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Bioremediation of swine wastewater with locally isolated microalgal strains

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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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Resumo

O crescimento exponencial da população humana tem vindo a colocar muita pressão na gestão adequada de recursos hídricos em vários sectores (ex.: agrícolas, domésticos e industriais). Em particular, a agropecuária tem vindo a ter um aumento de recursos hídricos e de nutrientes para tentar acompanhar o desenvolvimento social e económico.

Tendo em conta a complexidade destes problemas, esta tese tem como objetivo melhorar o tratamento de águas residuais de suinicultura, usando microalgas como uma ferramenta de tratamento de águas. A utilização de microalgas permite captar os nutrientes presentes nas águas residuais, podendo depois a biomassa produzida ser usada em diferentes indústrias. Por outro lado, as águas residuais tratadas apresentam uma carga de nutrientes reduzida ou nula, podendo ser reutilizadas ou descartadas em segurança.

Para o desenvolvimento desta tese, foram selecionadas duas microalgas, *Chlorella* sp. e *Scenedesmus* sp. Ambas as espécies de microalgas foram produzidas em três sistemas de produção distintos: fotobiorreatores de coluna de 2 L, *raceways* pilotos de 600 L e um *raceway* industrial de 110 m^3. No decorrer do trabalho, as condições de crescimento foram otimizadas para cada espécie nos sistemas mencionados, e a performance de ambas as microalgas foi comparada a nível de produção de biomassa e da eliminação de nutrientes de um efluente proveniente de uma suinicultura. Ambas as espécies, *Chlorella* sp. e *Scenedesmus* sp., cultivadas no efluente com uma diluição de 1:20 (v/v) nos fotobiorreatores de coluna, registaram concentrações de biomassa bastante relevantes, 2,5 ± 0,4 g/L e 4,6 ± 0,3 g/L, respetivamente. Contudo, as melhoras taxas de remoção de 100 % de amoníaco, 82,8 e 80,3 % de nitratos e 73,2 e 100 % de fosfatos, foram observadas em ambas as respetivas microalgas cultivadas neste sistema de produção, no efluente com uma diluição de 1:10 (v/v), num regime de *fed-batch*.

Nos *raceways* pilotos, ambas as microalgas foram cultivadas em regime de *fed-batch* em efluente diluído a 1:40 (v/v), sendo que a *Chlorella* sp. atingiu uma concentração de biomassa máxima de 0,77 g/L. Por sua vez, a espécie *Scenedesmus* sp. atingiu valores máximos de peso seco de 0,38 g/L quando cultivada em Nutribloom® Plus (condição controlo) e 0,24-0,33 g/L quando cultivada em efluente de suinicultura com uma diluição de 1:40 (v/v), em regime de *fed-batch*. Por fim, a microalga com melhor performance, a *Chlorella* sp., foi cultivada num *raceway* industrial em regime semi-contínuo, no qual atingiu um peso seco máximo de 0,96 g/L ao fim de 15 dias de ensaio. No decorrer do

ensaio, foram obtidas taxas de remoção de amoníaco, nitratos e fosfatos de 71,99 %, 63,81 % e 95,58 %, respetivamente.

Em termos de composição bioquímica, *Chlorella* sp. cultivada em efluente diluído 1:22 (v/v) no *raceway* industrial, apresentou 36,31 ± 1,27 % de proteínas, 5,58 ± 1,25 % de lípidos, 10,83 ± 0,37 % de cinzas e 48,34 ± 1,58 % de glícidos. Tendo em conta esta composição bioquímica, sugere-se que a biomassa apresenta potencial para aplicação no setor da agricultura, nomeadamente para a produção de biofertilizantes e bioestimulantes, como também para a produção de bioetanol, bio-hidrogénio e biogás a partir de fermentação alcoólica, fermentação no escuro e digestão anaeróbica, respetivamente.

Em conclusão, esta tese mostrou que o tratamento de efluentes com microalgas permite reduzir significativamente a carga de nutrientes dos efluentes de suinicultura com a produção simultânea de biomassa de microalgas com um perfil bioquímico relevante.

Palavras-chave:
Microalgas; Efluente de suinicultura; *Chlorella*; *Scenedesmus*; Tratamento de efluentes

Abstract

The exponential growth of the human population has put a lot of pressure on water resources management in various sectors, such as agricultural, domestic, and industrial. Particularly, the agriculture and livestock sectors have been experiencing increased water resources and nutrient usage to accompany social and economic development.

Considering the complexity of these problems, this thesis aims to improve swine wastewater treatment using microalgae as a wastewater treatment tool. Microalgae are able to capture the nutrients present in wastewater, and the biomass produced can then be used in different industries, such as the agricultural industry. On the other hand, treated wastewater have a reduced or absent nutrient load and can be safely reused or disposed.

For the development of this study, two microalgae strains, *Chlorella* sp. and *Scenedesmus* sp., were produced in three different production systems: 2 L-bubble column photobioreactors, 600 L-pilot raceways, and 110 m^3-industrial raceway. The growing conditions were optimized for each species in the mentioned systems. The performance of both microalgae strains was compared in terms of biomass production and nutrient reduction from the wastewater. Both *Chlorella* sp. and *Scenedesmus* sp. cultivated in 1:20 (v/v) wastewater in bubble column photobioreactors had the best biomass concentration results, 2.5 ± 0.4 g/L and 4.6 ± 0.3 g/L, respectively. However, the best removal rates (100 % of ammonium, 82.8 and 80.3 % of nitrates and 73.2 and 100 % of phosphates) in this production system were observed in both microalgae grown in fed-batch under 1:10 (v/v) diluted effluent.

In the pilot-scale raceways, both microalgae strains were cultivated in swine wastewater diluted to 1:40 (v/v). In these production systems, *Chlorella* sp. cultivated in 1:40 (v/v) wastewater in fed-batch regime reached a maximum biomass dry weight of 0.77 g/L. In contrast, *Scenedesmus* sp. reached a maximum dry weight value of 0.38 g/L when grown in Nutribloom® Plus (control conditions), and 0.24-0.33 g/L in diluted pig wastewater, in the same growth regime. Finally, the best-performing microalga, *Chlorella* sp., was cultivated in an industrial-scale raceway using 1:22 (v/v) diluted swine wastewater using a semi-continuous growth mode, reaching a maximum dry weight of 0.96 g/L after 15 days. During this period, maximum removal rates of ammonium, nitrates and phosphate of 71.99 %, 63.81 % and 95.58 %, respectively, were obtained.

In terms of biochemical composition, *Chlorella* sp. grown in 1:22 (v/v) wastewater in the 110 m^3-industrial raceway presented 36.31 ± 1.27 % of proteins, 5.58 ± 1.25 % of lipids, 10.83 ± 0.37 % of ashes and 48.34 ± 1.58 % of carbohydrates. The obtained biochemical composition suggests that the biomass holds potential for the development of biofertilizers and bio-stimulants, as well as produce bioethanol, biohydrogen and biogas, through alcoholic fermentation, fermentation in dark and anaerobic digestion, respectively.

