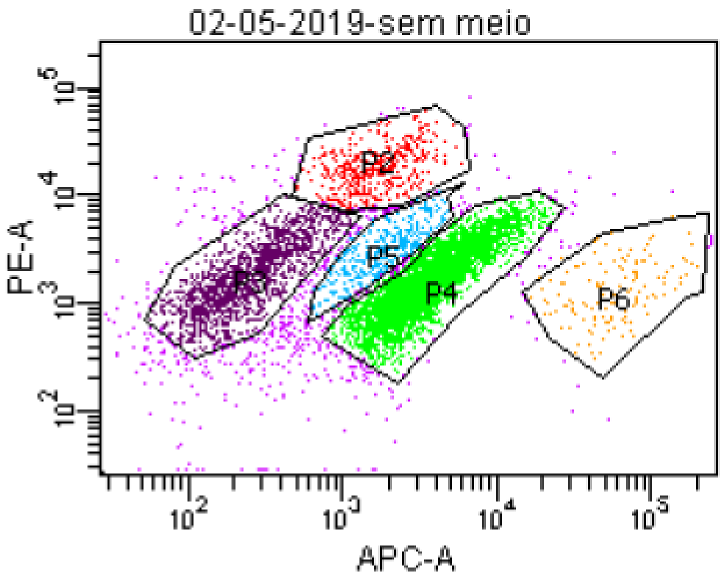

Figure 8 – Combination of fluorescence emission by PE-A and APC-A channels. Five clusters P2, P3, P4, P5 and P6 were drawn.

Cells from the clusters P2, P3, P4, P5 and P6 were sorted into 96-well plates containing algal medium (**Fig. 9 I**). Microscopical observations showed that isolates were mainly from the class *Chlorophyceae* (P4 cluster) (**Fig. 9 II and III**). Although observed under the microscope, it was not possible to isolate cells from the classes *Cyanophyceae* or *Bacillariophyceae* which could be related with the fact that the culture medium was not suitable for the growth of these classes.

The isolated monoalgal colonies were grown and maintained under axenic conditions. Therefore, approximately 25 mg of wet biomass was withdrawn for identification through 18S rDNA. Sequences were analyzed by Maximum Likelihood (ML) inference and phylogenetic trees were constructed. The sequence labeled as "clone C02" presented a probability of 0.86 of being from the specie *Desmodesmus abundans* (**Fig. 10**) and was therefore classified as *Desmodesmus abundans* GTM11 (**Fig. 9A**). The sequence labeled as clone E03 was classified as belonging to the genus *Scenedesmus* with a probability of 0.86 (**Fig. 11**) and further classified as the strain *Scenedesmus* sp. GTM2 (**Fig. 9B**). The sequence labeled as clone F02 belongs to the genus *Chlorella* with a posterior probability of 0.92 (**Fig. 12**); in the same way, two other isolates were classified as *Chlorella* sp. GTM4 and *Chlorella* sp. GTM5 (**Fig. 9C** and **Fig. 9D**).

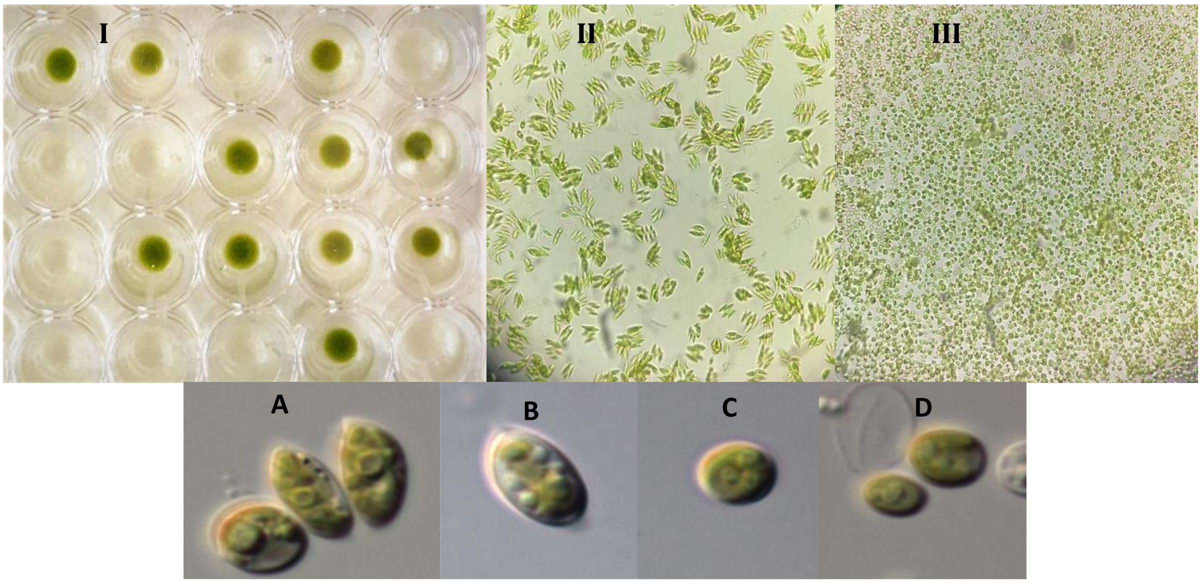

Figure 9 – Cells sorted into 96-well plates (**I**). Microscope observations (40x) from genera *Scenedesmus* (**II**) and *Chlorella* (**III**) isolated from the pilot PBRs. Microscope observations (100x) and identification of isolated strains: **A** – *Desmodesmus abundans* GTM11; **B** - *Scenedesmus* sp. GTM2; **C** - *Chlorella* sp. GTM4; **D** – *Chlorella* sp. GTM 5.

The isolated and identified strains from the genera *Chlorella* and *Scenedesmus* are the most commonly used microalgae in wastewater treatments due to their capability to grow and tolerate adverse environmental conditions, which is in agreement with cited literature (Zhou et al., 2012). The isolated strains from the effluent were maintained in optimal culture conditions, so that they can be used in future studies with wastewater treatment.

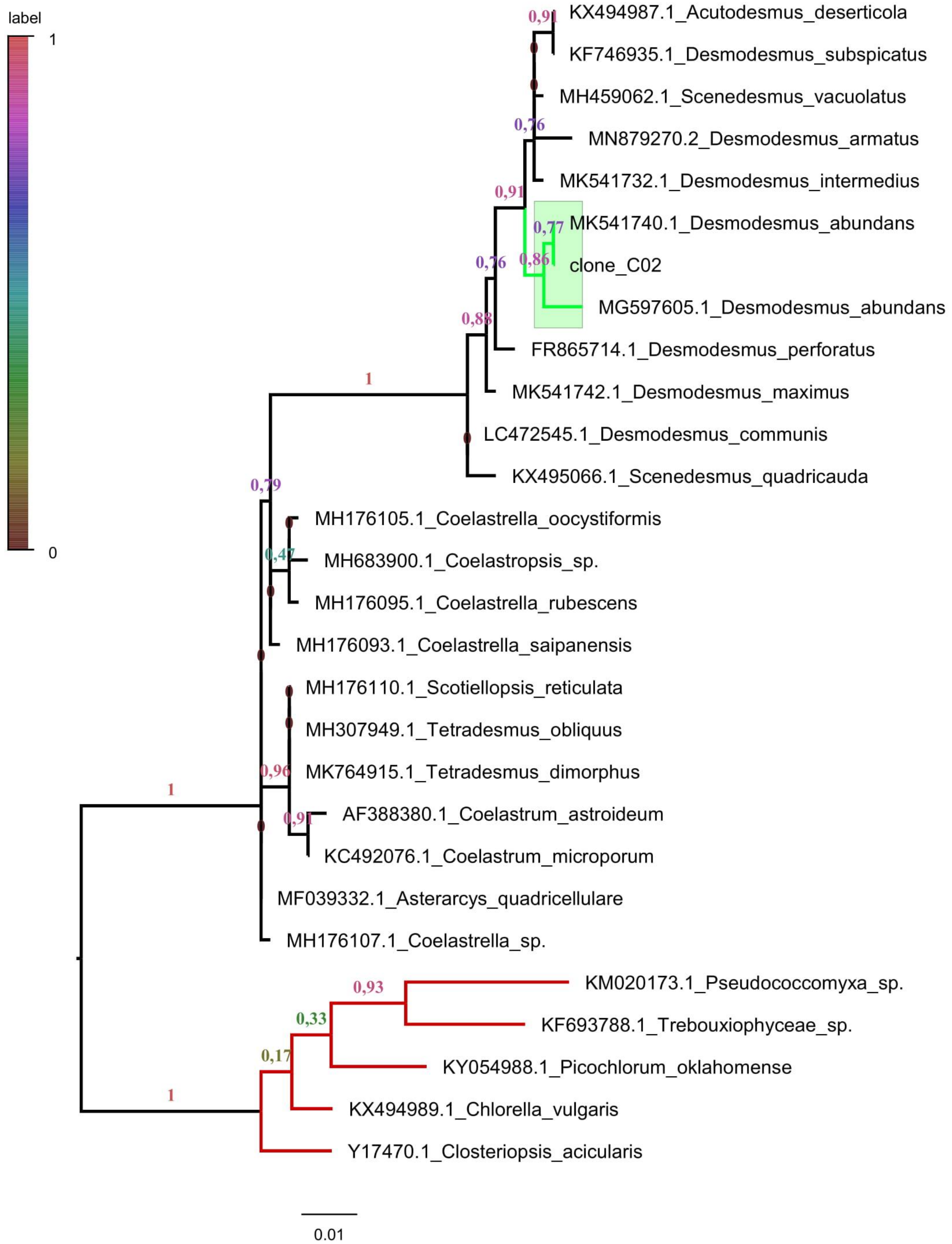

Figure 10 – Maximum-likelihood inference using 18S rDNA. Probabilities are indicated at the branches. The outgroup is represented by the red branches. The analyzed sequence C02 is indicated with green.

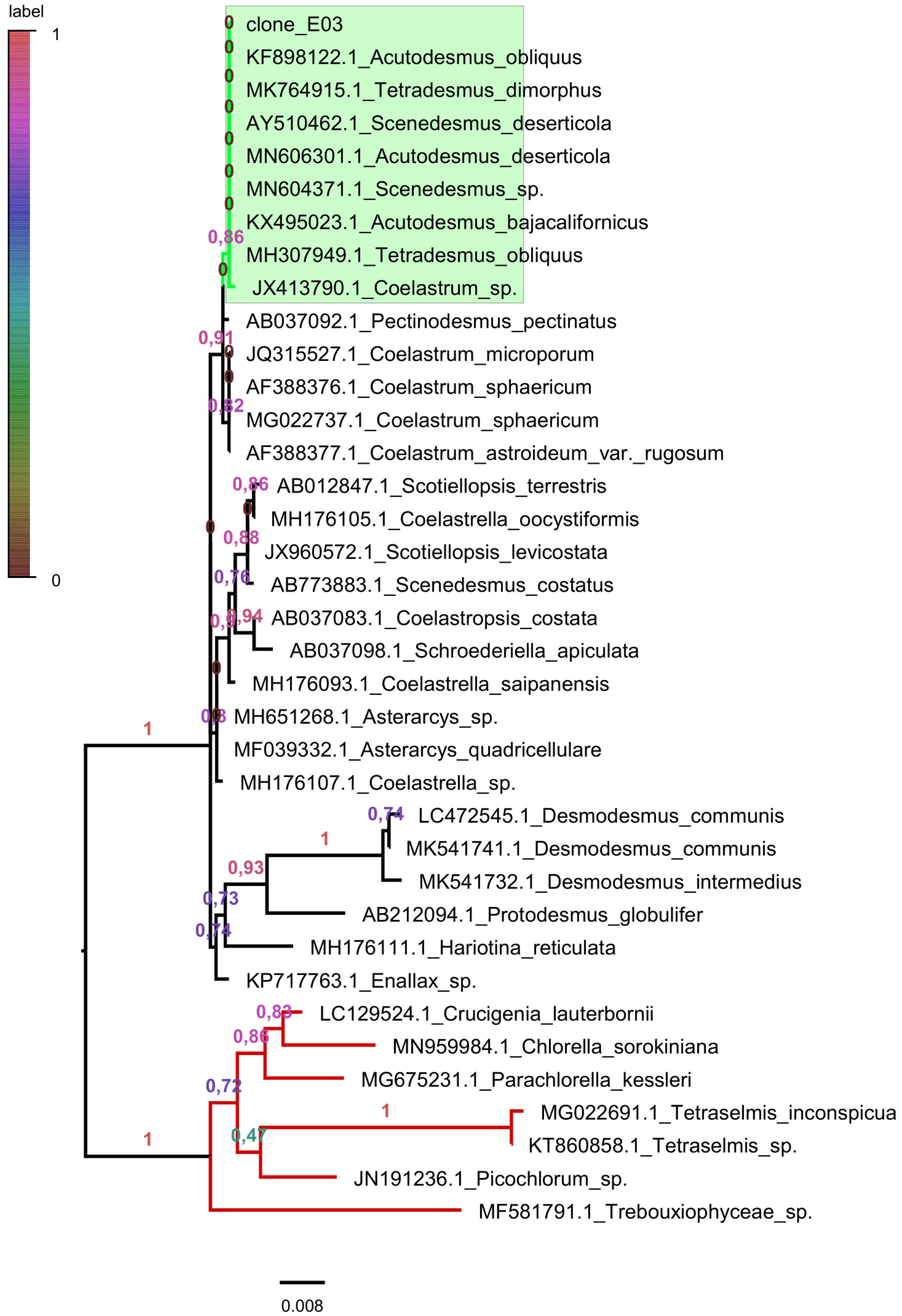

Figure 11 - Maximum-likelihood inference using 18S rDNA. Probabilities are indicated at the branches. The outgroup is represented by the red branches. The analyzed sequence E03 is indicated with green.

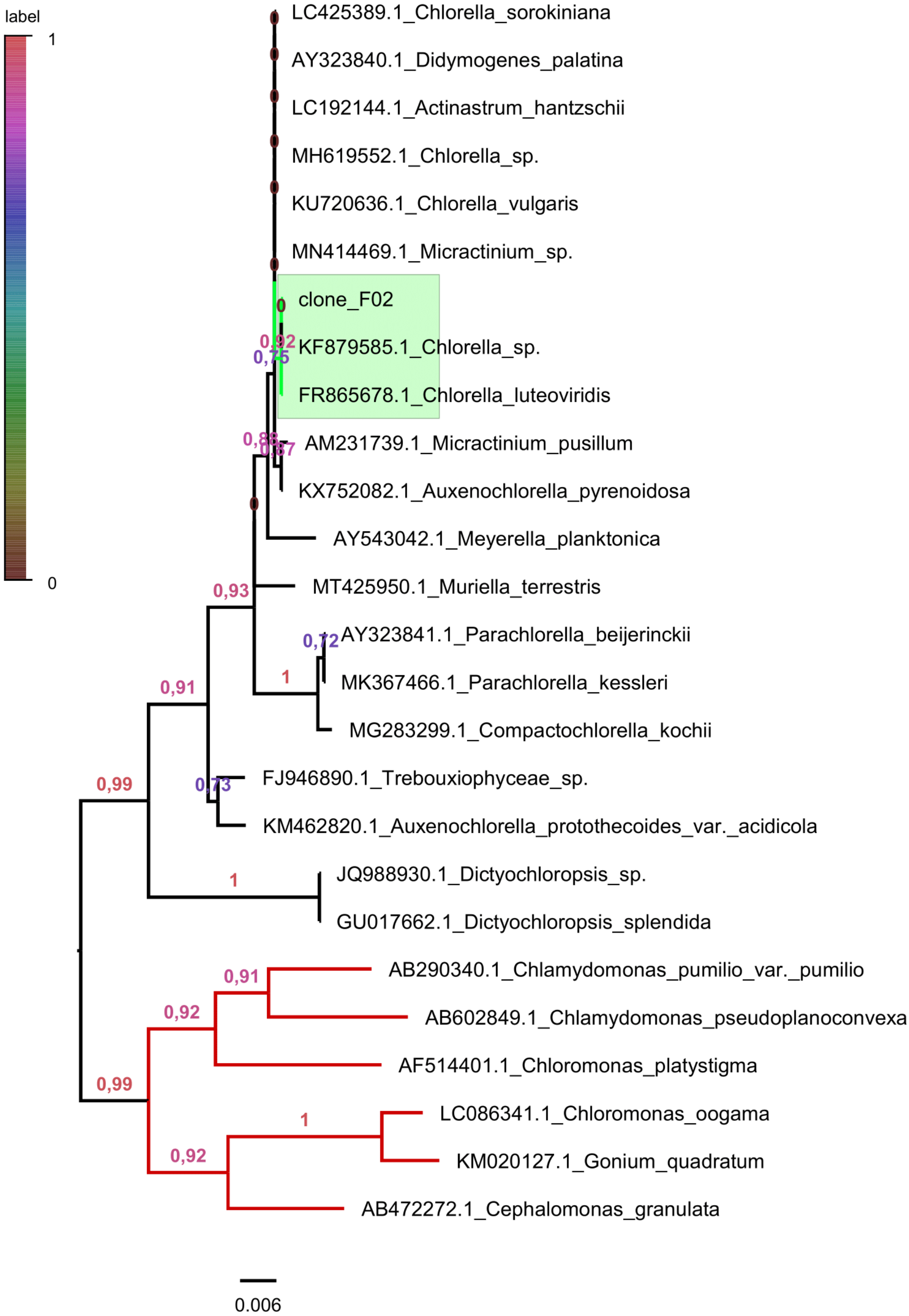

Figure 12 - Maximum-likelihood inference using 18S rDNA. Probabilities are indicated at the branches. The outgroup is represented by the red branches. The analyzed sequence F02 is indicated with green.