In conclusion, this work showed that the wastewater treatment using microalgae allows to significantly reduce the nutrient load of swine effluents with the simultaneous production of microalgae biomass with a relevant biochemical profile.

Keywords:
Microalgae; Swine Wastewater; *Chlorella*; *Scenedesmus*; Wastewater treatment

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List of Abbreviations

ATP – adenosine triphosphate

BOD – biochemical oxygen demand

CC – cell count

COD – chemical oxygen demand

DW – dry weight

IBC – intermediate bulk container

NADPH – nicotinamide adenine dinucleotide phosphate

NB^+ - Nutribloom® Plus

OD – optical density

PBR – photobioreactor

RW – raceway

SD – standard deviation

SS – solid suspended

TAG – triacylglycerids

tbcPBR – tubular bubble column photobioreactor

TN – total nitrogen

TP – total phosphorus

TSS – total solid suspended

WW – wastewater

1. Introduction

1.1. Wastewater

Over the past decades, the global population has been growing fast, followed by increasing demand for fresh water, energy, and food (Seitzinger *et al.*, 2005; Chai *et al*., 2021). This growth has led to an increased discharge of effluents or wastewaters (WW), which, most of the time, are unsafe for the public and environmental health (Seitzinger *et al.*, 2005; Gonçalves *et al.*, 2016; Yousuf, 2020).

Even though water covers 71 % of our planet's surface, 2.5 % of it is fresh (Khatri, 2015). From the total water available, only 0.007 % is available for human consumption, and if this resource gets contaminated, it will threaten all living organisms (Sonune and Ghate, 2004; Lorenzo and Kinzig, 2020).

Due to the scarcity of drinking and potable water, there is a need to develop efficient WW treatments so that the water used for human activities, such as agricultural activities and livestock production, can be recycled. Additionally, WW treatment can be complemented with efficient nutrients' removal from these WWs for agricultural activities (Martínez *et al.*, 2000; Lofrano and Brown, 2010; Rawat *et al.*, 2011; Mata *et al.*, 2012).

According to their source, WW can be classified into two major groups: urban and industrial. In turn, they are subdivided as domestic, commercial, urban/municipal, among others (Lofrano and Brown, 2010; Crini and Lichtfouse, 2019). Depending on the source, they are composed of different compounds, including inorganic and organic matter, heavy metals, pesticides, pharmaceuticals, hormonal residues, and many other hazardous elements (Martínez *et al*., 2000; Ho *et al*., 2012; McGinn *et al.*, 2012; Cheng *et al.*, 2019). Hence, depending on the WW composition, different treatments can be applied to clean the contaminated water to be safely discharged or reused (Mishra *et al*., 2018).

The standard WW treatment is generally organized in five successive steps: i) pre-treatment, based on the removal of heavy inorganic solids; ii) primary treatment, which includes grit removal, screening, and sedimentation; iii) secondary treatment, where the oxidation of dissolved organic matter occurs; iv) tertiary treatment, for nutrient removal, and chemical (oxidation, precipitation or disinfection) and physical (pumping, screening sedimentation or filtration) methods; and v) sludge treatment, which is the treatment of the waste generated along with all the other steps (Fig. 1.1).

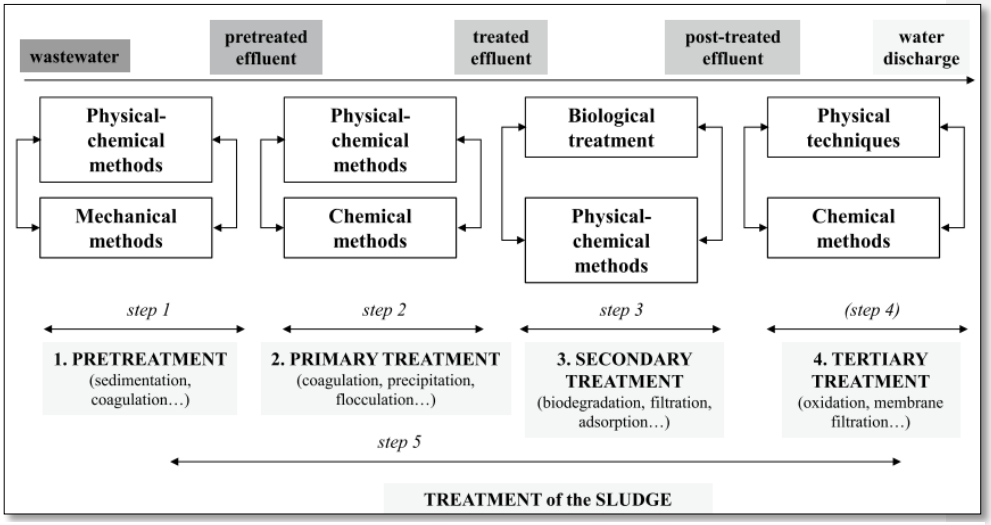


Figure 1.1: Conventional wastewater treatment steps. The treatment methods can be classified into physical (screening), chemical (chemical precipitation) and biologic (activated sludge process), where in every steps, sludge is generated (Crini and Lichtfouse, 2019).

The preliminary treatment step aims to remove coarse solids and other large materials often found, such as sand and gravel in raw WW, through screening and/or grit chamber (Sonune and Ghate, 2004; Kumar *et al.*, 2019). This step does not depend much on the WW source or composition, mainly separating visible solids from the contaminated water (Kumar *et al.*, 2019).

After removing the heavy solids, the primary treatment step removes the organic and inorganic solids by physical processes such as precipitation and flocculation (Sonune and Ghate, 2004). The purpose of this treatment step is to remove as many of the suspended solids as possible. Depending on the WW source and composition, it is possible to remove about 25-50 % of biochemical oxygen demand (BOD), 50-70 % of the total suspended solids (TSS), and 65 % of oil and grease, while colloidal and dissolved

constituents are not affected (Sonune and Ghate, 2004; Kumar *et al.*, 2019). This step can be processed with a combination of physical-chemical (pumping, screening, sedimentation, or filtration with oxidation, precipitation, or disinfection) and mechanical treatments. These treatments can be different according to the company and/or the type of water (Hendricks, 2006; Rawat *et al.*, 2011; Mishra *et al*., 2018). The WW resulting from the primary treatment consists mainly of colloidal, dissolved organic, and inorganic solids (Sonune and Ghate, 2004).

The secondary treatment is used to remove the residual organics and suspended solids (Sonune and Ghate, 2004; Kumar *et al.*, 2019). This treatment can be processed with physical-chemical methods or biological treatment, such as the activated sludge and anaerobic processes (Sonune and Ghate, 2004). The performance of this step is mainly measured in terms of BOD and suspended solids (SS) removal, reaching an efficiency of removal between 85 % to 95 % (Sonune and Ghate, 2004; Kumar *et al*., 2019). However, the cost of this treatment is about 3 to 4 times higher than the primary treatment, which only removes 30 to 40 % of N and P (Wu, 1999).

The primary and secondary treatment removes the majority of BOD and total suspended solids (TSS) found in the WW but, nowadays, it has been proved that both steps are not enough to protect the receiving waters or provide reusable water for industrial and/or domestic usage. Because of this, the WW treatment can be continued by the implementation of three other treatments: tertiary treatment, physicochemical treatment, and combined biological-physical treatment depending on the WW composition, after the secondary treatment step (Sonune and Ghate, 2004). These advanced WW treatments can be used for the additional removal of organic and SS, nutrients, and toxic materials (Sonune and Ghate, 2004). A WW of better quality can be obtained with these added treatments than that achieved using the traditional secondary treatment (Sonune and Ghate, 2004).

Nevertheless, to achieve an efficient waste removal process and use a proper WW treatment, each WW must be previously studied and characterized (Hendricks, 2006; Rawat *et al.*, 2011; Mishra *et al*., 2018).

1.2. Swine Wastewater Treatment

The human protein requirements are estimated to be 0.8-2 g/Kg/d for adult men and women (Page *et al*., 2021). In order to meet the daily protein requirements, there are three major livestock activities practised worldwide: poultry, cattle, and pork, with the last one being the focus of this project.

Swine farming is one of the most important livestock activities to meet the increasing demand for proteins related to the expanding world population (Bohrer, 2019; Qu *et al.*, 2019). Nevertheless, swine production generates massive quantities of WW rich in ammonium and phosphorus from the urine and faeces, and in high chemical oxygen demand/biological oxygen demand (COD/BOD), resulting from pig excrements and cleaning of the hog house sheds (Nagarajan *et al.*, 2019; Qu *et al.*, 2019).

Nowadays, there are several conventional methods to dispose or treat swine WW, such as: i) disposal of animal manure directly as a cropland fertilizer; ii) waste stabilization ponds; iii) constructed wetlands; iv) usage of aquatic plant systems; v) aerobic systems; and vi) anaerobic digestion (Collos and Harrison, 2014; Nagarajan *et al.*, 2019). However, the processes mentioned above display significant disadvantages and challenges, such as using fossil fuels or risks associated with WW leaks into the surrounding environment.

Hence, developing an effective treatment for swine WW is of the utmost importance. In addition, most traditional WW processes are only focused on treating freshwater. Nonetheless, due to sustainability concerns, increasing attention is being given to recycling nutrients, specifically N and P present in different WW (Molinuevo-Salces *et al.*, 2016; Kabbe, 2018). When animal manure is disposed directly into the soil, there is the re-usage of freshwater and nutrients that can be uptake by the crops. Still, it usually contributes to soil contamination due to the presence of pathogens, pharmaceutical and hormonal residues, leading to the contamination of the crops and nearby water sources (Khoshnevisan *et al*., 2021).

1.3. The Need to Recycle Nutrients

In the last decades, the use of nutrients has been increasing to meet agricultural demands. N and P are key for agriculture since they are the main nutrients needed for plant growth. The dominant hotspots of agricultural N and P fertilizers were located in the United States of America and Western Europe, shifting to eastern Asia (N and P) and Brazil (P) (Xin *et al*., 2011). Surveys of country-level fertilizer input became available in the 60's, showing that the N/P ratio has increased 0.8 g/g per decade worldwide from 1961 to 2013 (Lu and Tian, 2017). In addition, during the same period, the usage of fertilizers rich in N and P increased nearly eight and three times, respectively (Lu and Tian, 2017). The current fertilizer requirements and their forecasted demands are related to human population growth and food demands (Grafton *et al*., 2015). Consequently, there is an increase in awareness of contaminated water and the future sustainability of N and P sources (Lu and Tian, 2017).

1.2.1. Nitrogen

Over the last century, the global cycling of reactive N has doubled due to the increasing human population and activities (Xu *et al.*, 2019; Beckinghausen *et al.*, 2020). In order to increase food production, this nutrient has been widely requested for crops and livestock activities. Although humans consume only 17 %, the remaining N is lost to freshwaters and the atmosphere (Beckinghausen *et al.*, 2020). The two main sources of NH_3 emissions are the volatilization from livestock manure and mineral fertilizer application (Xu *et al.*, 2019). The majority of N used for agricultural activities is achieved by the production of NH_3, obtained through the Haber-Bosch process. However, this method is an energy-intensive process that depends on fossil fuels, such as natural gas (Baltrusaitis, 2017; Kyriakou *et al.*, 2020). Normally, to produce one ton of anhydrous NH_3 fertilizer, about 949 m^3 of natural gas are required, and nearly 1.6 tons of CO_2 are released (Beckinghausen *et al.*, 2020). The supply of N by the Haber-Bosch process raised several environmental concerns, and better alternatives should be employed for fertilizer production (Baltrusaitis, 2017).

Alternative methods are being pursued, such as the production of green NH_4^+ through N photofixation (Li, Wang, and Gong, 2020) and treatment of livestock wastewaters, such as swine WW, to recover as many nutrients as possible (Molinuevo-Salces *et al.*, 2016; Nagarajan *et al.*, 2019; Wang *et al.*, 2019).

Depending on the size of the urban area, the European Urban Waste Water Treatment Directive set the maximum annual mean of total N (TN) concentration from 1.5 to 10 mg N/L (You *et al*., 2019).

1.2.2. Phosphorus

The supply of P for different industrial sectors is obtained through mining. However, significant concerns have been raised for modern agriculture due to the depletion of the global P reserves (Fig. 1.2) (Baltrusaitis, 2017; Barquet *et al.*, 2020). Over the years, the P paradox has been increasing: there is a lack of usable P, but there is an over-abundance of P in water bodies due to unsafe WW discharge and mines, damaging water quality (presenting an acyclic life) (Leinweber *et al.*, 2018; Barquet *et al.*, 2020). Because of this imbalance, the economic and political incentives for P recovery and reuse have increased. Unfortunately, several barriers need to be addressed, namely: 1) performance of technologies for phosphorous reuse across economic, agricultural, and environmental criteria remains understudied; 2) slow rate of change in the system that can support P recovery and create waste-derived products; 3) manure is an abundant yet under-utilized source of nutrient-rich material; and 4) at the global level, the importance of P management was long lacking in food and agriculture policy debate (Barquet *et al.*, 2020).

Depending on the size of the urban area, the European Urban Waste Water Treatment Directive set the maximum annual mean of total P (TP) concentration from 0.001 to 2 mg P/L. Still, conventional treatments such as chemical precipitation or biological removal cannot reach concentrations below 0.1 mg P/L (You *et al*., 2019).

Because of these uncertainties, and to improve the current recycling of nutrients, considering the circular economy principle, microalgae production seems to be a promising solution for the treatment of swine WW, with concomitant fixation of N and P in the microalgal biomass (Rawat *et al.*, 2011; Priyadarshani *et al*., 2012; Barquet *et al.*, 2020).