4.2. Treatment efficiency – seasonal effect

4.2.1. *Variation of pH*

The pH is one of the most important parameters for microalgae growth regulation, since it is influenced by inorganic carbon, where the most common forms are CO_2, HCO_3^- and CO_3^{2-} (Qiu et al., 2017). This inorganic carbon form is consumed by microalgae, thus increasing the pH in the system during photosynthesis. The results for the Autumn with HRT of 2d (A2), Winter with HRT of 2d (W2) and Winter with HRT of 1.12d (W1.12) experiments are shown along 30, 18, and 16 consecutive days, respectively. Although the pH changed during the experiments, but no significant variations of pH were observed between seasons ($p > 0.05$).

In experiment A2 the pH was, in average, 9.70 ± 0.73 and varied between 8.14 and 11 (**Fig. 13**). All lines showed similar trends with a decrease at t = 5 and t = 16d. The natural process in photobioreactors involves the consumption of inorganic carbon by microalgae, which causes the increase of the pH, while cells grow and multiply. If inorganic carbon is not consumed, then cell multiplication decreases, as well as pH. Accordingly, it was observed that pH oscillated in all lines for the 30 days of experiment, which indicated that microalgal photosynthetic activity may have been the cause for the variation in pH and possibly in cellular concentrations. In outdoor conditions, external factors such as sunlight irradiance or temperature are key factors in microalgae activity. Phototrophs are submitted to different light intensities or photoperiods and temperatures. High irradiance in sunny days or low irradiances due to shading by clouds leads to higher or lower photosynthetic activities, and subsequently, pH either increases or decreases, respectively. It was observed a decrease in pH from t = 20 that could have been induced by a reduction of daily light exposure (lower photoperiods). Another factor that may have contributed for pH oscillations was temperature that determined photosynthesis and consumption of inorganic carbon.

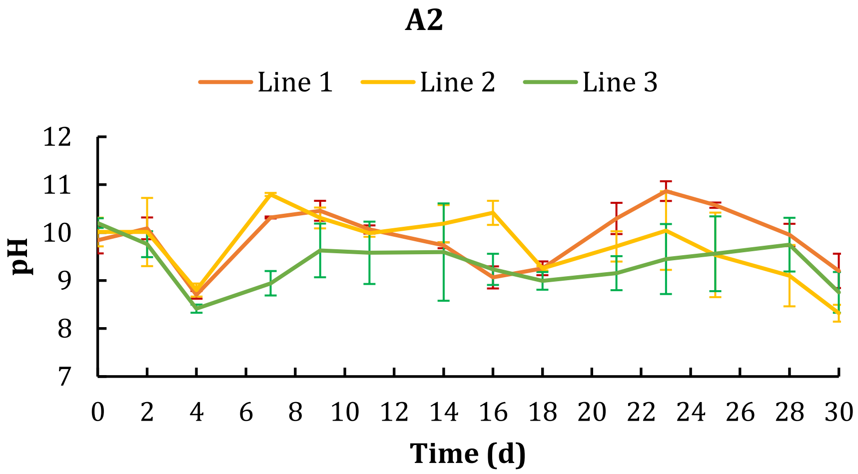

Figure 13 – Analysis of pH for line 1, line 2 and line 3 during the 30 days of the experiment in Autumn with an HRT of 2 days. The values for each line are represented as average values from three photobioreactors (mean ± SD).

The mean pH for W2 was 9.73 ± 0.98 varying between 7.7 and 10.99 (**Fig. 14**). Among lines in W2 there were similar trends. The range values did not show major differences compared to A2. Although, in the W2 experiment at t = 0 it is observed a constant increase until t = 10, then reaching stable values. The constant consumption of inorganic carbon during photosynthetic activity of microalgae may have been the factor responsible for this variation. Possibly, the reason for increased activity was due to increased sunlight irradiances along with temperature.

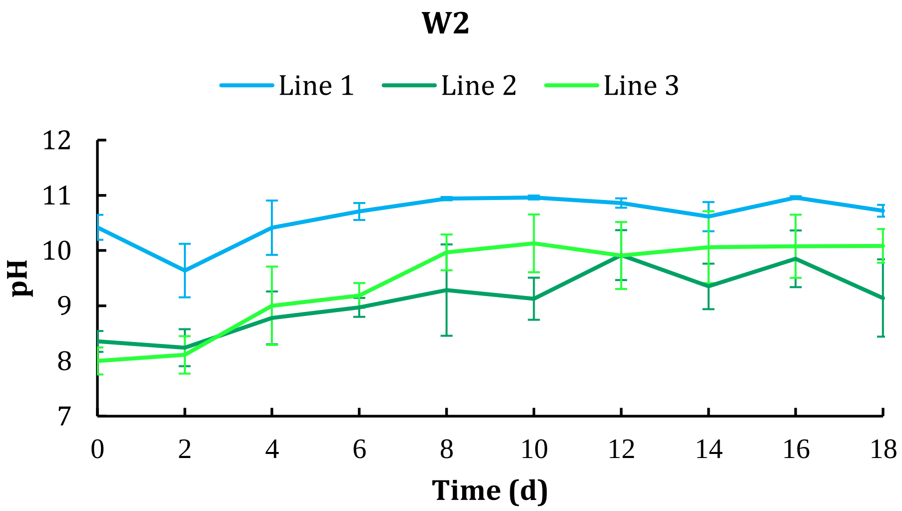

Figure 14 – Analysis of pH parameter for line 1, line 2 and line 3 during 18 days of experiment in Winter with an HRT of 2 days. The values for each line are represented as average values from three photobioreactors (mean ± SD).

In the W1.12 experiment the observed pH was 9.58 ± 1.08 varying between 7.7 and 1.07 (**Fig. 15**). These results were similar to the values in W2. However, contrary to W2, the tendency in W1.12 showed a stable pH at the beginning until t = 7 followed by a decrease until t = 16. This constant decrease could be related to lower photosynthetic activities leading to lower cellular concentrations in photobioreactors. On one hand, the observed results may be associated with lower light irradiances or registered temperatures. On the other hand, the HRT with 1.12d means greater water flows through the system, which consequently can originate washout condition which possibly means that microalgae exited the system quicker than its multiplication capacity. The washout may have led to lower inorganic carbon consumptions and subsequently a pH decrease.

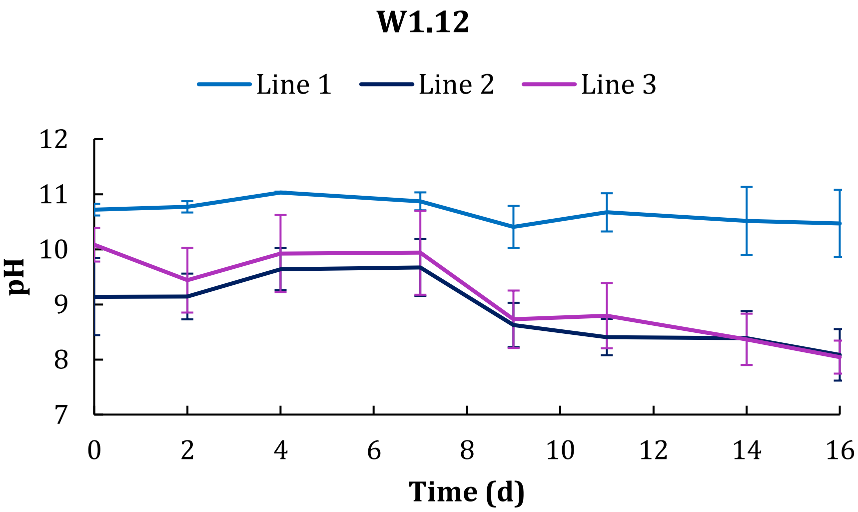

Figure 15 – Analysis of pH parameter for line 1, line 2 and line 3 during 16 days of experiment in Winter with an HRT of 1.12 days. The values for each line are represented as average values from three sequential photobioreactors (mean ± SD).

4.2.2. *Temperature*

Temperature varies with daily light as well as irradiance intensity that changes between seasons. It is a crucial factor that controls microalgae metabolic activity, enhancing photosynthesis and cellular division (Ras et al., 2013). In general, the optimal temperature that yields maximum growth rates for microalgae cultures in outdoor conditions ranges between 20 to 35°C (Gupta et al., 2019). For A2, W2, and W1.12 the mean temperatures were 24.81± 3.13°C, 20.48 ± 2.66°C, and 21.58 ± 1.34°C, respectively (**Fig. 16, 17 and 19**, respectively).

The data presented for the Autumn with HRT of 2 d (A2), Winter with HRT of 2d (W2) and Winter with HRT of 1.12d (W1.12) experiments are shown along 30, 18, and 16 consecutive days, respectively.

The A2 experiment had the highest temperature variation between 16.5 and 30.5°C and registered the maximum value, since the light intensity was also higher during Autumn in relation to Winter (**Fig. 16**). The temperature decreased during the 30 days of experiment. It is observed that temperature measured in the reactors follows the tendency of air temperature. Despite of the indication of the effect of temperature in the photosynthetic activity and subsequentially in pH, the statistical data did not show significant correlations between pH and temperature (r = 0.093, n = 12 p > 0.05).

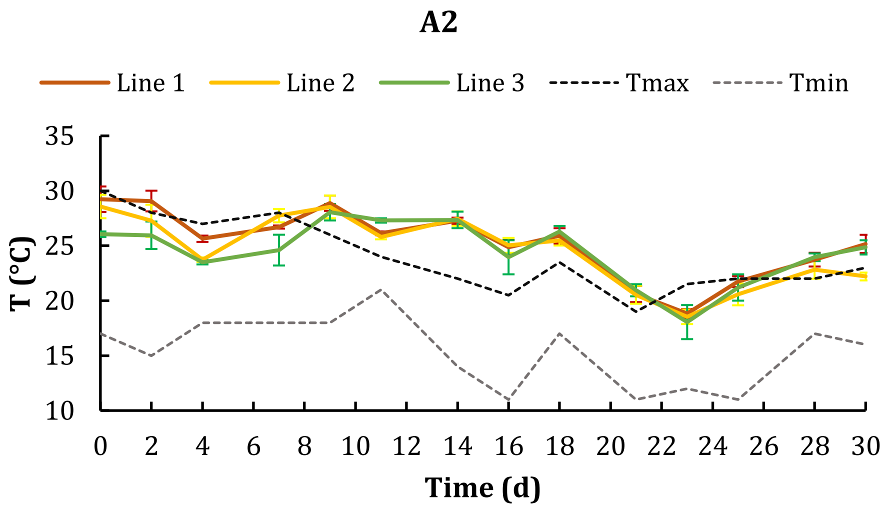

Figure 16 – Analysis of temperature for line 1, line 2 and line 3 during 30 days of experiment in Autumn with an HRT of 2 days. The values for each line are represented as average values from three sequential photobioreactors (mean ± SD). Tmax and Tmin corresponds to air

The temperature increased during the experiment at W2, whose similar tendency is also observed in pH (W2), registering oscillations between 15.2 and 28.0°C (**Fig. 17**). The lowest temperature was observed in W2 as it was early winter with lower irradiance. Lower temperature and irradiance decrease photosynthesis by microalgae. Correlation analysis showed a strong positive relation between the temperature and pH (r = 0.896, n = 8, p < 0.01), indicating a constant increase of temperature during W2, as well as inorganic carbon consumption by photosynthetic activity, which was observed by the increased pH.

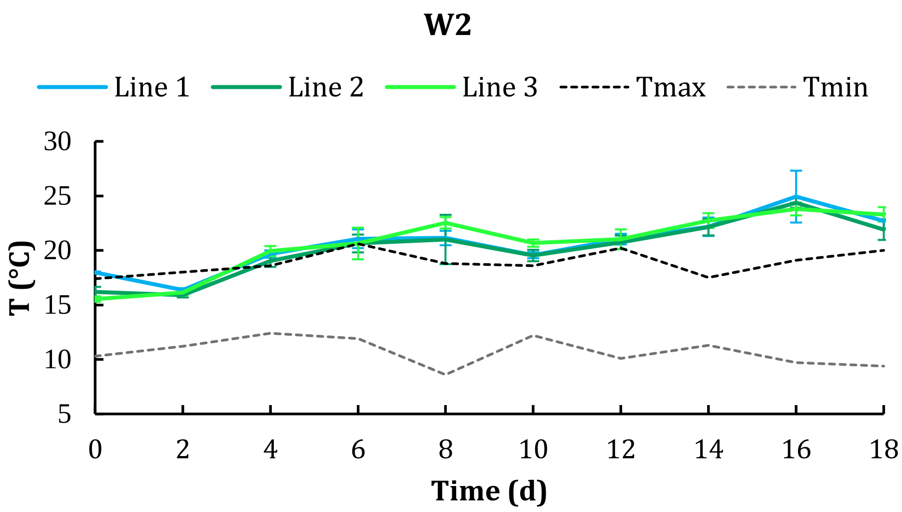

Figure 17 – Analysis of temperature for line 1, line 2 and line 3 during 18 days of experiment in Winter with an HRT of 2 days. The values for each line are represented as average values from three sequential photobioreactors (mean ± SD). Tmax and Tmin corresponds to air temperature between 27th of January, 2020 to 17th of February, 2020 (IPMA, 2020).

The temperature fluctuations at W1.12 were between 18.0 and 24.0°C (**Fig. 18**). The minimum temperature is higher than the one in W2 since it was late winter. The pattern of the curve also resembles the one showed in pH (W1.12). Data analysis showed a strong and positive correlation between temperature and pH (r = 0.913, n = 8, p < 0.01). The environmental temperature showed constant maximum values, but the minimal temperature decreased, which could have been the cause for temperature decrease inside the photobioreactors (**Fig. 18**). Another possibility could be sunlight irradiance. Lower temperature and light affect photosynthetic activities, decreasing nutrients consumption and cellular concentration, and thereafter, a decrease in pH of the culture.

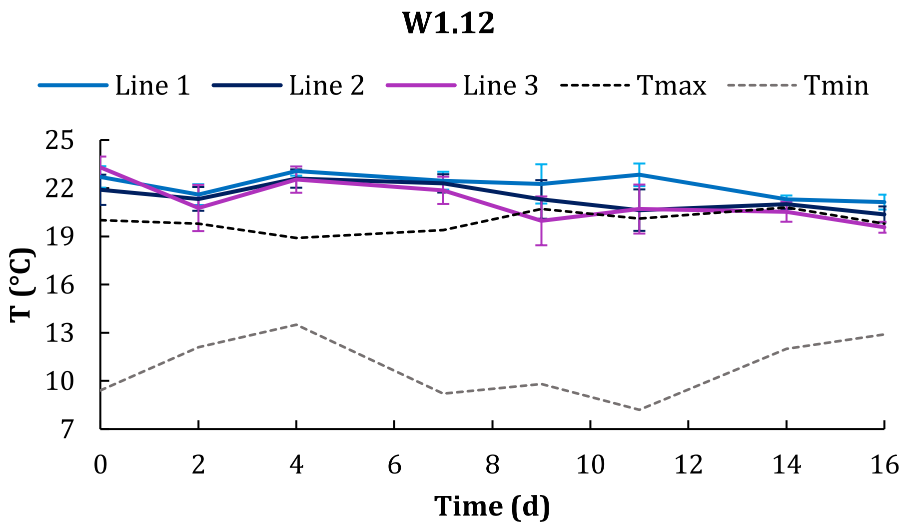

Figure 18 – Analysis of temperature for line 1, line 2 and line 3 during 16 days of experiment in Winter with an HRT of 1.12 days. The values for each line are represented as average values from three sequential photobioreactors (mean ± SD). Tmax and Tmin corresponds to air temperature between 17th of February, 2020 to 4th of March, 2020 (IPMA, 2020).

The temperature showed significant differences between the autumn and winter experiments ($p < 0.05$). However, the values were within the optimal range of temperature and occasionally the lowest optimal temperature was exceeded in Winter. Yet, both *Chlorella* and *Scenedesmus* strains found in photobioreactors are able to operate in a wide range of temperatures (15-30°C and 10-30°C, respectively) (González-Camejo et al., 2019; Xin et al., 2011). That way, the Algarve (Portugal) proved to have proper cultivation conditions even in the coldest season, which makes this Mediterranean region (37°01’09” N) suitable to future investments using microalgae.

4.2.3. *Biomass productivity*

Biomass concentration is a key variable as it is indicative of microalgal growth and biomass production rates. It is highly affected by culture conditions, such as light exposure, temperature, pH and nutrients load.

In the A2 experiment, the mean biomass concentration obtained was 0.069 ± 0.066 gL^{-1}. The biomass concentration during A2 fluctuated between 0 and 0.31 gL^{-1}, presenting peak concentrations at t = 7 and t = 18 (**Fig. 19**). Correlation analysis did not show significant correlation between cellular concentration and temperature ($r = 0.265$, n =11, $p > 0.05$), or pH ($r = 0.320$, n = 11, $p > 0.05$).