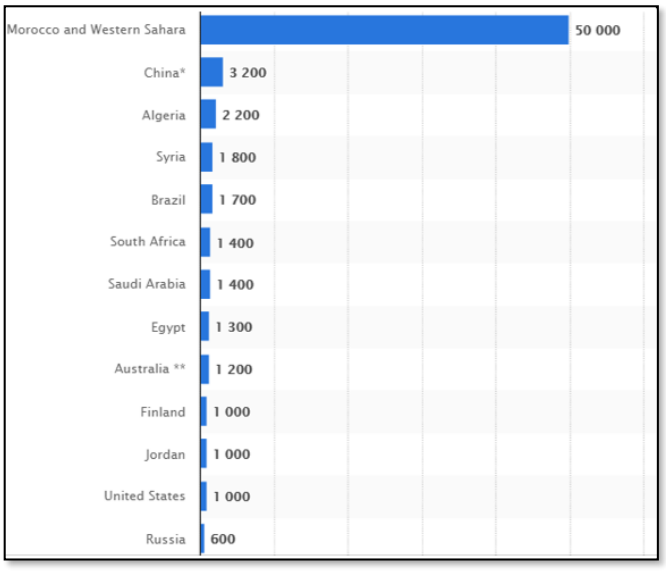


Figure 1.2: Phosphate rock reserves estimation in 2018 in million tonnes. (https://www.statista.com/statistics/681747/phosphate-rock-reserves-by-country/)

2. Microalgae-based Wastewater Treatment

Since the 50's, the usage of microalgae as a WW treatment step has been discussed (Oswald *et al*., 1957). In terms of nutrient removal, many authors have reported efficient usage of microalgae to treat different WWs (Wang *et al*., 2010; Kshirsagar, 2013; Andreotti *et al*., 2020; Wu *et al*., 2021).

Microalgae are considered a promising feedstock for biofuels and other commercial products. Coupling their production with swine WW treatment supports the potential development of the circular economy, making it more commercially feasible (Cheng *et al*., 2020). The treatment of WW using microalgae strains or consortium is a treatment step already achievable using municipal WW, which has low nutrient levels preventing toxicity of the microalgae, yet enough to generate microalgae biomass (Schulze et al., 2017, Li *et al*., 2019). Unfortunately, this treatment step still has many disadvantages, mainly in terms of operation costs, such as the high-energy input required to harvest microalgae cultures and the space needed to operate high volumes of WW (Cheng *et al*., 2020; Wu *et al*., 2021). Therefore, it is crucial to use competitive microalgae genus, such as *Chlorella* or *Scenedesmus*, because some bacteria (or other microorganisms) can

compete with microalgal growth (Chen *et al*., 2020). Nonetheless, microalgae can co-operate with bacteria, producing oxygen so that bacteria can remove organic matter. In contrast, microalgae can uptake nutrients from the WW, converting them into valuable resources and improving the overall biomass production (Cheng *et al*., 2015).

2.1. Microalgae

Microalgae are highly biodiverse unicellular microorganisms, with rapid exponential growth, and the ability to convert inorganic carbon and nutrients into different biochemical compounds through photosynthesis (Larsdotter, 2006; Sukla *et* al., 2019).

When microalgae production is compared with traditional crops, they display significant advantages over the cultivation of higher plants, such as higher photosynthetic efficiency, higher biomass productivity (40-50 %), and higher CO_2 fixation (1.83 CO_2/Kg of biomass) (Li *et al*., 2013; Gonzalez-Fernandez and Muñoz, 2017; Shahid *et al.*, 2020). In addition, microalgae can be cultivated on non-arable land using non-potable water and can double in number within hours under optimal conditions (Wang *et al.*, 2010; Ho *et al*.,2012; McGinn *et al.*, 2012; Cheng *et al.*, 2019; Sukla *et al.*, 2019).

Industrial production of photoautotrophic microalgae is successfully achieved in different production systems. On the one hand, they can be produced in open systems, such as raceways or open ponds that have the advantages of being less expensive and easier to process. On the other hand, they can be produced in closed systems, such as flat-panel and tubular photobioreactors, which ensure high biomass productivity and better control of environmental factors (e.g., bacterial or algal contamination, temperature and pH) (Larsdotter, 2006; Hulst, 2012; Yousuf, 2020).

In terms of industrial production, one reason that makes microalgae expensive is the culture medium (Rawat *et al.*, 2011; Acién *et al.*, 2012; Delrue *et al.*, 2012). This problem can be solved by replacing it with WW rich in N and P, reducing the environmental impact and microalgae production cost at the same time (Mata *et al.*, 2012, 2014; Wu *et al.*, 2012; Molinuevo-Salces *et al.*, 2016; Schulze *et al.*, 2017). After microalgal cultivation, the harvesting process represents another important and costly step, which can comprise 20-30 % of the total cost of biomass production (Rawat *et al.*, 2013).

Microalgae have a unique ability to adsorb, metabolize and/or accumulate harmful elements present in the WWs (Delrue *et al.*, 2012), being widely recognized as a promising phycoremediation tool (Rawat *et al.*, 2011; Mata *et al.*, 2012; Wu *et al.*, 2012;

Molinuevo-Salces *et al*., 2016). Since WWs are rich in inorganic compounds, it is feasible to use them as a microalgae cultivation medium (Larsdotter, 2006; Rawat *et al.*, 2011). In this way, microalgae play two key functions: the treatment of WWs, and the production of microalgal biomass with high applicability for several biotechnological applications, such as food, feed, biofertilizers, pharmaceutical, biofuels, among others (Wang *et al.*, 2010; Rawat *et al.*, 2011; Mata *et al.*, 2012).

Algae can be divided into 11 phyla: Cyanophyta, Dinophyta, Rhodophyta, Glaucophyta, Euglenophyta, Chlorarachniophyta, Charophyta, Cryptophyta, Haptophyta Heterokontophyta and Chlorophyta (Barkia *et al*., 2019). Chlorophyta comprises a group of green microalgae, which are considered an important source for sustainable production of high value-added chemicals (Baudelet *et al*., 2017).

2.1.1 Chlorophyta

The phylum Chlorophyta encompasses different strains, including the widely known genera *Chlorella*, *Scenedesmus*, *Tetraselmis*, and *Dunaliella*. Chlorophytes are the most common cultivated species of microalgae, used as additives for human foods and cosmetics, mainly *Chlorella* sp., *Dunaliella salina* and *Scenedesmus* sp. (Ambati *et al*., 2019; Sidari and Tofalo, 2019). Both *Chlorella* sp. (Figure 2.1A) and *Scenedesmus* sp. (Figure 2.1B) are the most used microalgal species from this class for N and P removal (Aravantinou and Manariotis, 2016). Chlorophytes are fast-growing unicellular freshwater algae that grow in a wide range of growth conditions and are known to accumulate high contents of valuable components (Kotrbáček *et al*., 2015; Ma *et al*., 2019). For this project, chlorophyte strains were chosen due to their resistance to high temperature, pH, and NH_4^+ concentration (Liu *et al*., 2019; Chuka-ogwude *et al*., 2020; Ye *et al*., 2020).