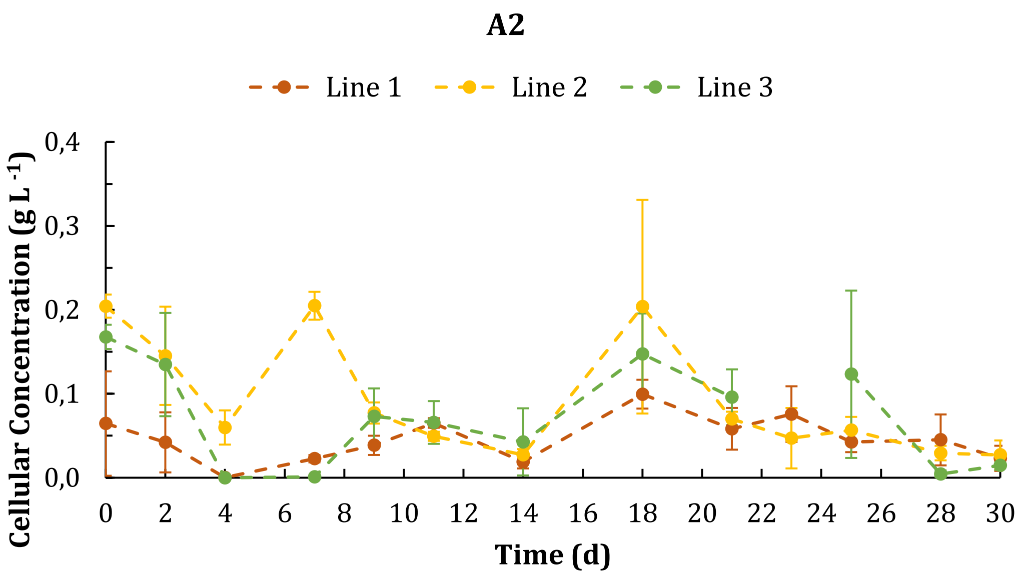

Figure 19 – Mean cellular concentration (gL^{-1}) ± SD (error bars) for 30 days in Autumn with HRT of 2 days for line 1, line 2, and line3. The values for each line are represented as average values from three sequential photobioreactors (mean ± SD).

The W2 experiment presented an average biomass concentration of 0.035 ± 0.057 g L^{-1}, varying between 0 and 0.27 g L^{-1} (**Fig. 20**). The biomass concentration increased continuously during the 18 days of the experiment and showed a positive significant correlation with pH (r = 0.774, n = 7, $p < 0.05$) and temperature (r = 0.791, n = 7, $p < 0.05$). The line 1 exhibited the highest concentrations compared to line 2 and line 3.

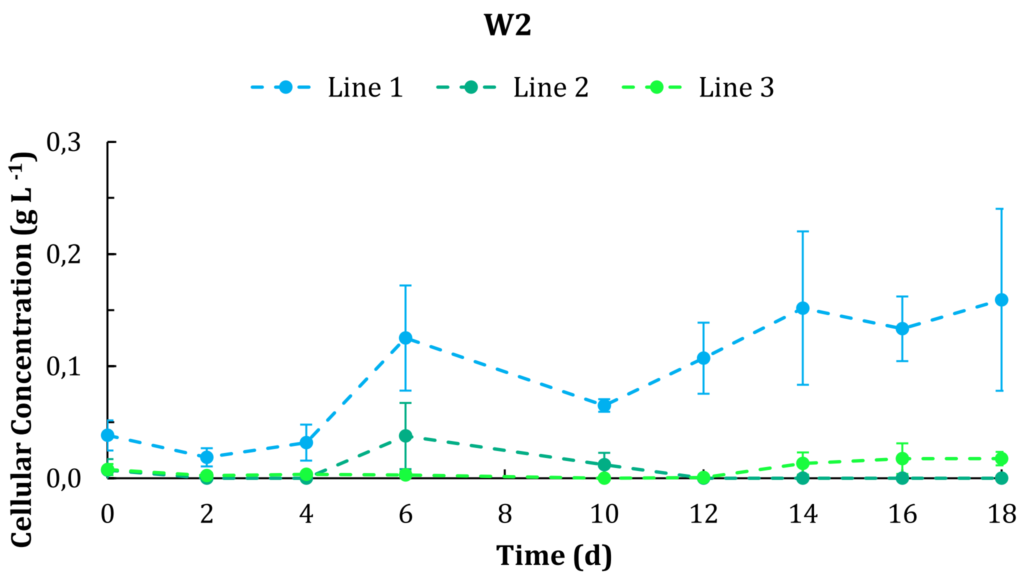

Figure 20 – Mean cellular concentration (g L^{-1}) ± SD (error bars) for 18 days in Winter with HRT of 2 days for line 1, line 2, and line3. The values for each line are represented as average values from three sequential photobioreactors (mean ± SD).

The highest registered values were observed at W1.12 experiment. The biomass concentration averaged 1.64 ± 2.23 gL^{-1} with variations between 0 and 10.12 gL^{-1} (**Fig. 21**). It was observed at t = 9 for line 2 and line 3, that the biomass concentration peaked. This parameter did not significantly correlate with pH (r = -0.638, n = 6, $p > 0.05$) and temperature (r = -0.647, n = 6, $p > 0.05$). However, the results were opposite to the observed in A2 and W2 experiments; in the experiment W1.12 higher cellular concentrations coincided with lower pH values. Also, biomass concentration should have increased because of cellular activity as well.

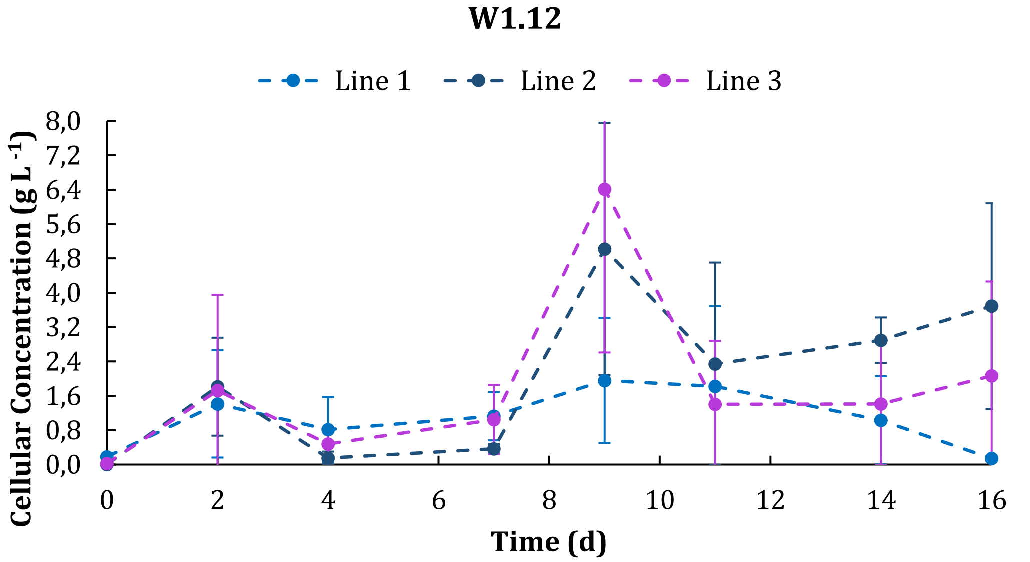

Figure 21 – During 16 days in Winter with HRT of 1.12 days, the mean cellular concentration (g L^{-1}) ± SD (error bars) were analyzed for line 1, line 2, and line3. The values for each line are represented as average values from three sequential photobioreactors (mean ± SD).

Statistical analysis showed that cellular concentrations varied significantly between experiments ($p < 0.05$). The experiment W2 registered the lowest productivity among A2 and W1.12 which was correlated with the lowest temperatures in winter. Discrepant results were observed in W1.12, in which higher amounts of biomass were observed. The increase of the influent flux supplied the system with higher amounts of nutrients. Thereafter, the increased biomass concentration in the experiment W1.12 could be explained by the increased nutrients concentration and availability when compared to A2 and W2. Such discrepancy in biomass concentration could be also explained by the methodological approach. On the water samples collected in the GDPBRs, the concentration was measured by the absorbance at 750 nm which is related with the amount of suspended material. The concentration between lines were similar in A2, line 1 presented higher cell concentrations in W2, and both line 2 and 3 were similar in W1.12. This could have indicated that the culture was dominated by different microorganisms either suspended in the medium and/or aggregated forming biofilms. The difference in productivities from A2 and W2 to W1.12 could be justified by the increase of turbulence and mixing rates inside GDPBR by changing the HRT from 2d to 1.12d, respectively, because it was observed less biofilms in photobioreactors (data not shown). The presence of higher amounts of suspended particles from previous formed biofilms were detected by absorbance at 750 nm and led to higher cellular concentration.

4.3. Nutrients removal from secondary wastewater

4.3.1. Total Suspended Solids

Total suspended solids (TSS) are non-dissolved particles present in waters that decreases water quality or purity. The analysis of TSS was only performed in the winter season. The concentration of TSS in W2 averaged 8.42 ± 4.85 mg L^{-1} and 48.69 ± 19.51 mg L^{-1} for E and T, respectively. Data analysis showed solids formation for 18 days (**Fig. 22**). These results demonstrated that the difference of solids between E and T were statistically significant ($p < 0.05$). The amount of solids did not significantly correlate with cellular concentration (r = -0.430, n = 7, $p > 0.05$), pH (r = 0.045, n = 7, $p > 0.05$) or temperature (r = -0.139, n = 7, $p > 0.05$). The legal limit for TSS in discharged wastewater in the Portuguese legislation (Decreto Lei 236-98, 1 de Agosto 1998) is 60 mg L^{-1}. However, the Environmental Portuguese Agency (APA) emitted a license certificate for this wastewater treatment plant (User title nº: L012942.2018.RH8, 1 de Agosto de 2018) of TSS of 35 mg L^{-1}. Overall, the concentration of TSS was above this limit.

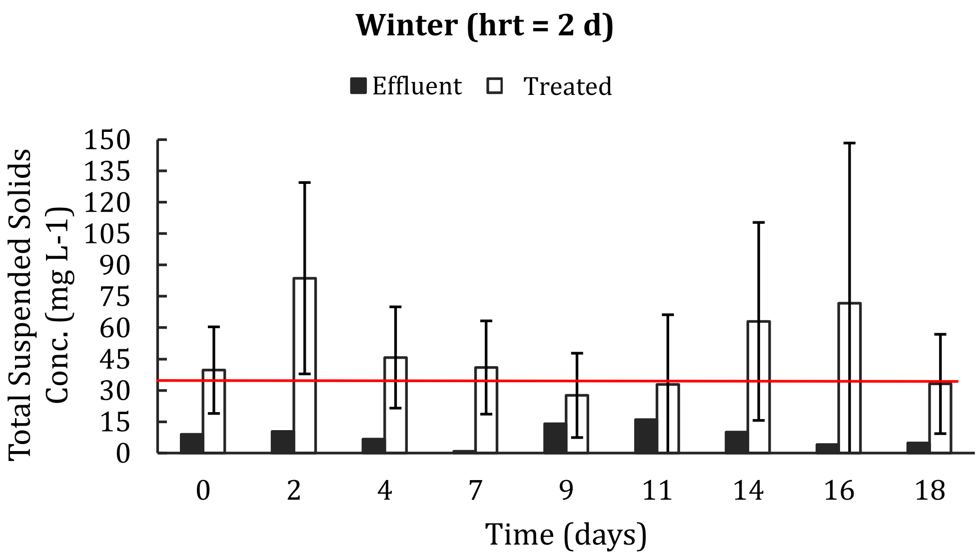

Figure 22 – Winter experiment with HRT of 2 days. Total suspended solids concentration (mg L^{-1}) ± SD (error bars) were analyzed for 18 days. The legislation limit for wastewater discharge is represented by the red line. The treated water was analyzed in triplicate.

The experiment W1.12 showed average concentrations for E and T of 6.71 ± 3.10 mg L^{-1} and 78.39 ± 32.10 mg L^{-1}, respectively. These results obtained in W1.12 were similar to W2 (**Fig. 23**). The statistical analysis showed significant differences between solids in E and T (p<0.05). Again, the increased solids were not significantly correlated with cellular concentration (r = -0.021, n = 6, p > 0.05), pH (r = 0.029, n = 6, p > 0.05) or temperature (r = 0.322, n = 6, p > 0.05). The concentration limit for discharging wastewaters for TSS was above the licensed limit of 35 mg L^{-1} .

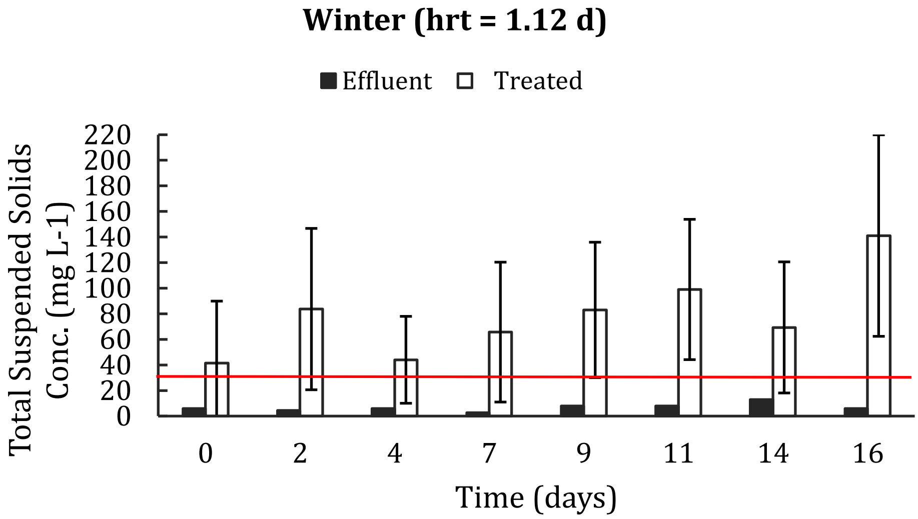

Figure 23 – Winter experiment with HRT of 1.12 days. Analysis of the total suspended solids concentration (mg L^{-1}) ± SD (error bars) were carried for 16 days. The legislation limit for wastewater discharge is represented by the red line. The treated water was analyzed in triplicate.

Treated water in both W2 and W1.12 experiments presented higher concentrations of solids compared to the influent (E). As shown in **Fig. 24**, the treated wastewater was collected from the bottom of the second settler where solid particles and/or microalgal biomass can still have accumulated. Although, the water samples are collected after filtration (F) this is probably was not efficient to remove the solids since high loads of microalgal biomass were also accumulated in the filter. Consequently, it was observed that water samples presented a greenish color due to the presence of chlorophyll pigments indicating the presence of microalgal cells that were accounted in the solids. For that reason, the removal of solids must be improved, either to not lose biomass or fulfill legislation limits. However, the limits are legislated for inorganic particles including bacteria, virus, or protozoa that may be pathogenic. On the contrary, no legislation is applicable to the quantification of microalgae as part of solid particles even though, microalgal biomass is a rich source of nutrients (N and P) that presents less environmental risks when compared to pathogens.

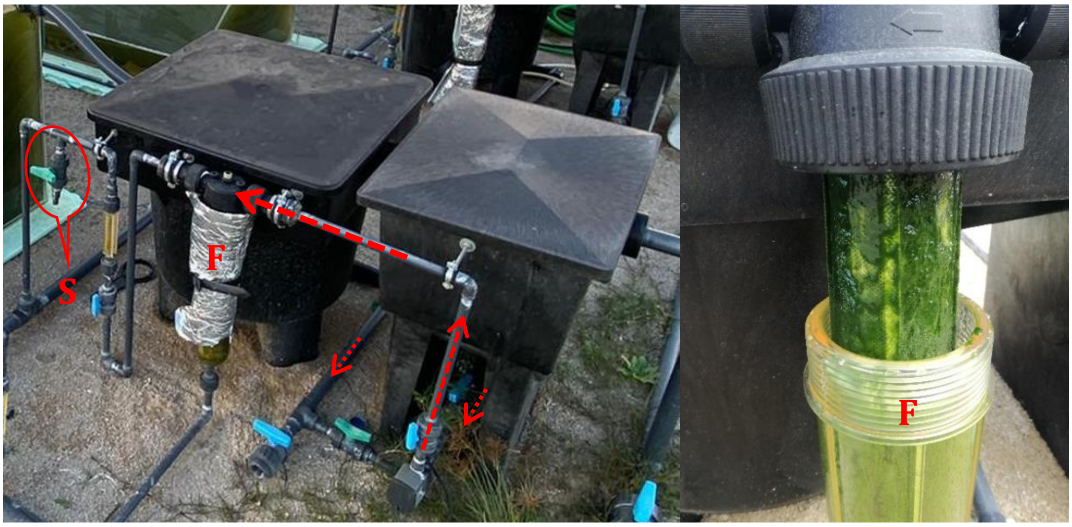

Figure 24 – Representation of settlers where biomass is accumulated (left), while treated water flows outside after filtration (right). Arrows – flux direction; F – filter; S – sampling.

4.3.2. *Total Nitrogen*

Nitrogen is a major nutrient that plays an important role in amino acid synthesis for microalgae growth. Its concentration can be derived by monitoring total nitrogen, that is the sum of ammonia, organic and reduced nitrogen, and nitrate-nitrite. The presence of high concentrations of those compounds in the environment can cause eutrophication in natural waters. For that reason, the treatment of wastewaters is required. Those waters can be used as nitrogen source for microalgae production and, simultaneously, reducing its excess.

TN analysis were performed only in the Winter experiment. The average concentration of TN in W2 experiment for the influent at the entrance of the GDPBR (E) and in the treated water (T) was 21.98 ± 5.04 mg N L^{-1} and 12.69 ± 3.36 mg N L^{-1}, respectively. Throughout the experiment, the removal rate (RE) was, in average, 41.97 % and oscillated between 26.50 and 54.60 %. In this experiment, the concentration of TN in the influent constantly increased until t = 18 (**Fig. 25**). Statistical analysis showed significant differences between TN at E and T ($p < 0.05$). The correlation was not significant between RE and pH ($r = 0.497$, n = 7, $p > 0.05$), temperature ($r = 0.340$, n = 7, $p > 0.05$) or biomass concentration ($r = -0.62$, n = 7, $p > 0.05$). The removal efficiency is in agreement with Lee et al. (2015) that showed 35.2-87.8 % removal using *Coelastrum microporum* in diluted sludge, and Lu et al. (2015) that observed 30.06-50.94 % removal of TN from industrial effluent (meat processing plant) using *Chlorella* sp.. The allowed concentration was surpassed occasionally but the average TN concentration in the treated water was not significantly different from the legal limit 15 mg N L^{-1} (Decreto Lei 236-98, 1 de Agosto 1998) or the licensed limit for this plant.