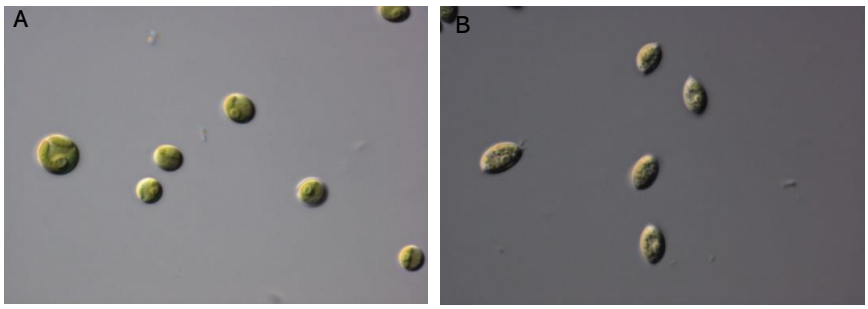


Figure 2.1: Microscopic observations of *Chlorella* sp. (A) and *Scenedesmus* sp. (B), using a differential interference contrast (DIC) microscopy and a 100 × lens with an additional 1.6 × amplification provided by an Optovar module.

2.2. Biochemical Composition

Depending on the microalgae species or strain, the biochemical profile can differ between growth phases or even among cultivation systems (Pereira *et al.*, 2019). For this reason, microalgae present high biochemical plasticity, accumulating different contents of intracellular macronutrients (proteins, lipids and carbohydrates) as a response to various biotic and abiotic conditions (Raja *et al.*, 2018; Shahid *et al.*, 2020).

2.2.1. Proteins

Proteins have an important role since they are present in the cells and tissues, and are needed for metabolism, hormones, antibodies, and the production of genetic material (Guedes *et al*., 2015). They are composed not only of carbon, oxygen, and hydrogen molecules, but they also contain N and, generally, sulphur in their composition (Guedes *et al*., 2015). On top of that, they also have both structural and metabolic functions.

The protein content and the amino acid profile helps to understand the nutritional value of microalgae for nutritional purposes (Williams and Laurens, 2010 and Guedes *et al*., 2015). The amino acids production by microalgae can vary depending on many external factors, as nutrient concentration, pH, turbulence, or salinity (Bondioli *et al.*, 2012). However, between species, both growth phase and light conditions do not relatively affect the protein amino acids composition during cultivation (Guedes *et* al., 2015; Sukla *et al.*, 2019).

Depending on the type of WW used and the microalgae species selected for the treatment of a given WW, different protein contents can be obtained. A review of the literature on this subject showed protein contents that range from 18.3 % to a maximum

of 67.0 % on *Botryococcus terribilis* (Cabanelas *et al*., 2013; Table 2.1). Michelon *et al.* (2019) and Dinnebier *et al*. (2021) reported that different strains of *Chlorella* grown in swine WW achieved 50.3 and 59.5 % of protein content, respectively. In other works, Perazzoli (2016) and Ferreira (2021) showed that *Scenedesmus* sp. presented 57.6 and 34.5 % of protein content, respectively. These high protein values can be responsible for the high N content present in the swine WW (Perazzoli *et al*., 2016).

2.2.2. Carbohydrates

Carbohydrates are mainly formed during the dark reaction of photosynthesis, where CO_2 is reduced during the Calvin Cycle, using energy resulting from NADPH and ATP, formed during the light reaction (Markou, Angelidaki and Georgakakis, 2012).

Similarly to proteins, carbohydrates also act as structural components in the cell walls (cellulose and insoluble polysaccharides) and have storage functions (plastids, mainly in the form of starch), serving as the starting point for the synthesis of biochemicals (Williams and Laurens, 2010; Markou, Angelidaki and Georgakakis, 2012).

The accumulation of carbohydrates is observed when the microalgal cells face environmental stress, typically nutrient limitation, which can be used during production as a strategy to maximize the intracellular content of carbohydrates in the biomass. However, other strategies can be implemented such as pH and temperature shifting, irradiance, and/or carbon source supplement (Chen *et al.*, 2013).

Different carbohydrate content can be obtained depending on the type of WW used and the microalgae species selected for its treatment. For example, a literature review on this subject showed maximum carbohydrate contents of 46.6 % in *Chlorella vulgaris* and 35.1 % in *Scenedesmus obliquus* when cultivated in swine WW (Wang *et al*., 2015; Ansari *et al*., 2017, Table 2.1).

2.2.3. Lipids

Lipids have a crucial role as energy reserves and are structural components present in the membranes of the cells. Microalgae lipids include neutral lipids, polar lipids, esters, sterols, carotenoids and quinines (prenyl derivates), and chlorophylls (pyrrol derivates) (Sharma *et al.*, 2012). Generally, they can be subdivided into two main groups: neutral lipids (mainly in the form of triacylglycerols (TAG)), which can be involved in the

production of the energy required for the cell, and polar lipids (mainly phospho- and glycolipids), being important structural components of organelles and cell membranes and can operate as signal molecules (Solovchenko, 2012; Yin *et al.*, 2020).

It is known that variations in temperature, pH, salinity and carbon/nitrogen (C:N) ratio in the culture media can have a stressful effect in the microalgae cells, resulting in the induction and accumulation of lipids, mainly in the form of TAG (Sharma *et al*., 2012; Chew *et al.*, 2017), which will be stored as energy reserves.

Table 2.1: Production of metabolites from microalgae grown in different wastewaters.

Species	Wastewater	Proteins (%)	Carbohydrates (%)	Lipid (%)	References
Ankistrodesmus falcatus	Aquaculture	30.59	33.88	35.9	Ansari *et al*., 2017
Botryococcus terribilis	Domestic	67	7.8	25	Cabanelas *et al*., 2013
Chlorella sorokiniana	Flocculated	36.6 to 25.5	15.4 to 20.0	8.8 to 19.8	Gupta *et al*., 2017
Chlorella sorokiniana	Aquaculture	28.81	35.4	31.85	Ansari *et al*., 2017
Chlorella sorokiniana LBA #39	Swine	59.5	23.4	3.1	Dinnebier *et al*., 2021
Chlorella spp.	Swine digestate	50.3	41	1.3	Michelon *et al*., 2019
Chlorella vulgaris	Domestic	65	3.9	27.3	Cabanelas *et al*., 2013
Chlorella vulgaris JSC-6	Swine	-	46.6	-	Wang *et al*., 2015
Chlorella zofingiensis	Swine	-	-	34.8	Zhu *et al*., 2013
Neochloris aquatica CL-M1	Swine	-	50.5	-	Wang *et al*., 2017
Parachlorella kessleri QWY28	Swine	-	56	-	Qu *et al*., 2019
Scenedesmus clone BF 063	Swine digestate	57.6	27.6	3.9	Perazzoli *et al*., 2016
Scenedesmus obliquus	Flocculated	37.4 to 28.5	13.6 to 20.4	8.6 to 16.0	Gupta *et al*., 2017
Scenedesmus obliquus	Brewery	31.4	30.2	17.9	Ferreira *et al*., 2019
Scenedesmus obliquus	Municipal	-	-	14.3	Ansari *et al*., 2018
Scenedesmus obliquus	Aquaculture	19.52	35.1	30.85	Ansari *et al*., 2017
Synechocystis sp.	Swine	40.8 - 42.6	19.6 - 19.8	23.9 - 26.3	Cheng *et al*., 2020
Tetradesmus obliquus	Swine	34.5	25.5	-	Ferreira *et al*., 2021
Tribonema sp.	Swine	18.3 - 19.6	11.9 – 11.1	40.7 - 42.4	Cheng *et al*., 2020

Over the past five decades, the interest in replacing conventional fossil fuels with eco-friendly fuel has increased, originating the third generation of fuels developed based

on microalgal biomass. Third generation (3G) fuels end up resolving the weakness of first-generation (1G) fuels, which directly compete with human and animal food production, and also the weakness of second-generation (2G) fuels, which require costly and modern innovations (Chowdhury and Loganathan, 2019). Nonetheless, 3G fuels still hold significant financial concerns (Chowdhury and Loganathan, 2019).

Nowadays, biofuels are being pursued through microalgal lipid production associated with CO_2 fixation studies and WW treatments to become a feasible and profitable product (Aravantinou and Manariotis, 2016; Schulze *et al.*, 2017; Cheng *et al.*, 2020).

The lipid content of microalgae cultivated in swine WW is similar to the carbohydrate content in some cases since the cells get stressed, and the metabolic response is to store energy for future days. This similarity depends on the light intensity, the N source, and/or the N depletion periods (Ho *et al.*, 2013). A literature review on this subject shows lipid contents ranging from 8.8 % to a maximum of 45.81 %, on *Chlorella zofigiensis* and 8.6 % to a maximum of 30.85 %, on *Scenedesmus obliquus* (Zhu *et* al., 2013; Gupta *et al*., 2017, Table 2.1).

3. Objectives

With the increase of WW production, many human and environmental health concerns have been discussed in the past decades. Achieving industrial microalgae production to treat these wastewaters can result in water that can be reused or safely discharged with simultaneous production of microalgal biomass, with high applicability for different biotechnological applications.

Having this in mind, the main objective of this project is to understand the potential of different microalgal strains for the treatment of swine wastewater. In order to reach this main goal, three major hypotheses must be answered:

i) Can microalgae remove the N and P present in swine wastewater?

ii) Which is the most effective microalgae strain for swine wastewater treatment?

iii) Can microalgae be used to treat swine wastewater at an industrial scale?

Accordingly, in order to answer these questions, different wastewater dilutions were tested using two different microalgal strains from laboratory to industrial scale using different production systems.

4. Materials and Methodology

4.1. Swine Wastewater

The swine WW used in this work was supplied by Valorgado - Agricultura e Pecuária, Lda. (Sarilhos grandes, Montijo) in the scope of the national project ALGAVALOR - *MicroALGAs: produção integrada e VALORização da biomassa e das suas diversas aplicações*. All studies and WW characterization were made in Necton's facilities.

4.2. Microalgae Cultivation

Two microalgae strains were used to evaluate the treatment of WW in the present work, *Scenedesmus* sp. and *Chlorella* sp. The strain of *Scenedesmus* sp. was isolated in the industrial microalgae production unit of Allmicroalgae (Pataias, Portugal) and supplied by the company. The *Chlorella* sp. strain was isolated in the scope of the ALGAVALOR project in a lagoon adjacent to the swine WW and was supplied by the MarBiotech laboratory (Centre of Marine Sciences, Portugal). Upon the arrival of the species, cultures were kept in 500 mL Erlenmeyer' flasks under controlled conditions in the inocula room of Necton S.A., and the scale-up process began.

Microalgal cultures were scaled from 500 mL Erlenmeyer flasks to 5 L bottles using Nutribloom® Plus (NB^+) culture media to a final NO_3^- concentration of 4 mM. Cultures were kept under constant aeration, bubbled with a mixture of air and CO_2, filtered through a 0.22 µm PTFE filter at a constant temperature of 19 ± 1 ºC. Later, when the cultures reached high cellular concentrations, they were used for the needed trials.

4.2.1. Cultivation Systems for Wastewater Treatment

4.2.1.1. Tubular Bubble Column Photobioreactors

At the beginning of this project, the ammonium concentration through optimal dilution in the microalgal culture medium was optimized in outdoor 2 L-tubular bubble column photobioreactors (tbcPBR; Fig. 4.1A) for each microalgal strain. The air was supplied from the bottom of the tbcPBR, and adjusted to promote an intense mixing of the liquid phase. Microalgal cultures were grown using a batch system, using four different WW dilutions (1:5, 1:10, 1:20, and 1:40, v/v) and one control condition (NB^+,

final NO_3^- concentration of 4 mM). Afterwards, both *Scenedesmus* sp. and *Chlorella* sp. were grown simultaneously in a fed-batch regime, where 1:40 (v/v) of WW was supplied on days 0, 3, 6, and then every 3 days, until day 19. The trials were conducted between February and April 2020.

4.2.1.2. Pilot-Raceway

The WW concentration optimized for each microalgal strain in the tbcPBR was later used in 0.6 m^3 raceways (RW). The RW were operated using a fed-batch growth regime, by adding 1:40 (v/v) of WW every 3 days, without achieving values higher than 100 mg/L of ammonium. The pilot-scale RWs were operated with a water column of 20 cm (600 L), and the culture flow velocity was set to 0.3 m/s. The pH of the cultures was controlled by an automated system that injected CO_2 when pH was higher than the set-point, pH 8 (Fig. 4.1B). The trials were conducted between November and December of 2020.

Figure 4.1: Microalgae production systems used to carry out the experimental trials: Tubular bubble column photobioreactors (A) and pilot-scale raceway pond (B)

4.2.1.3. Industrial Raceway

At the end of the study, the microalgae species with the best growth performance (*Chlorella* sp.) was selected and grown in an industrial-scale RW (110 m^3, Fig. 4.2) under the same conditions (water column height and pH range) of the pilot-scale RW. The industrial RW was grown in a batch regime in the first 10 days, followed by a semi-continuous growth regime (further explained below). In order to inoculate the industrial RW, *Chlorella* sp. was first cultivated in a 19 m^3 tubular PBR to produce enough inoculum for the industrial RW. The tubular PBR was composed of horizontal acrylic tubes, with a degassing zone, and the microalgal culture was mixed with the aid of a

pump. The PBR was operated for two weeks, using NB^+ as culture medium, with a pH set-point of 8.2, until achieving a maximum DW of 1.95 g/L.