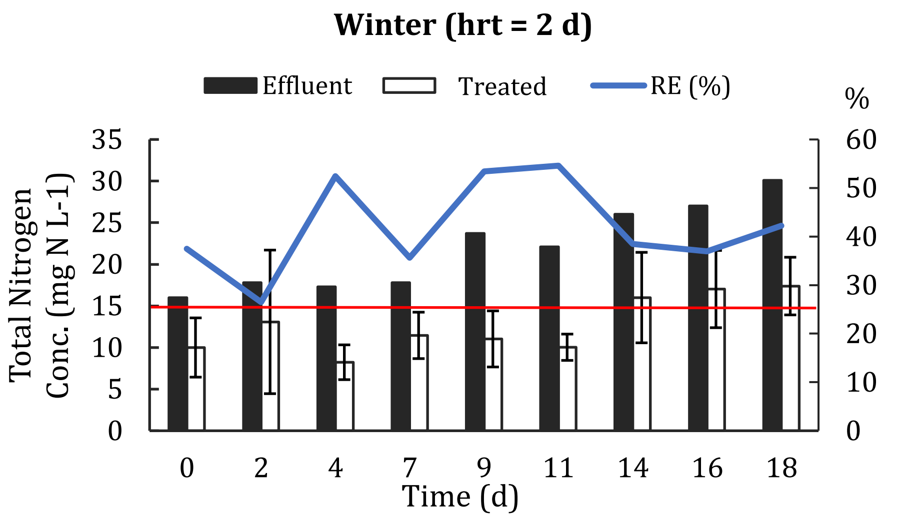

Figure 25 – Total nitrogen concentration (mg N L^{-1}) ± SD (error bars) through 18 days in winter with HRT of 2 days. Blue line represents the percentage of TN removal. Red line shows the discharge limits for wastewater. The treated water was analyzed in triplicate.

In the W1.12 experiment, TN presented an average concentration of 26.90 ± 2.50 mg N L^{-1} and 19.28 ± 2.78 mg N L^{-1} for E and T, respectively. The removal efficiency averaged 27.48 % with a variation of between 5.45 and 50.00 % (**Fig. 26**). There were significant differences between E and T ($p < 0.05$). Statistical data showed positive correlations between RE and pH ($r = 0.792$, n = 6, $p < 0.05$) and temperature ($r = 0.809$, n = 6, $p < 0.05$). RE was not correlated with biomass concentration ($r = -0.648$, n = 6, $p > 0.05$). W1.12 was able to reduce TN to concentrations below admissible discharge limits only until t = 7. After day 7, there was a decrease in the RE and TN was always above legislation limits in the treated water (Decreto Lei 236-98, 1 de Agosto 1998). The treatment with HRT of 1.12d was not able to completely remove nitrogen.

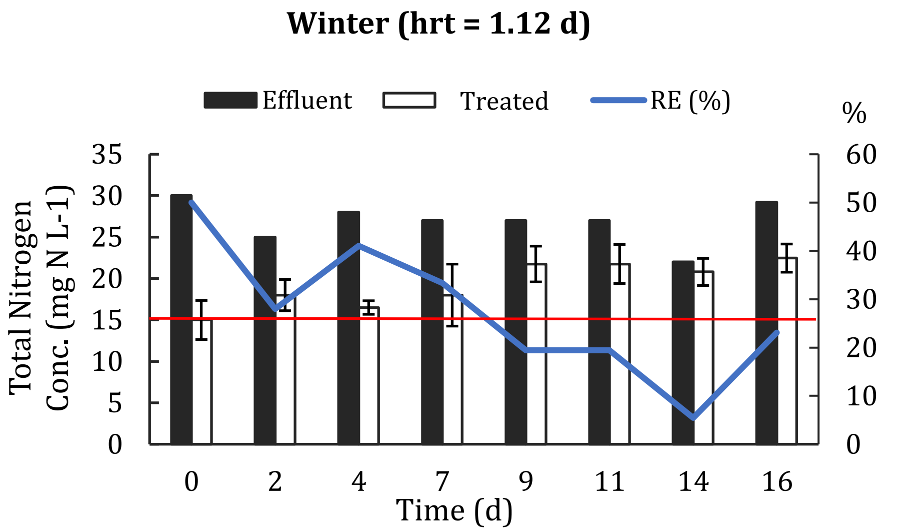

Figure 26 – Winter experiment with HRT of 1.12 days. Total nitrogen concentration (mg N L^{-1}) ± SD (error bars) was analyzed for 16 days. Blue line represents the percentage of TN removal. Red line shows the discharge limits for wastewater. The treated water was analyzed in triplicate.

The results showed elevated concentration of TN in treated waters despite significant differences with E. TN was analyzed in non-filtered samples hence the concentration of TN could have been influenced by the presence of nitrogen in microalgal cells as confirmed by the presence of higher TSS in the treated water caused by a defective sedimentation method.

4.3.3. *Ammonium*

One of the most common nitrogen sources in urban wastewater is ammonium. It is present in natural waters, or in excessive levels in wastewaters. The ammonium nitrogen can be simultaneously present as free ammoniacal nitrogen (NH_3) and ammonium ions (NH_4^+). These compounds can be directly assimilated by the microalgae used to treat effluents (J. Wang et al., 2019).

The A2 experiment registered an average concentration of 17.39 ± 8.83 mg NH_4 L^{-1} in the influent (E) and 8.43 ± 5.19 mg NH_4 L^{-1} in the treated water (T), which resulted in 30.67 % removal rate with a variation that ranged 0 % and 77.57 %. (**Fig. 27**). Higher or lower concentrations of ammonium could be dependent on increased or decreased anthropogenic activities and its seasonality. No significant correlations were observed between RE and pH (r = 0.100, n = 10, p > 0.05), temperature (r = 0.318, n = 10, p > 0.05) or cell concentration (r = 0.022, n = 10, p > 0.05). Statistical analysis showed significant differences between N-NH_4 at E and T (p < 0.05). The ammonium was removed to below admissible discharge limits of 10 mg NH_4 L^{-1} (Decreto Lei 236-98, 1 de Agosto 1998). Occasionally the concentration of ammonium at T surpassed the legal limit concentrations. These results agreed with the 54 % removal of NH_4^+ using *Chlorella vulgaris* to treat diluted piggery effluent (Kumar et al., 2010).

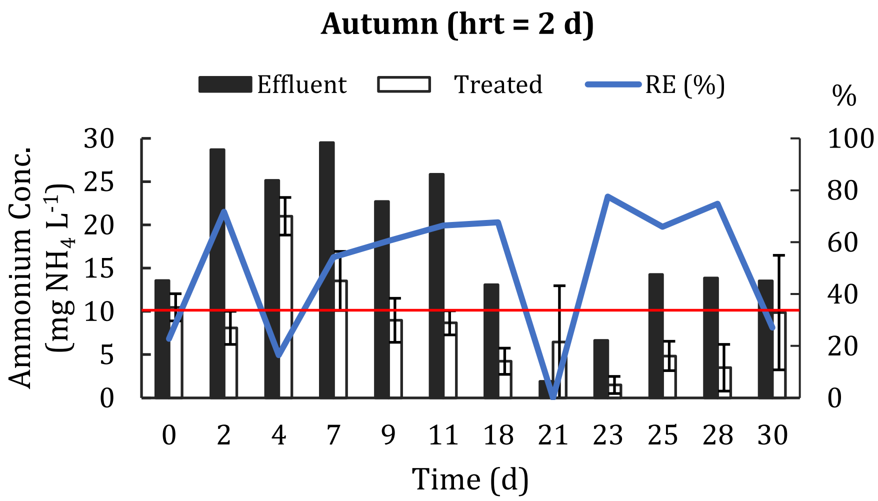

Figure 27 – Ammonium concentration (mg NH_4 L^{-1}) ± SD (error bars) analyzed for 30 days in autumn with HRT of 2 days. Blue line represents the percentage of NH_4 removal. Red line shows the permissible legislation limit for wastewater discharge. The treated water was analyzed in triplicate.

The experiment W2 showed mean concentrations of 12.43 ± 4.14 mg NH_4 L^{-1} and 2.26 ± 0.89 mg NH_4 L^{-1} for E and T, respectively, resulting in a reduction of 81.12 % with oscillation between 67-88.60 % (**Fig. 28**). No significant correlations were observed between RE and pH (r = 0.024, n = 7, $p > 0.05$), temperature (r = 0.003, n = 7, $p > 0.05$) or cell concentration (r = -0.045, n = 7, $p >$ 0.05). Statistical analysis showed significant differences between N-NH_4 at E and T ($p < 0.05$). The ammonium concentration throughout W2 remained below 10 mg NH_4 L^{-1} at T (Decreto Lei 236-98, 1 de Agosto 1998). The pH also increased which could indicate augmented metabolic activity by microalgae. This was reflected in the removal of ammonium keeping the levels below discharge limits for wastewater.

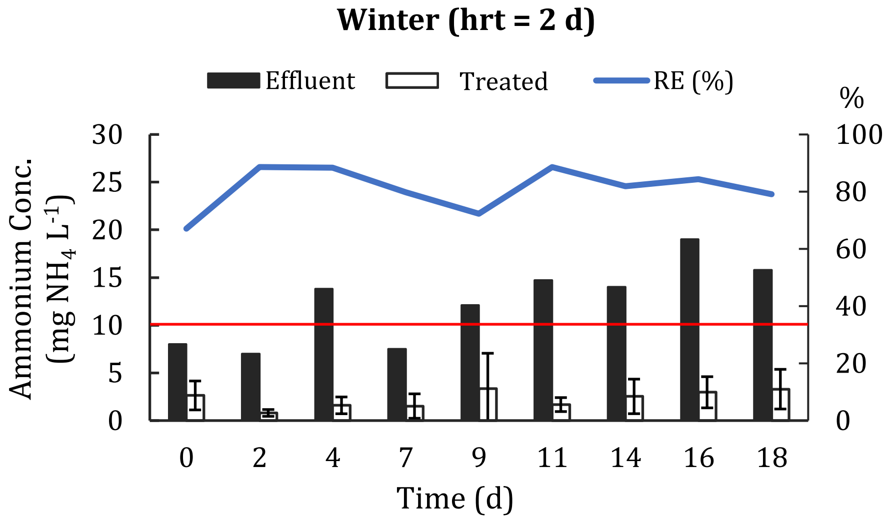

Figure 28 – Ammonium concentration (mg NH_4 L^{-1}) ± SD (error bars) through 18 days in winter with HRT of 2 days. Blue line represents the percentage of NH_4 removal. Red line shows the legislation limit. The treated water was analyzed in triplicate.

The experiment W1.12 demonstrated average ammonium concentrations of 21.65 ± 2.55 mg NH_4 L^{-1} and 5.58 ± 1.24 mg NH_4 L^{-1} at E and T, respectively. The efficiency was 74.12 % of reduction that varied within 67-82.33 % (**Fig. 29**). Statistical analysis showed significant differences for N-NH_4 at E and T (p < 0.05). A positive correlation between NH_4 and TN was observed (r = 0.747, n = 6, p < 0.05) but no significant correlations were obtained between RE and pH (r = 0.363, n = 6, p > 0.05), temperature (r = 0.462, n = 6, p > 0.05), or biomass concentration (r = -0.637, n = 6, p > 0.05). The ammonium concentration of the wastewater in W1.12 was higher than W2. Even with higher concentrations of ammonium, the pH decreased during 16 days of experiment as a possible consequence of increasing the flux of wastewater with lower pH levels inside photobioreactors or limited CO_2 consumption. Yet, the removal of ammonium to concentrations within discharge limits of wastewater was attained.

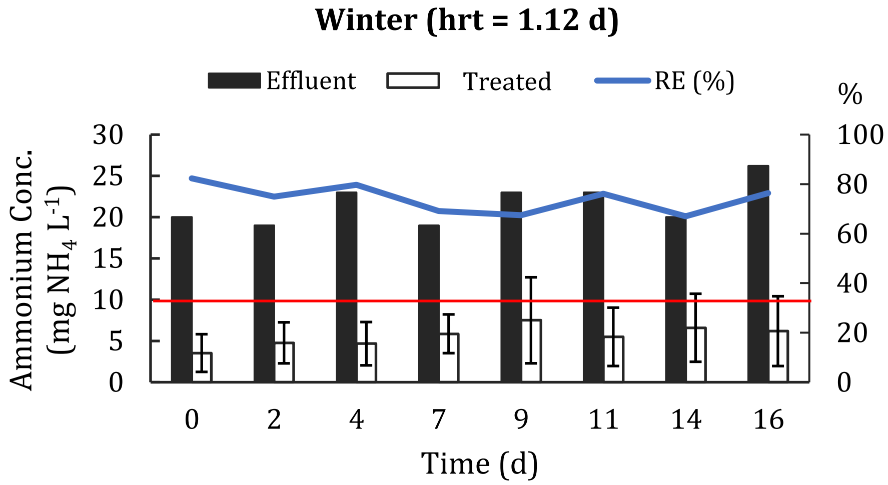

Figure 29 – Analysis of ammonium concentration (mg NH_4 L^{-1}) ± SD (error bars) for 16 days in winter with HRT of 1.12 days. Blue line represents the percentage of NH_4 removal. Red line shows the legislation limit. The treated water was analyzed in triplicate.

In agreement with other studies, both RE of the experiments W2 and W1.12 were similar to the results obtained by Ruiz-Marin et al. (2010) that showed 96.6-100 % and 60.1-80.0 % removal of NH_4^+ from a secondary effluent by *Scenedesmus obliquus* and *Chlorella vulgaris*, respectively.

Contrary to TN, filtered samples were used to analyze ammonium present as soluble form. The increase of biomass productivity from W2 to W1.12 could have been associated with an increase of nutrients input because the inflow of wastewater increased. Unfortunately, statistical analysis did not support this hypothesis as a significant positive correlation between N-NH_4 removal and cellular concentration was not observed in none of the experiments. It was observed only a correlation between RE of N-NH_4 and TN in W1.12, which demonstrated that the removal of ammonium was affected by the concentration of total nitrogen. This may be related to the increased input of N-NH_4 into photobioreactors.

In all experiments, the system was able to decrease the N-NH_4 concentration. However, the wastewater treatment plant of Quinta do Lago has license certificate (User title nº: L012942.2018.RH8, 1 de Agosto de 2018) in which the analysis of ammonium levels are not mandatory.

4.3.4. *Nitrate*

Nitrate is also commonly found in natural waters or wastewaters with excessive amounts. The assimilation of nitrate by photosynthetic eukaryotes requires energy usage for transport and reduction into ammonium that is incorporated into microalgal cells.

In this study nitrates were analyzed in W2 and W1.12. The mean concentration of nitrate in the influent and treated water of W2 was 36.61 ± 9.22 mg NO_3 L^{-1} and 16.30 ± 5.27 mg NO_3 L^{-1} ($p < 0.05$), respectively, with a removal efficiency of 49.87 % that ranged within 0-80.32% (**Fig. 30**). Correlation analyses did not show significant relationships between RE of nitrate and pH (r = 0.476, n = 7, $p > 0.05$) or temperature (r = 0.475, n = 7, $p > 0.05$), but the correlation was significant and positive with cellular concentration (r = 0.734, n = 7, $p < 0.05$). After t = 10 it was registered higher biomass concentration which could indicate increased consumption of nitrates During the experimental time nitrates concentration remained below 50 mg NO_3 L^{-1} at T (Decreto Lei 236-98, 1 de Agosto 1998).

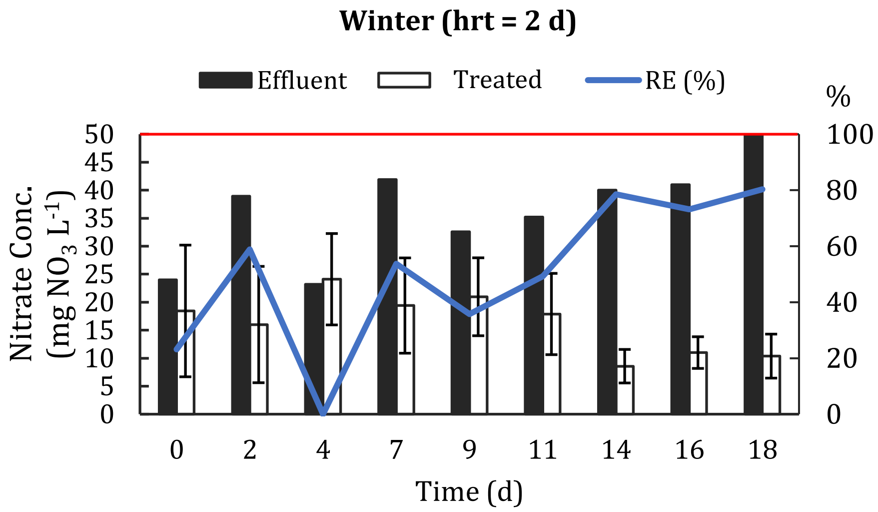

Figure 30 – Nitrate concentration (mg NO_3 L^{-1}) ± SD (error bars) in winter with HRT of 2 days were analyzed for 18 days. Blue line represents the percentage of NO_3 removal. Red line shows the legislation limit. The treated water was analyzed in triplicate.

Nitrate concentration in W1.12 was 31.90 ± 5.67 mg NO_3 L^{-1} and 20.13 ± 7.70 mg NO_3 L^{-1} for E and T ($p < 0.05$), respectively, corresponding to an average removal of 34.42 % with variation between 0-71.46 % (**Fig. 31**). Nitrates remained below the legal limit of 50 mg NO_3 L^{-1} at T (Decreto Lei 236-98, 1 de Agosto 1998). Statistical analysis showed positive correlations between RE of nitrates and pH (r = 0.907, n = 6, p < 0.05), temperature (r = 0.827, n = 6, p < 0.05), and RE of TN (r = 0.815, n = 6, p < 0.05). The temperature decreased during W1.12 resulting in lower photosynthetic activity, and subsequently, pH decreased, and a lower consumption of nitrates was observed.

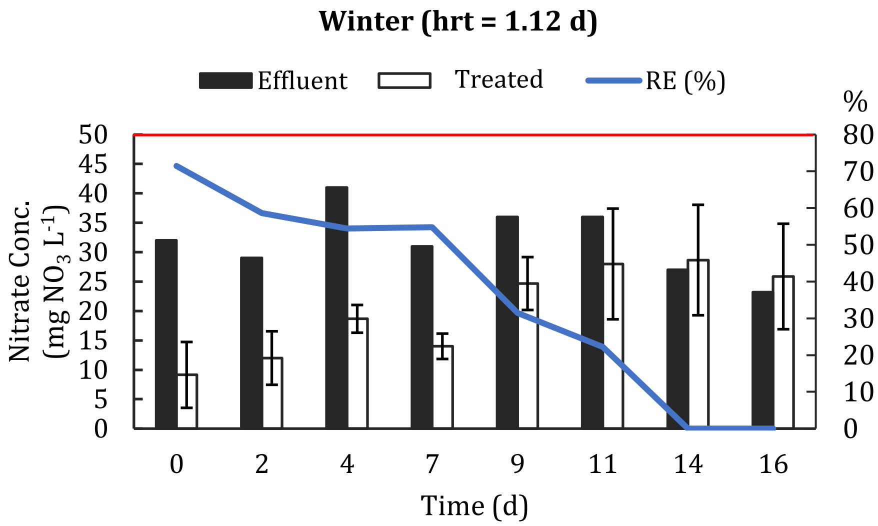

Figure 31 – Analysis of nitrate concentration (mg NO_3 L^{-1}) ± SD (error bars) for 16 days in winter with HRT of 1.12 days. in Blue line represents the percentage of NO_3 removal. Red line shows the legislation limit. The treated water was analyzed in triplicate.

The wastewater treatment plant of Quinta do Lago has a license certificate (User title nº: L012942.2018.RH8, 1 de Agosto de 2018) where the analysis of nitrates are not mandatory, similarly to ammonium. The legislation is suitable for conventional treatment that considers suspended inorganic solids with elevated bacterial loads. In this study, microalgae were accounted and also considered part of suspended solids. Ammonium showed higher removal efficiencies compared to nitrates. The RE of nitrate in W1.12 dropped until null values, which could have been a consequence of preferential consumption of ammonium. However, the concentration of NO_3 at T could have resulted in a higher concentration of total nitrogen, where its removal failed due to poor sedimentation efficiency. Yet, the treated water showed N-NH_4 below legislated limits and NO_3 despite being in higher concentrations is less harmful than N-NH_4 for the environment.

4.3.5. *Total Phosphorus*

Phosphorus is a limiting nutrient that defines microalgae growth. Total phosphorus is commonly measured because it contains both phosphorus and phosphates. The major sources include runoff waters from agricultural lands or wastewaters.

The concentration of TP in A2 showed average values of 6.32 ± 4.54 mg P L^{-1} and 3.06 ± 2.33 mg P L^{-1} for E and T, respectively. The RE during the experiment was not constant and presented a mean value of 41.59 % with a variation between 0 and 98.50 % (**Fig. 32**). The RE dropped until null values from t = 0 to t = 4 and from t = 10 to t = 28. The difference between E and T in A2 was not statistically significant. However, it was observed a positive correlation between temperature and TP removal (r = 0.707, n = 7, $p < 0.05$), as seen by a constant drop in the temperature from t = 10 until the end of the study. The legislation for TP concentration applied to discharged wastewater from the treatment plant (Quinta do Lago, Algarve) within the Natural Park of Ria Formosa (Algarve, Portugal) must be below 3 mg P L^{-1} (Decreto Lei 236-98, 1 de Agosto de 1998). However, the discharge license (User title nº: L012942.2018.RH8, 1 de Agosto de 2018) considers the limit of 10 mg P L^{-1}. The concentration of TP in A2 remained below the licensed limit.

The TP concentration in the effluent presented high variations that could have influenced the rate of TP removal in the treatment. The cause of such discrepancy in A2 could have been a consequence of operational conditions at WWTP of Quinta do Lago. These results contrast with the constant concentrations of TP observed in W2 and W1.12.

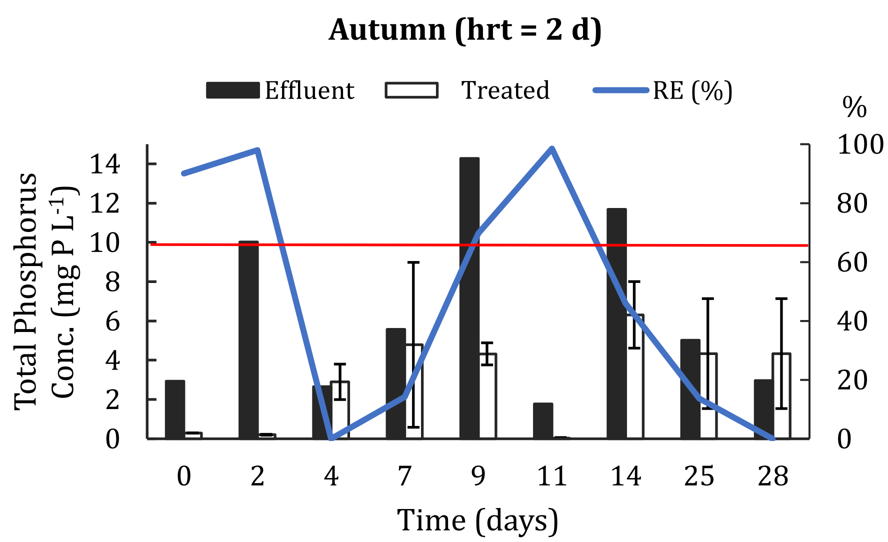

Figure 32 – The analysis of total phosphorus concentration (mg P L^{-1}) ± SD (error bars) in autumn with HRT of 2 days were performed for 30 days. The percentage of TP removal is represented by the blue line, and the legislation limit for wastewater discharge by the red line. The treated water was analyzed in triplicate.

The concentration of TP presented in W2 experiment were 4.90 ± 0.75 mg P L^{-1} and 3.02 ± 1.11 mg P L^{-1}, for E and T, respectively. The RE constantly increased for 18 consecutive days, presenting a mean of 34.63 % removal that oscillated between 0 and 66.19 % (**Fig. 33**). Both TP concentration at E and T were below 10 mg P L^{-1} ($p < 0.05$), thus fulfilling the discharged limits for wastewaters in the treatment plant of Quinta do Lago (User title nº: L012942.2018.RH8, 1 de Agosto de 2018). Statistical analysis showed direct significant correlations between RE of TP and pH (r = 0.964, n = 7, $p < 0.01$), temperature (r = 0.906, n = 7, $p < 0.01$), and biomass concentration

(r = 0.811, n = 7, p < 0.01). The increase of cellular concentration resulted from increased temperature and inorganic C consumption which caused the increase of pH. Another possibility for TP removal is also the consumption of inorganic P or P removal by precipitation and deposition at the bottom of photobioreactor as a consequence of increased pH (Cai et al., 2013). The correlation was stronger between TP removal and pH than with temperature or cell concentration, which possibly showed that pH was an important factor.

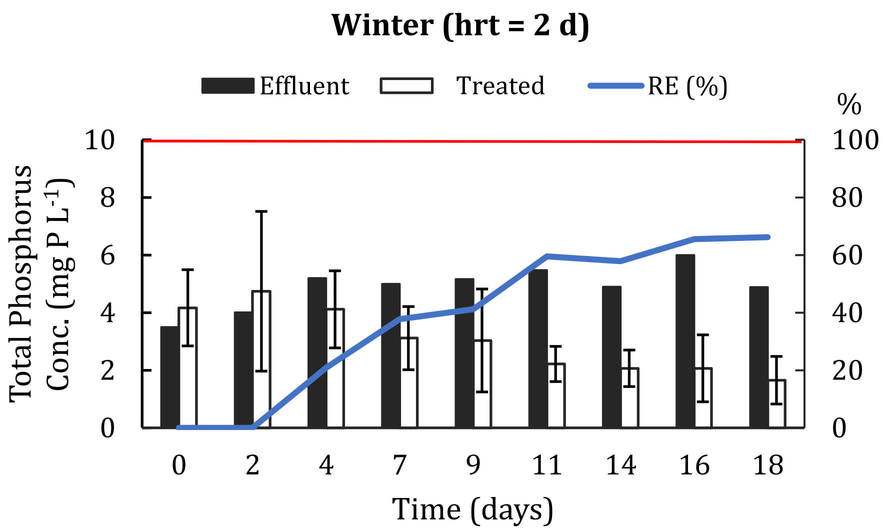

Figure 33 – Total phosphorus concentration (mg P L^{-1}) ± SD (error bars) analysis. The winter experiment with HRT of 2 days lasted 18 days. The percentage of TP removal is represented by the blue line, and the legislation limit for wastewater discharge by the red line. The treated water was analyzed in triplicate.

The W1.12 experiment showed TP concentrations of 4.98 ± 0.81 mg P L^{-1} and 3.14 ± 1.89 mg P L^{-1}, for E and T, respectively. The RE constantly decreased over the experiment. The results showed an average removal rate of 37.76 % with fluctuations between 0 and 70.92 %, which were higher than W2 (**Fig. 34**). TP concentration at E and T remained below the legal limit of 10 mg P L^{-1} for wastewaters discharge (User title nº: L012942.2018.RH8, 1 de Agosto de 2018). Statistical analysis showed significant differences between E and T (p < 0.05). The RE of TP was directly correlated with pH (r = 0.846, n = 6, p < 0.01) and temperature (r = 0.779, n = 6, p < 0.05). The decrease of temperature could have contributed to less photosynthetic activity resulting in a pH decrease. In those conditions, the deposition of phosphorus probably did not occur from t = 8 until t = 16 since pH was below 9. Statistical analysis also demonstrated a positive correlation between TP and NO_3 (r = 0.969, n = 6, p < 0.01) and TN concentration (r = 0.707, n = 6, p < 0.05). Similar to nitrogen, TP analysis also performed in non-filtered samples. For that reason, the presence of microalgal cells could have influenced TP concentration at T as a cause of poor sedimentation of suspended solids.

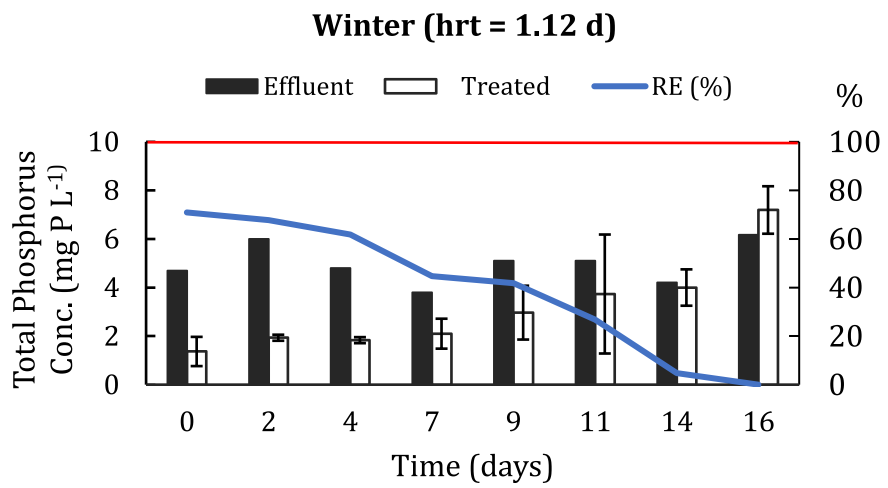

Figure 34 – Total phosphorus concentration (mg P L^{-1}) ± SD (error bars) analysis. The winter experiment with HRT of 1.12 days lasted 16 days. The percentage of TP removal is represented by the blue line, and the legislation limit for wastewater discharge by the red line. The treated water was analyzed in triplicate.

4.3.6. *Phosphate*

Phosphorus is also measured as ortho-phosphates, which are soluble reactive compounds that are directly assimilated by microalgae. In experiment A2, the E and T averaged a concentration of 8.17 ± 3.44 mg P L^{-1} and 3.08 ± 2.36 mg P L^{-1}, respectively ($p < 0.05$). The mean RE was 54.45 % and variated within 0 and 97.66 % (**Fig. 35**). No significant correlations were observed between RE of P-PO_4 and pH ($r = 0.429$, n = 12, $p > 0.05$), temperature ($r = 0.198$, n = 12, $p > 0.05$), cellular concentration ($r = -0.039$, n = 12, $p > 0.05$), or TP ($r = 0.424$, n = 12, $p > 0.05$). The results on the removal of phosphates presented relative significant standard deviations what could have difficulted the correlation analysis. There is no legislation that includes the analysis of phosphates (Decreto Lei 236-98, 1 de Agosto de 1998).

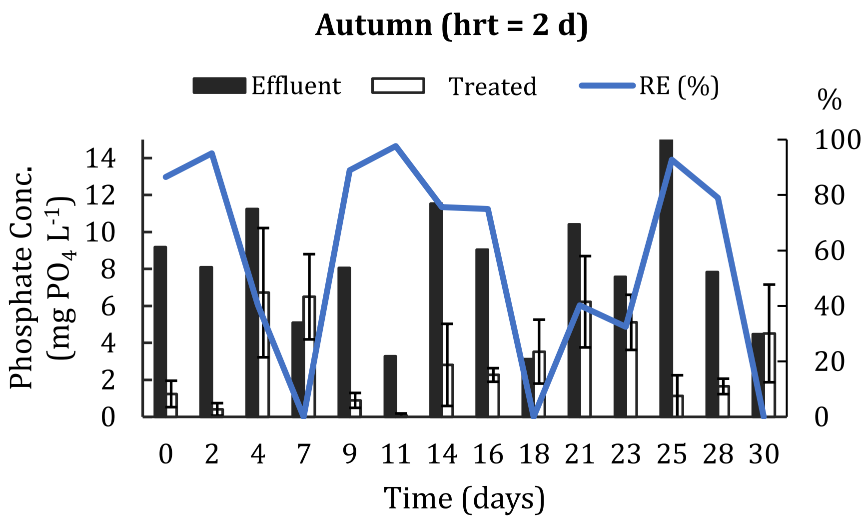

Figure 35 – Phosphate concentration (mg PO_4 L^{-1}) ± SD (error bars) is represented in 30 days of autumn experiment with HRT of 2 days. The percentage of PO_4 removal is represented by the blue line, and the legislation limit for wastewater discharge by the red line. The treated water was analyzed in triplicate.

Along 18 days in W2 experiment, the average concentration for E and T were 4.90 ± 0.75 mg P L^{-1} and 1.63 ± 1.04 mg P L^{-1}, respectively ($p < 0.05$). The average RE was 63.07 % and fluctuated between 1.90 and 88.89 % (**Fig. 36**). Statistical analysis showed positive correlations between RE of P-PO_4 and pH ($r = 0.866$, n = 7, $p < 0.01$), temperature ($r = 0.861$, n = 7, $p < 0.01$), and cellular concentration ($r = 0.742$, n = 7, $p < 0.05$). Two mechanisms might be occurring: 1) the direct uptake of soluble forms of phosphate increased as a consequence of increased photosynthetic activity due to higher temperatures, or 2) the removal of phosphate occurred by deposition caused by increased pH.

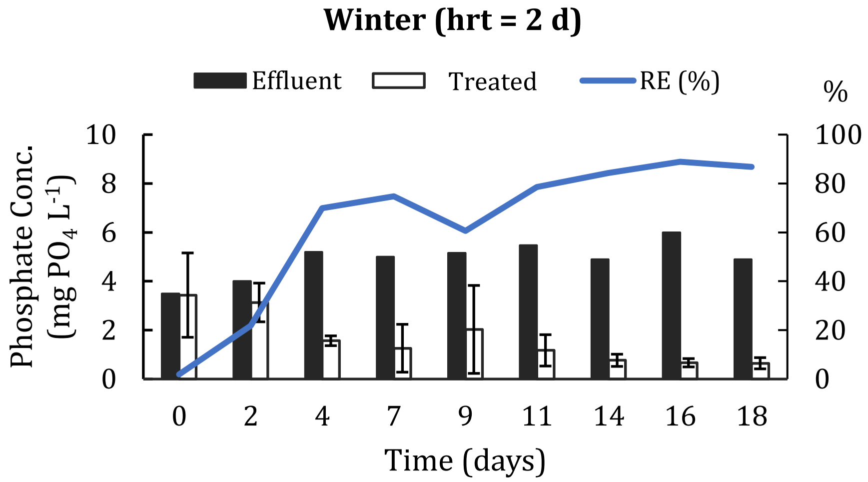

Figure 36 – Phosphate concentration (mg PO_4 L^{-1}) ± SD (error bars) analyzed for 18 days in winter experiment with HRT of 2 days. The percentage of PO_4 removal is represented by the blue line, and the legislation limit for wastewater discharge by the red line. The treated water was analyzed in triplicate.