After that, the RW was filled with 85 m^3 of water, and the 20 m^3 microalgal culture was transferred to the RW. In order to complete the inoculation, 5 m^3 of swine WW was added to the culture to reach a final total volume of 110 m^3, which corresponds to a dilution of 1:22 (v/v). Additional swine WW was added to the RW after 10 days, around 1-2 m^3 every day until the end of the trial (day 22), resulting in a total of 17 m^3 of swine WW during the assay. Biomass was harvested by centrifugation for 11 days, where 1-2 m^3 of culture were processed. At the end of the trials a total of 75 kg of wet microalgal paste, equivalent to more than 15 kg of dry *Chlorella* were harvested. The obtained biomass was packed and stored at -20 ºC for further biochemical analysis and to be used for further studies related to ALGAVALOR project. This industrial RW was operated during April 2021.

Figure 4.2: 110 m^3-industrial raceway inoculated with *Chlorella* sp.

4.3. Culture Monitoring

In each trial, samples were collected daily to monitor the microalgal growth for each condition. The growth was monitored by measuring:

4.3.1. Optical Density

The optical density (OD) was measured by transferring the culture samples to a plastic cuvette and measured at 540 nm in a spectrophotometer UVmini-1240 UV/VIS

Spectro (Shimadzu, Kyoto, Japan), using freshwater as a blank, every day. Samples were diluted when the OD measured was higher than 0.8.

4.3.2. Cellular Concentration

Cellular concentration (CC) was assessed every two days using a Neubauer chamber according to the manufacturer's procedure. If the cell count exceeded 300 cells per field, the sample was diluted. The CC was obtained with the following formula:

$$\mathrm{CC} = \text{Mean cell count} \times \text{dilution} \times 10^4 \ (\text{cells/mL})$$

4.3.3. Dry Weight

Dry weight (DW) was determined by filtration. Briefly, several filters were dried at 60 ºC for at least 4 hours. After that, they were placed in a desiccator for 10 minutes, weighed, and stored. Then, the filters were placed in a filtration system, and a known volume of microalgal samples was filtered. The filters were then washed with the same volume of distilled water and dried in an incubator at 60 ºC for 72 hours. The filters were put in the desiccator for 10 minutes and weighed when dried. The DW was determined using the following equation:

$$\mathrm{DW}\ (g/L) = \frac{(\text{Final filter weight (g)} - \text{initial filter weight (g)})}{\text{Sample volume (L)}}$$

4.3.4. Ammonium Concentration

The NH_4^+ concentration was performed using the Spectroquant® Ammonium Reagent Kit Test (Merck, Germany). Samples were centrifuged at 2800 g over 10 minutes, then 5 mL of the supernatant was filtered through a 0.22 µm filter and mixed with the reagents according to the manufacturer's procedure, at room temperature. After the reaction time (5 minutes), the sample was placed in a spectrophotometer UV-mini 1240 UV/VIS Spectro (Shimadzu, Kyoto, Japan) at 690 nm, and the concentration of ammonium was calculated using a previously established calibration curve. Samples were diluted with distilled water, whenever the OD was higher than 2 (a.u.) to prevent quantification errors.

4.3.5. Nitrate Concentration

The determination of nitrates was performed with a Spectroquant® Nitrate Cell Kit Test (Merck, Germany). Samples were centrifuged and filtered as previously described, following the kit instructions. The reagents were prepared and added to 1.5 mL of the supernatant. After the reaction time (10 minutes), the sample was measured at 525 nm, and the concentration of NO_3^- was calculated through a previously established calibration curve. Samples were diluted with distilled water if the OD was higher than 2, to prevent quantification errors.

4.3.6. Phosphate Concentration

The determination of phosphate concentration was performed with Spectroquant® Phosphate Reagent Kit Test (Merck, Germany). Samples were centrifuged and filtered as previously described, following the kit instructions, were added to 5 mL of the filtrated sample. After the reaction time (5 min), the sample was measured at 480 nm, and the concentration of phosphate was calculated through a previously established calibration curve. Samples were diluted with distilled water if the OD was higher than 1.5, to prevent quantification errors.

4.4. Biochemical Composition

The biochemical composition of the biomass produced in the pilot-scale RW and industrial RW was assessed. Samples from pilot RW were centrifuged at 2800 *g* for 10 min, and from the industrial RW, samples were centrifuged at around 9000 rpm for 2 hours. The samples were later lyophilized (LyoQuest Telstar, Terrassa, Spain) and stored at -20 ºC until further analysis.

4.4.1. Proteins

Protein content was determined using elemental analysis of C, H, and N by measuring the total N value. For this analysis, 1 mg of lyophilized biomass was weighed and stored in small aluminium caps and analysed using a Vario EL III (Elementar Analyser systems GmbH, Germany), according to the manufacturer's procedure. The obtained value of total N was multiplied by a conversion factor of 4.78 to obtain the total protein content (Lourenço *et al*., 2004).

4.4.2. Lipids

Total lipid content was determined using a modified protocol of Bligh & Dyer (1959) method (Pereira *et al*., 2018). The lyophilized biomass (10 to 20 mg) was weighed into tubes for lipid extraction, and 0.8 mL of distilled water was added to it. For each sample, 2 mL of methanol and 1 mL of chloroform was added and homogenised using an IKA Ultra-turrax disperser (Merck, Germany) at maximum speed (25000 rpm) for 60 seconds on ice. Afterwards, 1 mL of chloroform was added, and samples were again homogenised for 30 seconds on ice. In the end, 1 mL of distilled water was added to each sample and further homogenised for 30 seconds. All samples were centrifuged at 2800 g for 10 minutes for phase separation. Later, using a Pasteur pipette, the organic phase (chloroform layer) was transferred into new tubes and from these, a known volume (0.7 mL) was pipetted to previously weighed lipid tubes. These tubes were then put in a dry bath at 60 ºC until total evaporation of the chloroform. After this, the tubes were put in the desiccator until cooled and later weighed.

The percentage of total lipids were calculated using the following formula:

$$\%\ \text{total lipids} = \frac{\left[\frac{[(FW\ -\ IW)\ \times\ total\ volume\ of\ chloroform]}{evaporated\ volume\ of\ chloroform}\right]}{\text{sample weight}} \times 100$$

FW: final weight; IW: initial weight

4.4.3. Ashes

Ash content was determined through incineration, using 50 mg of lyophilized biomass of each sample. Weighed biomass was placed on labelled thermal crucibles and then placed in the oven (Nabertherm Controller B170, Nabertherm, Germany) for 8 hours at 525 ºC. On the following day, these crucibles were removed and stored in a desiccator for at least 10 minutes and then weighed.

The percentage of ashes was calculated using the following formula (Wibdom, 1984):

$$\%\ \text{total ash}\ = \frac{(\text{FW}\ -\ \text{IW})}{\text{Sample weight}} \times 100$$

4.4.4. Carbohydrates

The carbohydrates content was obtained by difference of the remaining macronutrients using the following formula:

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

4.4.5. Statistical Analysis

Statistical analyses were performed using IBM SPSS, version 26 (V Armonk, NY: IBM Corp.) using one-way ANOVA followed by Tukey tests ($p \leq 0.05$).