In W1.12 experiment the mean phosphate concentration was 4.98 ± 0.81 mg P L^{-1} and 1.55 ± 1.11 mg P L^{-1} for E and T, respectively ($p < 0.05$). The average RE was 69.11 % and oscillated between 31.37 and 88.65 % (**Fig. 37**). When compared to W2, the RE was higher and the variation was lower. Statistical analysis showed a direct significant correlation between P-PO_4 removal with pH ($r = 0.746$, n = 6, $p < 0.05$), TP ($r = 0.739$, n = 6, $p < 0.05$), TN ($r = 0.723$, n = 6, $p < 0.05$) and NO_3 ($r = 0.785$, n = 6, $p < 0.05$). The removal rates of phosphates during all the three experiments were similar to the results obtained with TP. The removal was performed either by deposition or direct uptake.

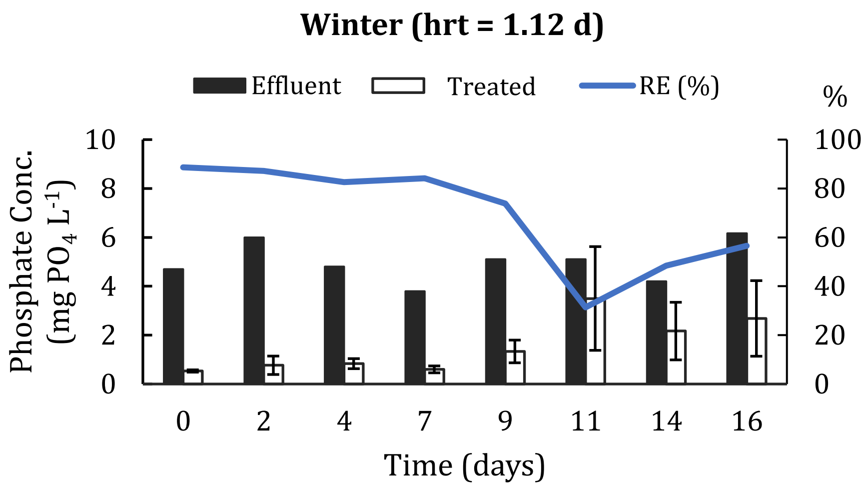

Figure 37 – Phosphate concentration (mg PO_4 L^{-1}) ± SD (error bars) analyzed for 16 days in winter experiment with HRT of 1.12 days. The percentage of PO_4 removal is displayed by the blue line, and the legislation limit for wastewater discharge by the red line. The treated water was analyzed in triplicate.

The N/P ratios were analyzed in A2, W2, and W1.12 experiments and showed means of 2.9±2.1, 4.5±0.8, and 5.5±0.9, respectively (**Fig. 38**). In autumn, the ratio oscillated more than in winter. A2 demonstrated the lowest average N/P ratio which was indicated by a decrease of nitrogen and an increase of phosphates from the day 18, which led to lower biomass concentration. The lower ratio could have been caused by less anthropogenic activities in a tourist region characteristic from Algarve (Portugal). W2 was relatively constant throughout the experience. W1.12 showed the highest mean ratio and, simultaneously, higher biomass concentrations because the increased flux resulted in an increased input of nutrients greater than A2 and W2.

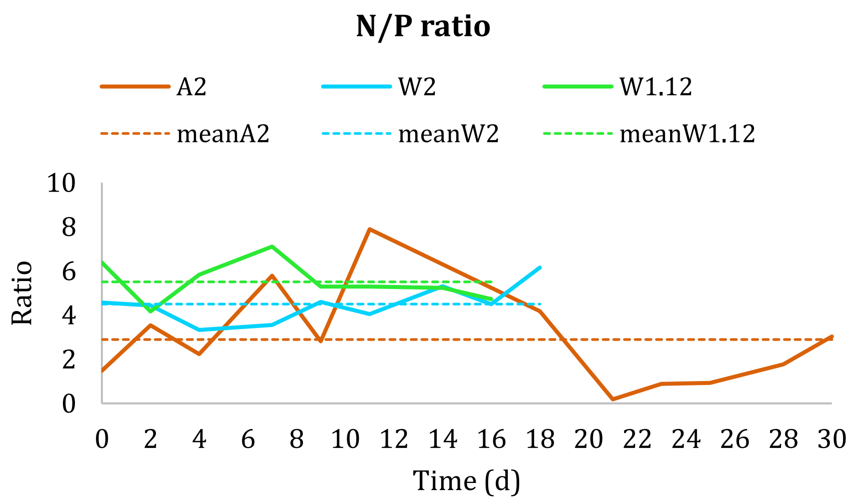

Figure 38 – Relative N/P ratio and associated averages represented for A2, W2, and W1.12 experiments.

4.4. Biomass Composition

The composition of the microalgal biomass was determined in what concerns proteins, lipids, carbohydrates, and ashes content in A2, W2 and W1.12 experiments. The uptake of nitrogen is important for amino acid synthesis and is directly implicated in protein formation, whose concentration depends on the composition of the effluent. The mean protein in A2, W2, and W1.12 showed percentages of 27.99±4.89%, 29.36±4.26%, and 31.98±4.06%, respectively (p > 0.05) (**Fig. 39**). It is important to highlight that the analyzed biomass is composed by microalgae-bacteria consortia from a tertiary treatment (treatment of secondary effluent). The protein content was relatively higher than those reported by Hernández et al. (2013) that showed 8.6 % and 26.6 % protein contents of microalgae-bacteria consortia from the treatment of piggery effluent and potato processing wastewater (secondary effluents), respectively. The results reported in Samorì et al. (2013) showed a protein content of 39.2 % for a consortium (several *Desmodesmus* sp. strains) produced in synthetic primary effluent. Different characteristics of the effluent could be responsible for the different biomass composition.

The lipids produced by microalgae are a rich source of energy that could be exploited for biodiesel production. The content of lipids in the biomass from A2, W2, and W1.12 had percentages of 8.12±1.92%, 7.13±1.67%, and 6.61±0.96%, respectively (p > 0.05) (**Fig. 39**). These results were similar to those reported in. Beltrán-Rocha et al. (2017) which showed 0.5 to 4.3 % of lipids in selected consortia (not specified) from a secondary municipal effluent treatment; and Samorì et al. (2013) 1.4 to 9.3 % of lipids content in different st of *Desmodesmus communis* and a consortium treating urban effluent (synthetic, primary or secondary wastewater).

Carbohydrates are accumulated in plastid cells of microalgae mainly as a reserve material (i.e., glucose and starch) or as a component of cell walls (i.e., cellulose and pectin). This study showed an average content in carbohydrates of 31.62±5.99%, 37.50±3.26%, and 33.39±2.68% for A2, W2, and W1.12, respectively (**Fig. 39**). The carbohydrate content was relatively higher than that reported in Gouveia et al. (2016), whose results showed a content of 20.6 % in biomass of a natural consortium used to treat urban effluent.

The presence of bacteria and other suspended solids in waste effluents contributes for the total amount of ash, which is represented by the composition of total minerals in the biomass. In this experiment, the ash content showed average percentages of 32.61±7.63%, 25.63±5.86%, and 28.02±1.77% for A2, W2, and W1.12, respectively (p > 0.05) (**Fig. 39**). These results were in agreement with those reported in Roberts et al. (2013), that demonstrated 29 % of ash in the treatment of municipal wastewater using a natural consortium. Beltrán-Rocha et al. (2017) also reported ash contents between 29.3 and 53.0 % in a selected consortia treating secondary municipal wastewater.

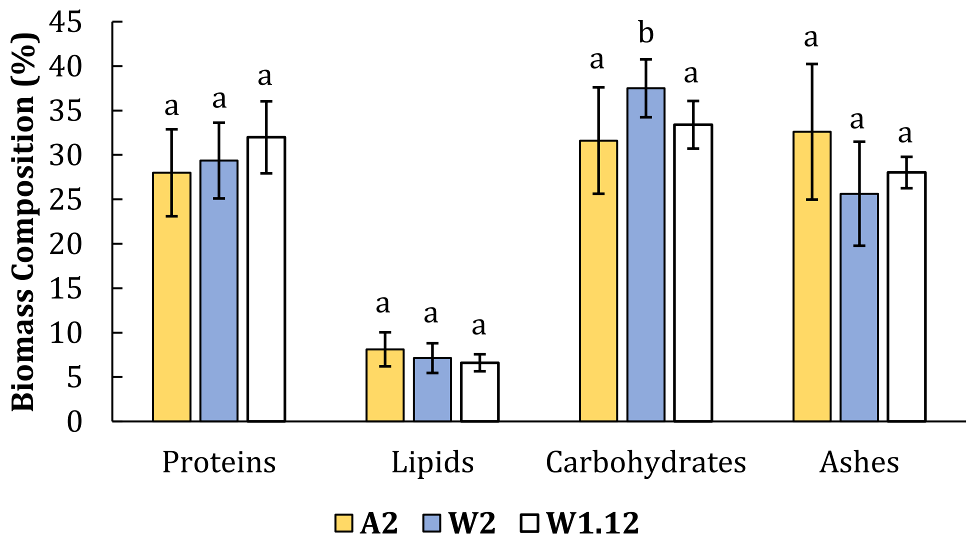

Figure 39 – Proteins, lipids, carbohydrates, and ashes are represented as mean percentages ± SD (error bars) A2, W2, and W1.12 experiments. Columns labelled with different letters are significantly different ($p < 0.05$).

Because of the high ash content in the biomass (25.63-32.61 %), the mineral profile was also analyzed. **Table 3** shows the concentration of minerals (mg/g) in the biomass from the winter experiments. Twelve elements, including Co, Cr, Mo, Ni, Se, Sb, As, Be, Cd, Ag, Tl, and Sn, had their contents near to or less than the method detection limits. The results indicated that minerals varied minimally between W2 and W1.12. Macro minerals Ca Na, Fe, Mg, K, and Al in W2 and W1.12 were the most abundant. Among micro/trace minerals, Cu, Mn, V, and Zn were present in less concentrations in W2 and W1.12. Regarding toxic heavy metals (Sb, As, Be, Cd, Pb, Ag, Tl, and Sn), only Pb was present in W2 with mean 0.017 mg/g dried biomass.

The main forms of inorganic carbon consumed by microalgae are carbon dioxide (CO_2), carbonate (CO_3^-), and bicarbonate (HCO_2^-). The calcium was the most predominant mineral in biomass, due to accumulation of crystal forms of calcium carbonate ($CaCO_3$) in microalgal cells which could either have occurred naturally, or as a consequence of adsorption of salt-based coagulants/flocculants in biomass (Liu, 2017).

Table 3 – Chemical elements composition of dried microalgal biomass (mg/g ± SD) for the experiments W2 and W1.12. The elements that were present in less concentrations or not detected were represented as < 0.01.

<table>
<tr><th>Element (mg/g)</th><th>W2</th><th>W1.12</th></tr>
<tr><td>Calcium (Ca)</td><td>25.969 ± 3.993</td><td>27.481 ± 4.564</td></tr>
<tr><td>Sodium (Na)</td><td>2.385 ± 0.355</td><td>2.444 ± 0.306</td></tr>
<tr><td>Iron (Fe)</td><td>2.655 ± 0.560</td><td>2.814 ± 0.167</td></tr>
<tr><td>Magnesium (Mg)</td><td>2.917 ± 1.522</td><td>2.366 ± 0.452</td></tr>
<tr><td>Potassium (K)</td><td>2.576 ± 0.673</td><td>2.608 ± 0.189</td></tr>
<tr><td>Aluminum (Al)</td><td>0.825 ± 0.431</td><td>0.979 ± 0.035</td></tr>
<tr><td>Barium (Ba)</td><td>0.039 ± 0.020</td><td>0.059 ± 0,004</td></tr>
<tr><td>Cobalt (Co)</td><td colspan="2">< 0.01</td></tr>
<tr><td>Chromium (Cr)</td><td colspan="2">< 0.01</td></tr>
<tr><td>Copper (Cu)</td><td>0.032 ± 0.017</td><td>0.032 ± 0.003</td></tr>
<tr><td>Manganese (Mn)</td><td>0.343 ± 0.130</td><td>0.513 ± 0.020</td></tr>
<tr><td>Molybdenium (Mo)</td><td colspan="2">< 0.01</td></tr>
<tr><td>Nickel (Ni)</td><td colspan="2">< 0.01</td></tr>
<tr><td>Selenium (Se)</td><td colspan="2">< 0.01</td></tr>
<tr><td>Vanadium (V)</td><td>0.148 ± 0.056</td><td>0.179 ± 0.009</td></tr>
<tr><td>Zinc (Zn)</td><td>0.200 ± 0.072</td><td>0.256 ± 0.010</td></tr>
<tr><td>Antimony (Sb)</td><td colspan="2">< 0.01</td></tr>
<tr><td>Arsenic (As)</td><td colspan="2">< 0.01</td></tr>
<tr><td>Beryllium (Be)</td><td colspan="2">< 0.01</td></tr>
<tr><td>Cadmium (Cd)</td><td colspan="2">< 0.01</td></tr>
<tr><td>Lead (Pb)</td><td>0.017 ± 0.008</td><td>< 0.01</td></tr>
<tr><td>Silver (Ag)</td><td colspan="2">< 0.01</td></tr>
<tr><td>Thallium (Tl)</td><td colspan="2">< 0.01</td></tr>
<tr><td>Tin (Sn)</td><td colspan="2">< 0.01</td></tr>
</table>

These results are common in biomass from microalgae produced in wastewater. Usually, the biomass presents high-protein (>30% DW), low-lipid (<10% DW), and high-ash (>25% DW) contents (Huang et al., 2016).

The analysis of biomass in this study showed the lowest contents for lipids (6.61-8.12 %). In contrast, high protein (27.99-31.98 %) and carbohydrates content (31.62-37.50 %) were observed in the biomass determining its suitability for biofuels production, including biogas, biomethane, or bioethanol. The results reported in Hernández et al. (2013) showed that the lipids content could determine the potential methane yield. However, the anaerobic digestion of microalgal substrate has shown two main problems, such as biodegradability of cell walls by bacteria and low C/N ratio (Ramos-Suárez & Carreras, 2014).

The results of C/N ratios of the analyzed biomass averaged 6.2±0.65 (data not shown) which is lower than the optimal C/N ratio (25-30) indicated in studies referenced in (Wang et al., 2014). On the other hand, solutions using enzymatic or acid pretreatments for cell disruption have been referred and cited in (Ramos-Suárez & Carreras, 2014). For example, the application of extraction methods resulted in a substantial increase in methane yield of *Scenedesmus* residues generated after amino acid or lipid extraction compared to the raw biomass (Ramos-Suárez & Carreras, 2014). The pretreatment through high pressure thermal hydrolysis also shown to enhance methane yield (Keymer et al., 2013). Moreover, combining high mineral content (25.63-32.61 %), reported in the present study, with N (indication of protein) along with P suggests that biomass could be suitable and recycled as biofertilizers for supplementation with nutrients and minerals.

5. Conclusion

This study reports the first pilot trial of the GDPBRs in urban wastewater tertiary treatment in two different seasonal conditions: autumn and winter. The treatment was performed by a natural bloom of microalgae in a consortium composed mainly of *Cyanophyceae*, *Chlorophyceae*, *Bacillariophyceae*, as observed under the microscope. Flow cytometry coupled with cell sorting was used to isolated some of the present strains. Four strains from the class *Chlorophyceae* were isolated, namely *Desmodesmus abundans* GTM11, *Scenedesmus* sp. GTM2, *Chlorella* sp. GTM4 and *Chlorella* sp. GTM 5. It was not possible to isolated strains from the classes *Cyanophyceae* and *Bacillariophyceae*, probably due to the use of inappropriate cultivation medium.

The cellular concentration was slightly different between the PBR lines (replicates) which could have been related to different microalgae-bacteria consortia characteristics defined by suspended cells or biofilms formation. The pilot was efficient in the removal of $N\text{-}NH_4$ and nitrates concentration remained below legal limits in the treated water in all seasons reflecting the potential of the GDPBRs for wastewater treatment. The presence of solids (probably microalgal cells) in the treated water, caused by poor sedimentation efficiency (operational conditions) reflected in high TN and TP concentrations. Nonetheless, all the limits imposed by the Portuguese legislation were abide. Both the hydraulic retention times tested, 2d and 1.12d, can be applied in autumn and winter conditions, which, coupled with the high volume/area ratio of the GDPBRs allows the efficient treatment of wastewater with less land requirements. The biochemical composition of the microalgal biomass demonstrated elevated protein and carbohydrates content with promising applications for biofuel production (biogas, biomethane, bioethanol). Biodiesel should not be considered due to the low levels of lipids in the biomass. The mineral composition showed that the produced biomass is rich in essential nutrients which are an important supply in agriculture applications as biofertilizers. From economic and environmental standpoints, the highlights of the GreenDune-PBR are the removal efficiency in 2d, or less, of operation using a working capacity of 450L per 1 m^2 of land and the lower carbon footprint as the emissions green-house gas are minute and the capture of CO2 is stimulated by the use of photoautotrophic organisms for the wastewater treatment. Future studies should address the advantage of adding GDPBRs as modules in order to assess and evaluate different operational conditions (e.g. HRT < 1.12 d) and efficacy.

6. References

6.1. Acién, F. G., Gómez-Serrano, C., Morales-Amaral, M. M., Fernández-Sevilla, J. M., & Molina-Grima, E. (2016). Wastewater treatment using microalgae: how realistic a contribution might it be to significant urban wastewater treatment? Applied Microbiology and Biotechnology, 100(21), 9013–9022. https://doi.org/10.1007/s00253-016-7835-7

6.2. Ajayan, K. V., Selvaraju, M., Unnikannan, P., & Sruthi, P. (2015). Phycoremediation of Tannery Wastewater Using Microalgae Scenedesmus Species. International Journal of Phytoremediation, 17(10), 907–916. https://doi.org/10.1080/15226514.2014.989313

6.3. Alam, M. A., Wan, C., Zhao, X. Q., Chen, L. J., Chang, J. S., & Bai, F. W. (2015). Enhanced removal of Zn2+ or Cd2+ by the flocculating Chlorella vulgaris JSC-7. Journal of Hazardous Materials, 289, 38–45. https://doi.org/10.1016/j.jhazmat.2015.02.012

6.4. Arashiro, L. T., Ferrer, I., Rousseau, D. P. L., Van Hulle, S. W. H., & Garfí, M. (2019). The effect of primary treatment of wastewater in high rate algal pond systems: Biomass and bioenergy recovery. *Bioresource Technology, 280*(January), 27–36. https://doi.org/10.1016/j.biortech.2019.01.096

6.5. Arias, D. M., Solé-Bundó, M., Garfí, M., Ferrer, I., García, K. & Uggetti, E. (2018). Integrating microalgae tertiary treatment into activated sludge systems for energy and nutrients recovery from wastewater. Bioresource Technology, 247, 513-519, https://doi.org/10.1016/j.biortech.2017.09.123.

6.6. Austic, R. E., Mustafa, A., Jung, B., Gatrell, S., & Lei, X. G. (2013). Potential and limitation of a new defatted diatom microalgal biomass in replacing soybean meal and corn in diets for broiler chickens. Journal of Agricultural and Food Chemistry, *61*(30), 7341–7348. https://doi.org/10.1021/jf401957z

6.7. Awad, H., Alalm, M. G., & El-etriby, H. K. (2019). Environmental and cost life cycle assessment of different alternatives for improvement of wastewater treatment plants in developing countries. Science of the Total Environment, 660, 57–68. https://doi.org/10.1016/j.scitotenv.2018.12.386

6.8. Batista, A. P., Ambrosano, L., Graça, S., Sousa, C., Marques, P. A. S. S., Ribeiro, B., … Gouveia, L. (2015). Combining urban wastewater treatment with biohydrogen production - An integrated microalgae-based approach. Bioresource Technology, 184, 230–235. https://doi.org/10.1016/j.biortech.2014.10.064

6.9. Binnal, P., & Babu, P. N. (2017). Optimization of environmental factors affecting tertiary treatment of municipal wastewater by Chlorella protothecoides in a lab scale photobioreactor. Journal of Water Process Engineering, 17(April), 290–298. https://doi.org/10.1016/j.jwpe.2017.05.003

6.10. Bradley, I. M., Sevillano-Rivera, M. C., Pinto, A. J., & Guest, J. S. (2019). Impact of solids residence time on community structure and nutrient dynamics of mixed phototrophic wastewater treatment systems. Water Research, 150, 271–282. https://doi.org/10.1016/j.watres.2018.11.065
6.11. Cai, T., Park, S. Y., & Li, Y. (2013). Nutrient recovery from wastewater streams by microalgae : Status and prospects. Renewable and Sustainable Energy Reviews, *19*, 360–369. https://doi.org/10.1016/j.rser.2012.11.030
6.12. Caporgno, M. P., Taleb, A., Olkiewicz, M., Font, J., Pruvost, J., Legrand, J., & Bengoa, C. (2015). Microalgae cultivation in urban wastewater: Nutrient removal and biomass production for biodiesel and methane. Algal Research, 10, 232–239. https://doi.org/10.1016/j.algal.2015.05.011
6.13. Chisti, Y. (2007). Biodiesel from microalgae. Biotechnology Advances, 25, 3, 294-306, https://doi.org/10.1016/j.biotechadv.2007.02.001.
6.14. Chen, C. Y., Zhao, X. Q., Yen, H. W., Ho, S. H., Cheng, C. L., Lee, D. J., … Chang, J. S. (2013). Microalgae-based carbohydrates for biofuel production. *Biochemical Engineering Journal*, *78*, 1–10. https://doi.org/10.1016/j.bej.2013.03.006
6.15. Chen, Z., Shao, S., He, Y., Luo, Q., Zheng, M., Zheng, M., … Wang, M. (2020). Nutrients removal from piggery wastewater coupled to lipid production by a newly isolated self-flocculating microalga Desmodesmus sp. PW1. Bioresource Technology, 302(December 2019), 122806. https://doi.org/10.1016/j.biortech.2020.122806
6.16. Chew, K. W., Yap, J. Y., Show, P. L., Suan, N. H., Juan, J. C., Ling, T. C., … Chang, J. S. (2017). Microalgae biorefinery: High value products perspectives. Bioresource Technology, 229, 53–62. https://doi.org/10.1016/j.biortech.2017.01.006
6.17. Choi, H. J., & Lee, S. M. (2015). Effect of the N/P ratio on biomass productivity and nutrient removal from municipal wastewater. Bioprocess and Biosystems Engineering, 3*8*(4), 761–766. https://doi.org/10.1007/s00449-014-1317-z
6.18. Chojnacka, K. & Marquez-Rocha, F. (2004). Kinetic and Stoichiometric Relationships of the Energy and Carbon Metabolism in the Culture of Microalgae. Biotechnology, 3: 21-34.
6.19. Christenson, L. and Sims, R. (2011) Production and Harvesting of Microalgae for Wastewater Treatment, Biofuels, and Bioproducts. Biotechnology Advances, 29, 686-702. https://doi.org/10.1016/j.biotechadv.2011.05.015
6.20. Costa, J. A. V., Freitas, B. C. B., Moraes, L., Zaparoli, M., & Morais, M. G. (2020). Progress in the physicochemical treatment of microalgae biomass for value-added product recovery. Bioresource Technology, 301(December 2019), 122727. https://doi.org/10.1016/j.biortech.2019.122727
6.21. Cuellar-Bermudez, S. P., Aleman-Nava, G. S., Chandra, R., Garcia-Perez, J. S., Contreras-Angulo, J. R., Markou, G., … Parra-Saldivar, R. (2017). Nutrients utilization and contaminants removal. A review of two approaches of algae and cyanobacteria in wastewater. Algal Research, 24, 438–449. https://doi.org/10.1016/j.algal.2016.08.018
6.22. Deblonde, T., Cossu-leguille, C., & Hartemann, P. (2015). Emerging pollutants in wastewater : A review of the literature. International Journal of Hygiene and Environmental Health, *214(6),* 442–448. https://doi.org/10.1016/j.ijheh.2011.08.002
6.23. Delgadillo-Mirquez, L., Lopes, F., Taidi, B., & Pareau, D. (2016). Nitrogen and phosphate removal from wastewater with a mixed microalgae and bacteria culture. Biotechnology Reports, 11, 18–26. https://doi.org/10.1016/j.btre.2016.04.003
6.24. Di Caprio, F. (2020). Methods to quantify biological contaminants in microalgae cultures. Algal Research, 49, 101943. https://doi.org/10.1016/j.algal.2020.101943
6.25. Duarte, J. H., Fanka, L. S., & Costa, J. A. V. (2016). Utilization of simulated flue gas containing CO2, SO2, NO and ash for Chlorella fusca cultivation. *Bioresource Technology*, *214*(x), 159–165. https://doi.org/10.1016/j.biortech.2016.04.078
6.26. Escapa, C., Coimbra, R. N., Paniagua, S., García, A. I., & Otero, M. (2015). Bioresource Technology Nutrients and pharmaceuticals removal from wastewater by culture and harvesting of Chlorella sorokiniana. Bioresource Technology, *185*, 276–284. https://doi.org/10.1016/j.biortech.2015.03.004

6.27. Escapa, C., Coimbra, R. N., Paniagua, S., García, A. I., & Otero, M. (2016). Comparative assessment of diclofenac removal from water by different microalgae strains. ALGA*L*, *18*, 127–134. https://doi.org/10.1016/j.algal.2016.06.008
6.28. European commission directive, 1998. Off. J. Eur. Commun. 29e30, 98/15/EC of 27 February.
6.29. Farhan, A., Udaiyappan, M., Abu, H., & Sobri, M. (2017). A review of the potentials, challenges and current status of microalgae biomass applications in industrial wastewater treatment . Journal of Water Process Engineering. 20(June), 8–21. https://doi.org/10.1016/j.jwpe.2017.09.006
6.30. P. Foladori, S. Petrini, G. Andreottola (2018). Evolution of real municipal wastewater treatment in photobioreactors and microalgae-bacteria consortia using real-time parameters. Chemical Engineering Journal, 345, 507-516, https://doi.org/10.1016/j.cej.2018.03.178.
6.31. Fu, F., & Wang, Q. (2011). Removal of heavy metal ions from wastewaters: A review. Journal of Environmental Management, *92*(3), 407–418. https://doi.org/10.1016/j.jenvman.2010.11.011
6.32. Gao, F., Li, C., Yang, Z. H., Zeng, G. M., Feng, L. J., Liu, J. zhi, … Cai, H. wen. (2016). Continuous microalgae cultivation in aquaculture wastewater by a membrane photobioreactor for biomass production and nutrients removal. Ecological Engineering, *92*, 55–61. https://doi.org/10.1016/j.ecoleng.2016.03.046
6.33. García, D., Posadas, E., Blanco, S., Acién, G., García-Encina, P., Bolado, S., & Muñoz, R. (2018). Evaluation of the dynamics of microalgae population structure and process performance during piggery wastewater treatment in algal-bacterial photobioreactors. *Bioresource Technology, 248,* 120–126. https://doi.org/10.1016/j.biortech.2017.06.079
6.34. Gonçalves, A. L., Pires, J. C. M., & Simões, M. (2017). A review on the use of microalgal consortia for wastewater treatment. Algal Research, 24, 403–415. https://doi.org/10.1016/j.algal.2016.11.008
6.35. González-camejo, J., Aparicio, S., Ruano, M. V, Borrás, L., Barat, R., & Ferrer, J. (2019). Bioresource Technology E ff ect of ambient temperature variations on an indigenous microalgae- nitrifying bacteria culture dominated by Chlorella. Bioresource Technology, 290(May), 121788. https://doi.org/10.1016/j.biortech.2019.121788
6.36. Gouveia, L., Graça, S., Sousa, C., Ambrosano, L., Ribeiro, B., Botrel, E. P., Neto, P. C., Ferreira, A. F. & Silva, C. M. (2016). Microalgae biomass production using wastewater: Treatment and costs: Scale-up considerations. Algal Research, 16, 167-176, https://doi.org/10.1016/j.algal.2016.03.010.
6.37. Guo, Y., Yuan, Z., Xu, J., Wang, Z., Yuan, T., Zhou, W., … Liu, S. (2017). Metabolic acclimation mechanism in microalgae developed for CO2 capture from industrial flue gas. Algal Research, 26(2), 225–233. https://doi.org/10.1016/j.algal.2017.07.029
6.38. Gupta, S., Pawar, S. B., Gajanan, R. A. P., & Satish, S. K. (2019a). Outdoor microalgae cultivation in airlift photobioreactor at high irradiance and temperature conditions : effect of batch and fed-batch strategies , photoinhibition , and temperature stress. Bioprocess and Biosystems Engineering, 42(2), 331–344. https://doi.org/10.1007/s00449-018-2037-6
6.39. Gupta, S., Pawar, S. B., & Pandey, R. A. (2019b). Science of the Total Environment Current practices and challenges in using microalgae for treatment of nutrient rich wastewater from agro-based industries. Science of the Total Environment, 687, 1107–1126. https://doi.org/10.1016/j.scitotenv.2019.06.115
6.40. Gutierrez, J., Kwan, T. A., Zimmerman, J. B., & Peccia, J. (2016). Ammonia inhibition in oleaginous microalgae. ALGAL, 19, 123–127. https://doi.org/10.1016/j.algal.2016.07.016
6.41. Hernández, D., Riaño, B., Coca, M., & García-González, M. C. (2013). Treatment of agro-industrial wastewater using microalgae-bacteria consortium combined with anaerobic digestion of the produced biomass. Bioresource Technology, 135, 598–603. https://doi.org/10.1016/j.biortech.2012.09.029
6.42. Hindersin, S., Leupold, M., Kerner, M., & Hanelt, D. (2013). Irradiance optimization of outdoor microalgal cultures using solar tracked photobioreactors. Bioprocess Biosyst Eng

(2013) 36:345–355 DOI 10.1007/s00449-012-0790-5
6.43. Holdmann, C., Schmid-staiger, U., & Hirth, T. (2019). Outdoor microalgae cultivation at di ff erent biomass concentrations — Assessment of di ff erent daily and seasonal light scenarios by modeling. *Algal Research, 38*(December 2017), 101405. https://doi.org/10.1016/j.algal.2018.101405
6.44. Ji, M. K., Yun, H. S., Park, Y. T., Kabra, A. N., Oh, I. H., & Choi, J. (2015). Mixotrophic cultivation of a microalga Scenedesmus obliquus in municipal wastewater supplemented with food wastewater and flue gas CO2 for biomass production. *Journal of Environmental Management*, *159*, 115–120. https://doi.org/10.1016/j.jenvman.2015.05.037
6.45. Keymer, P., Ruffell, I., Pratt, S., & Lant, P. (2013). High pressure thermal hydrolysis as pre-treatment to increase the methane yield during anaerobic digestion of microalgae. *Bioresource Technology*, *131*, 128–133. https://doi.org/10.1016/j.biortech.2012.12.125
6.46. Kumar, A., Ergas, S., Yuan, X., Sahu, A., Zhang, Q., Dewulf, J., … van Langenhove, H. (2010). Enhanced CO2 fixation and biofuel production via microalgae: Recent developments and future directions. Trends in Biotechnology, 28(7), 371–380. https://doi.org/10.1016/j.tibtech.2010.04.004
6.47. Lakaniemi, A., Hulatt, C. J., Wakeman, K. D., Thomas, D. N., & Puhakka, J. A. (2012). Bioresource Technology Eukaryotic and prokaryotic microbial communities during microalgal biomass production. *124*, 387–393. https://doi.org/10.1016/j.biortech.2012.08.048
6.48. Lee, C. S., Lee, S., Ko, S., Oh, H., & Ahn, C. (2014). ScienceDirect Effects of photoperiod on nutrient removal , biomass production , and algal-bacterial population dynamics in lab-scale photobioreactors treating municipal wastewater. *8*. https://doi.org/10.1016/j.watres.2014.10.029
6.49. Leong, Y. K., & Chang, J. S. (2020). Bioremediation of heavy metals using microalgae: Recent advances and mechanisms. Bioresource Technology, 30*3*(December 2019), 122886. https://doi.org/10.1016/j.biortech.2020.122886
6.50. Lewkowska, P., Cieślik, B., Dymerski, T., Konieczka, P., & Namieśnik, J. (2016). Characteristics of odors emitted from municipal wastewater treatment plant and methods for their identification and deodorization techniques. Environmental Research, 1*51*, 573–586. https://doi.org/10.1016/j.envres.2016.08.030
6.51. Li, K., Liu, Q., Fang, F., Luo, R., Lu, Q., Zhou, W., … Ruan, R. (2019). Bioresource Technology Microalgae-based wastewater treatment for nutrients recovery : A review. Bioresource Technology, 291(July), 121934. https://doi.org/10.1016/j.biortech.2019.121934
6.52. Liu, K. (2017). Characterization of ash in algae and other materials by determination of wet acid indigestible ash and microscopic examination. *Algal Research, 25*(February), 307–321. https://doi.org/10.1016/j.algal.2017.04.014
6.53. Liu, K. (2019). Effects of sample size, dry ashing temperature and duration on determination of ash content in algae and other biomass. *Algal Research, 40*(March), 101486. https://doi.org/10.1016/j.algal.2019.101486
6.54. López-serna, R., Posadas, E., García-encina, P. A., & Muñoz, R. (2019). Science of the Total Environment Removal of contaminants of emerging concern from urban wastewater in novel algal-bacterial photobioreactors. Science of the Total Environment, 662, 32–40. https://doi.org/10.1016/j.scitotenv.2019.01.206
6.55. Lourenço, S. O., Barbarino, E., De-paula, J. C., Otávio, L., Pereira, S., & Marquez, U. M. L. (2002). *Amino acid composition , protein content and calculation of nitrogen-to-protein conversion factors for 19 tropical seaweeds*. (November 2017). https://doi.org/10.1046/j.1440-1835.2002.00278.x
6.56. Lu, Q., Zhou, W., Min, M., Ma, X., Chandra, C., Doan, Y. T. T., … Ruan, R. (2015). Bioresource Technology Growing Chlorella sp . on meat processing wastewater for nutrient removal and biomass production. *BIORESOURCE TECHNOLOGY*, *198*, 189–197. https://doi.org/10.1016/j.biortech.2015.08.133
6.57. Lv, J., Liu, Y., Feng, J., Liu, Q., Nan, F., & Xie, S. (2018). Nutrients removal from undiluted cattle farm wastewater by the two-stage process of microalgae-based wastewater treatment.

Bioresource Technology, 264(April), 311–318. https://doi.org/10.1016/j.biortech.2018.05.085
6.58. Markou, G., Dao, L. H. T., Muylaert, K., & Beardall, J. (2017). Influence of different degrees of N limitation on photosystem II performance and heterogeneity of Chlorella vulgaris. Algal Research, 26(July), 84–92. https://doi.org/10.1016/j.algal.2017.07.005
6.59. Markou, G., Depraetere, O., & Muylaert, K. (2016). Effect of ammonia on the photosynthetic activity of Arthrospira and Chlorella: A study on chlorophyll fluorescence and electron transport. Algal Research, *16*, 449–457. https://doi.org/10.1016/j.algal.2016.03.039
6.60. Markou, G., & Muylaert, K. (2016). Bioresource Technology Effect of light intensity on the *degree of ammonia toxicity on PSII activity of Arthrospira platensis and Chlorella vulgaris. Bioresource Technology, 216*, 453–461. https://doi.org/10.1016/j.biortech.2016.05.094
6.61. Markou, G., Wang, L., Ye, J., & Unc, A. (2018). Using agro-industrial wastes for the cultivation of microalgae and duckweeds: Contamination risks and biomass safety concerns. Biotechnology Advances, 36(4), 1238–1254. https://doi.org/10.1016/j.biotechadv.2018.04.003
6.62. Martínez, C., Bernard, O., & Mairet, F. (2017). Maximizing microalgae productivity by shading outdoor cultures. *IFAC-PapersOnLine*, *50*(1), 8734–8739. https://doi.org/10.1016/j.ifacol.2017.08.1725
6.63. Matamoros, V., Gutiérrez, R., Ferrer, I., García, J., & Bayona, J. M. (2015). Capability of microalgae-based wastewater treatment systems to remove emerging organic contaminants : A pilot-scale study. Journal of Hazardous Materials, 288, 34–42. https://doi.org/10.1016/j.jhazmat.2015.02.002
6.64. Michelon, W., Pirolli, M., Soares, H. M., Michelon, W., Mezzari, M. P., & Busi da Silva, M. L. (2019). Residual sugar from microalgae biomass harvested from phycoremediation of swine wastewater digestate. *Water Science and Technology*, *79*(11), 2203–2210. https://doi.org/10.2166/wst.2019.226
6.65. Muñoz, I., Gómez, M. J., Molina-díaz, A., Huijbregts, M. A. J., Fernández-alba, A. R., & García-calvo, E. (2008). Chemosphere Ranking potential impacts of priority and emerging pollutants in urban wastewater through life cycle impact assessment. Chemosphere, 74(1), 37–44. https://doi.org/10.1016/j.chemosphere.2008.09.029
6.66. Naidoo, S., & Olaniran, A. O. (2014). Treated Wastewater Effluent as a Source of Microbial Pollution of Surface Water Resources. Int. J. Environ. Res. Public Health, 11(1), 249-270. https://doi.org/10.3390/ijerph110100249
6.67. N. Nirmalakhandan, T. Selvaratnam, S.M. Henkanatte-Gedera, D. Tchinda I.S.A. Abeysiriwardana-Arachchige, H.M.K. Delanka-Pedige, S.P. Munasinghe-Arachchige, Y. Zhang, F.O. Holguin, P.J. Lammers (2019). Algal wastewater treatment: Photoautotrophic vs. mixotrophic processes, Algal Research, 41, https://doi.org/10.1016/j.algal.2019.101569.
6.68. Oyebamiji, O. O., Boeing, W. J., Holguin, F. O., Ilori, O., & Amund, O. (2019). Green microalgae cultured in textile wastewater for biomass generation and biodetoxification of heavy metals and chromogenic substances. *Bioresource Technology Reports*, *7*(November 2018), 100247. https://doi.org/10.1016/j.biteb.2019.100247
6.69. Paskuliakova, A., Tonry, S., & Touzet, N. (2016). Phycoremediation of landfill leachate with chlorophytes: Phosphate a limiting factor on ammonia nitrogen removal. Water Research, 99, 180–187. https://doi.org/10.1016/j.watres.2016.04.029
6.70. Pereira, H., Barreira, L., Mozes, A., Florindo, C., Polo, C., Duarte, C. V., … Varela, J. (2011). Microplate-based high throughput screening procedure for the isolation of lipid-rich marine microalgae. *Biotechnology for Biofuels*, *4*(1), 61. https://doi.org/10.1186/1754-6834-4-61
6.71. Pereira, H., Schulze, P. S. C., Schüler, L. M., Santos, T., Barreira, L., & Varela, J. (2018). Fluorescence activated cell-sorting principles and applications in microalgal biotechnology. *Algal Research*, *30*(August 2017), 113–120. https://doi.org/10.1016/j.algal.2017.12.013
6.72. Pires, J. C. M., Alvim-Ferraz, M. C. M., Martins, F. G., & Simões, M. (2013). Wastewater treatment to enhance the economic viability of microalgae culture. *Environmental Science and Pollution Research*, *20*(8), 5096–5105. https://doi.org/10.1007/s11356-013-1791-x
6.73. Posadas, E., Morales, M. del M., Gomez, C., Acién, F. G., & Muñoz, R. (2015). Influence of pH

and CO 2 source on the performance of microalgae-based secondary domestic wastewater treatment in outdoors pilot raceways. Chemical Engineering Journal, 265, 239–248. https://doi.org/10.1016/j.cej.2014.12.059

6.74. Quiroz, C. E., Peebles, C., & Bradley, T. H. (2015). Scalability of combining microalgae-based biofuels with wastewater facilities : A review. *ALGAL*, *9*, 160–169. https://doi.org/10.1016/j.algal.2015.03.001

6.75. Ramesh Kumar, B., Deviram, G., Mathimani, T., Duc, P. A., & Pugazhendhi, A. (2019). Microalgae as rich source of polyunsaturated fatty acids. *Biocatalysis and Agricultural Biotechnology*, *17*(November 2018), 583–588. https://doi.org/10.1016/j.bcab.2019.01.017

6.76. Ramos-Suárez, J. L., & Carreras, N. (2014). Use of microalgae residues for biogas production. *Chemical Engineering Journal*, *242*, 86–95. https://doi.org/10.1016/j.cej.2013.12.053

6.77. Ranganathan, P., & Savithri, S. (2019). Techno-economic analysis of microalgae-based liquid fuels production from wastewater via hydrothermal liquefaction and hydroprocessing. *Bioresource Technology*, *284*(March), 256–265. https://doi.org/10.1016/j.biortech.2019.03.087

6.78. Rashid, N., Ryu, A. J., Jeong, K. J., Lee, B., & Chang, Y. K. (2019). Co-cultivation of two freshwater microalgae species to improve biomass productivity and biodiesel production. *Energy Conversion and Management*, *196*(June), 640–648. https://doi.org/10.1016/j.enconman.2019.05.106

6.79. Rasouli, Z., Valverde-Pérez, B., D'Este, M., De Francisci, D., & Angelidaki, I. (2018). Nutrient recovery from industrial wastewater as single cell protein by a co-culture of green microalgae and methanotrophs. *Biochemical Engineering Journal*, *134*, 129–135. https://doi.org/10.1016/j.bej.2018.03.010

6.80. Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M. C., … Fatta-Kassinos, D. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. Science of the Total Environment, *447*, 345–360. https://doi.org/10.1016/j.scitotenv.2013.01.032

6.81. Roberts, G. W., Fortier, M. O. P., Sturm, B. S. M., & Stagg-Williams, S. M. (2013). Promising pathway for algal biofuels through wastewater cultivation and hydrothermal conversion. *Energy and Fuels*, *27*(2), 857–867. https://doi.org/10.1021/ef3020603

6.82. Robles, A., Seco, A., Ferrer, J., & Ruano, M. V. (2020). On-line monitoring of photosynthetic activity based on pH data to assess microalgae cultivation. *276*(August). https://doi.org/10.1016/j.jenvman.2020.111343

6.83. Rossi, S., Díez-Montero, R., Rueda, E., Castillo Cascino, F., Parati, K., García, J., & Ficara, E. (2020). Free ammonia inhibition in microalgae and cyanobacteria grown in wastewaters: Photo-respirometric evaluation and modelling. Bioresource Technology, 305(January), 123046. https://doi.org/10.1016/j.biortech.2020.123046

6.84. Saini, R. K., & Keum, Y. S. (2018). Omega-3 and omega-6 polyunsaturated fatty acids: Dietary sources, metabolism, and significance — A review. *Life Sciences*, *203*(April), 255–267. https://doi.org/10.1016/j.lfs.2018.04.049

6.85. Samorì, G., Samorì, C., Guerrini, F., & Pistocchi, R. (2013). Growth and nitrogen removal capacity of Desmodesmus communis and of a natural microalgae consortium in a batch culture system in view of urban wastewater treatment: Part I. *Water Research*, *47*(2), 791–801. https://doi.org/10.1016/j.watres.2012.11.006

6.86. Santos, L. H. M. L. M., Gros, M., Rodriguez-mozaz, S., Delerue-matos, C., Pena, A., Barceló, D., & Montenegro, M. C. B. S. M. (2013). Science of the Total Environment Contribution of hospital ef fl uents to the load of pharmaceuticals in urban wastewaters : Identi fi cation of ecologically relevant pharmaceuticals. Science of the Total Environment, *The*, *461–462*, 302–316. https://doi.org/10.1016/j.scitotenv.2013.04.077

6.87. Sayre, R. (2010). Microalgae: The Potential for Carbon Capture. BioScience, 60(9), 722–727. https://doi.org/10.1525/bio.2010.60.9.9

6.88. Schulze, P. S. C., Carvalho, C. F. M., Pereira, H., Gangadhar, K. N., Schüler, L. M., Santos, T. F., … Barreira, L. (2017). Urban wastewater treatment by Tetraselmis sp. CTP4 (Chlorophyta).

Bioresource Technology, 223, 175–183. https://doi.org/10.1016/j.biortech.2016.10.027
6.89. Sharma, J., Kumar, S. S., Kumar, V., Malyan, S. K., Mathimani, T., Bishnoi, N. R., & Pugazhendhi, A. (2020). Upgrading of microalgal consortia with CO2 from fermentation of wheat straw for the phycoremediation of domestic wastewater. Bioresource Technology, 305(December 2019), 123063. https://doi.org/10.1016/j.biortech.2020.123063
6.90. Solovchenko, A. E., Ismagulova, T. T., Lukyanov, A. A., Vasilieva, S. G., Konyukhov, I. V, Pogosyan, S. I., … Gorelova, O. A. (2019). Luxury phosphorus uptake in microalgae. Journal of Applied Phycology (2019) 31:2755–2770 https://doi.org/10.1007/s10811-019-01831-8
6.91. Solovchenko, A., Verschoor, A. M., Jablonowski, N. D., & Nedbal, L. (2016). Phosphorus from wastewater to crops : An alternative path involving microalgae. Biotechnology Advances, 34(5), 550–564. https://doi.org/10.1016/j.biotechadv.2016.01.002
6.92. Sonune,A. & Ghate, R. (2004). Developments in wastewater treatment methods, Desalination, 167, 55-63, https://doi.org/10.1016/j.desal.2004.06.113.
6.93. Sutherland, D. L., Turnbull, M. H., Broady, P. A., & Craggs, R. J. (2014). Wastewater microalgal production, nutrient removal and physiological adaptation in response to changes in mixing frequ*ency. Water Research, 61*, 130–140. https://doi.org/10.1016/j.watres.2014.05.011
6.94. Takabe, Y., Hidaka, T., Tsumori, J. & Mizuhiko Minamiyama (2016). Effects of hydraulic retention time on cultivation of indigenous microalgae as a renewable energy source using secondary effluent, Bioresource Technology, 207, 399-408, https://doi.org/10.1016/j.biortech.2016.01.132.
6.95. Teijon, G., Candela, L., Tamoh, K., Molina-díaz, A., & Fernández-alba, A. R. (2010). Occurrence of emerging contaminants , priority substances (2008 / 105 / CE) and heavy metals in treated wastewater and groundwater at Depurbaix facility (Barcelona , Spain). Science of the Total Environment, 408(17), 3584–3595. https://doi.org/10.1016/j.scitotenv.2010.04.041
6.96. Varela, A. R., Ferro, G., Vredenburg, J., Yanik, M., Vieira, L., Rizzo, L., … Manaia, C. M. (2013). Vancomycin resistant enterococci: From the hospital effluent to the urban wastewater treatment plant. Science of the Total Environment, *450–451*, 155–161. https://doi.org/10.1016/j.scitotenv.2013.02.015
6.97. Julia M. Valigore, Peter A. Gostomski, David G. Wareham, Aisling D. O'Sullivan (2012). Effects of hydraulic and solids retention times on productivity and settleability of microbial (microalgal-bacterial) biomass grown on primary treated wastewater as a biofuel feedstock, Water Research, 46, 9, 2957-2964, https://doi.org/10.1016/j.watres.2012.03.023.
6.98. Wang, H., Xiong, H., Hui, Z., & Zeng, X. (2012). Mixotrophic cultivation of Chlorella pyrenoidosa with diluted primary piggery wastewater to produce lipids. Bioresource Technology, 104, 215–220. https://doi.org/10.1016/j.biortech.2011.11.020
6.99. Wang, J., Zhou, W., Chen, H., Zhan, J., He, C., & Wang, Q. (2019). Ammonium nitrogen tolerant Chlorella strain screening and its damaging effects on photosynthesis. *Frontiers in Microbiology*, *10*(JAN), 1–13. https://doi.org/10.3389/fmicb.2018.03250
6.100. Wang, X., Lu, X., Li, F., & Yang, G. (2014). Effects of temperature and Carbon-Nitrogen (C/N) ratio on the performance of anaerobic co-digestion of dairy manure, chicken manure and rice straw: Focusing on ammonia inhibition. PLoS ONE, *9*(5), 1–7. https://doi.org/10.1371/journal.pone.0097265
6.101. Wang, Yinghui, Liu, J., Kang, D., Wu, C., & Wu, Y. (2017). Removal of pharmaceuticals and personal care products from wastewater using algae-based technologies: a review. Reviews in Environmental Science and Biotechnology, *16*(4), 717–735. https://doi.org/10.1007/s11157-017-9446-x
6.102. Wang, Yue, Ho, S., Cheng, C., Guo, W., Nagarajan, D., & Ren, N. (2016). Bioresource Technology Perspectives on the feasibility of using microalgae for industrial wastewater treatment. Bioresource Technology, *222*, 485–497. https://doi.org/10.1016/j.biortech.2016.09.106
6.103. Xin, C., Addy, M. M., Zhao, J., Cheng, Y., Ma, Y., Liu, S., … Ruan, R. (2018). Waste-to-biofuel integrated system and its comprehensive techno-economic assessment in wastewater

treatment plants. Bioresource Technology, 250(November 2017), 523–531. https://doi.org/10.1016/j.biortech.2017.11.040

6.104. Xin, L., Hong-ying, H., Ke, G., & Ying-xue, S. (2010). Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga Scenedesmus sp. Bioresource Technology, 101(14), 5494–5500. https://doi.org/10.1016/j.biortech.2010.02.016

6.105. Xin, L., Hong-ying, H., & Yu-ping, Z. (2011). Bioresource Technology Growth and lipid accumulation properties of a freshwater microalga Scenedesmus sp . under different cultivation temperature. Bioresource Technology, 102(3), 3098–3102. https://doi.org/10.1016/j.biortech.2010.10.055

6.106. Xiong, J., Kurade, M. B., Abou-shanab, R. A. I., Ji, M., Choi, J., Oh, J., & Jeon, B. (2016). Bioresource Technology Biodegradation of carbamazepine using freshwater microalgae Chlamydomonas mexicana and Scenedesmus obliquus and the determination of its metabolic fate. BIORESOURCE TECHNOLOGY, *205*, 183–190. https://doi.org/10.1016/j.biortech.2016.01.038

6.107. Yang, J. S., Cao, J., Xing, G. L., & Yuan, H. L. (2015). Lipid production combined with biosorption and bioaccumulation of cadmium, copper, manganese and zinc by oleaginous microalgae Chlorella minutissima UTEX2341. *Bioresource Technology*, *175*, 537–544. https://doi.org/10.1016/j.biortech.2014.10.124

6.108. Yunlong Luo, Pierre Le-Clech, Rita K. Henderson (2020). Characterisation of microalgae-based monocultures and mixed cultures for biomass production and wastewater treatment, Algal Research, 49, https://doi.org/10.1016/j.algal.2020.101963.

6.109. Yoshida, H., Clavreul, J., Scheutz, C., & Christensen, T. H. (2014). Influence of data collection schemes on the Life Cycle Assessment of a municipal wastewater treatment plant. *Water Research, 56, 29*2–303. https://doi.org/10.1016/j.watres.2014.03.014

6.110. Zhang, T. Y., Wu, Y. H., Zhu, S. feng, Li, F. M., & Hu, H. Y. (2013). Isolation and heterotrophic cultivation of mixotrophic microalgae strains for domestic wastewater treatment and lipid production under dark condition. *Bioresource Technology*, *149*, 586–589. https://doi.org/10.1016/j.biortech.2013.09.106

6.111. Zhou, W., Hu, B., Li, Y., Min, M., Mohr, M., Du, Z., … Ruan, R. (2012). Mass Cultivation of Microalgae on Animal Wastewater : a Sequential Two-Stage Cultivation Process for Energy Crop and Omega-3-Rich Animal Feed Production. 348–363. https://doi.org/10.1007/s12010-012-9779-4

6.112. Zou, S., Kanimba, E., Diller, T. E., Tian, Z., & He, Z. (2018). Modeling assisted evaluation of direct electricity generation from waste heat of wastewater via a thermoelectric generator. Science of the Total Environment, 635, 1215–1224. https://doi.org/10.1016/j.scitotenv.2018.04.201