5. Results and Discussion

5.1 Swine wastewater characterization

The WW used during this experimental period was collected from the Valorgado's retention basin, which before each trial was characterized regarding the key nutrients, namely ammonium, nitrate and phosphate (Table 5.1). The level of nutrients present in the 20 L-jugs' WW used for the tbcPBRs ranged from 1886.6 to 2589.6 mg/L of ammonium, 129.2 to 505.5 mg/L of nitrates and 28.1 to 56.4 mg/L of phosphates.

From the 1 m^3-IBC used in the 600 L-pilot raceways, the ammonium concentration presented a variation during the trials, from 1775.1 to 2889.5 mg/L. Nitrate concentrations were lower than the WW used in the tbcPBRs, varying between 9.7 to 40.3 mg/L, while phosphates were higher than the WW used in the previous system, from 73.9 to 164.5 mg/L.

The WW collected from the 20 m^3-tanker truck presented ammonium concentrations with lower ranges, from 1915.3 to 1989.7 mg/L, nitrate concentrations from 35.2 to 56.5 mg/L and phosphate from 55.6 to 96.0 mg/L. Several samples were analysed during the industrial raceway trial before each WW addition.

Table 5.1: Wastewater nutrient values estimated for each system trial. Values are presented as mean ± standard deviation.

System	NH_4^+ (mg/L)		NO_3^- (mg/L)		PO_4^{3-} (mg/L)	
	Mean	SD	Mean	SD	Mean	SD

tbcPBR	2095.02	235.10	377.48	120.08	41.58	13.20
Pilot RW	2294.45	326.12	26.87	12.45	121.00	0.38
Industrial RW	1959.28	31.82	43.12	9.51	71.28	17.69

5.2 Ammonium Optimization using Tubular Bubble Column Photobioreactors

5.2.1. Cultivation of *Scenedesmus* sp. under different wastewater dilutions

In the first stage of this work, *Scenedesmus* sp. was cultivated in 2 L-tbcPBRs under outdoor conditions, with different WW dilutions (1:5, 1:10, 1:20, and 1:40), to evaluate the toxicity of the effluent. The obtained growth curves show that the maximum DW was obtained in the cultures grown with a swine WW dilution of 1:20 after 6 days, achieving 4.6 ± 0.3 g/L, followed by cultures grown at a dilution of 1:40, 1:10, Control (NB^+) and dilution 1:5 (Figure 5.1). Cultures grown with a dilution of 1:10 achieved a maximum DW of 3.6 ± 0.6 g/L after 9 days, while cultures grown at a dilution of 1:5 collapsed after 6 days of operation, due to the excess NH_4^+ concentration.

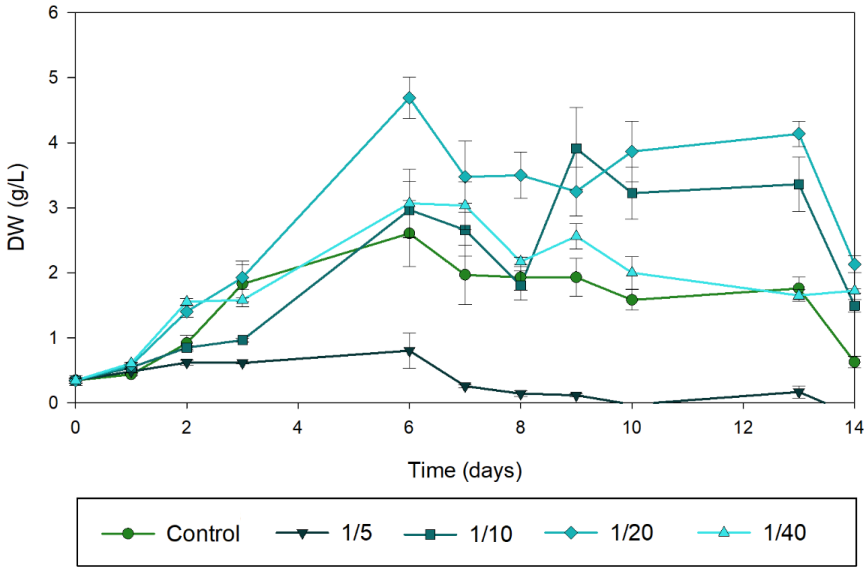


Figure 5.1: Growth performance, in dry weight (g/L), of *Scenedesmus* sp. grown in 2 L-tubular bubble column photobioreactors, under different dilution rates of swine wastewater (n=3).

At the beginning of the trial, nutrient concentration varied between treatments. For the control, NB^{+} was used at a final NO_3^- concentration of 4 mM, containing 124.0 mg/L of nitrates and 9.5 mg/L of phosphates. In the WW treatments, NH_4^+ concentration varied between 50.0 and 400.1 mg/L, while nitrates and phosphates varied between 12.6 and 124.0 mg/L, and 0.7 and 9.5 mg/L, respectively. At the end of the trial, NH_4^+ was only detected in the dilution of 1:5, at a concentration of 164.7 mg/L. Nitrates were found at a lower concentration, ranging from 5.5 to 9.5 mg/L, except for the treatment of 1:5 dilution (186.5 mg/L). Phosphates were absent or detected at residual concentrations, except for the 1:5 dilution (Table 5.2).

Comentado [HP1]: Confirmar se está bem

The decreasing NH_4^+ and PO_4^{3-} values could not be directly correlated with the cell's uptake, since the pH of the cultures was not controlled. The lack of pH control might have led to NH_4^+ stripping and phosphate precipitation, meaning that instead of being 100 % absorbed, the NH_4^+ could have been converted to NH_3 under alkaline pH, being volatile and easily dissipated from the cultures (Wang *et al*., 2021). Additionally, PO_4^{3-} is known to precipitate faster at pH higher than 12 (Gracida-Valdepeña *et* al., 2019; Wang *et al*., 2019; Lee *et al*., 2021; Khalil *et al*., 2021). Through this assay, it was possible

to see that *Scenedesmus* sp. had a higher nutrient consumption, along with higher growth performance, at a dilution of 1:20 followed by 1:10 and 1:40. In the present study, 4.6 times more biomass was obtained with swine WW, when compared to Doria *et al.* (2011), which only reached a maximum DW of 1 g/L after 20 days using *Scenedesmus acutus* cultivated with urban WW in a vertical tubular PBR. Ji *et al*. (2013) used filtered undiluted swine WW (520 mg/L NH_4-N) in a conical flask, and observed maximum growth on day 40, slightly above 0.6 g/L.

Table 5.2: Nutrient consumption of *Scenedesmus sp.* cultures throughout the trial. n.d - not detected

Nutrient	Condition	t_0	t_{14}	Consumption
NH_4^+ (mg/L)	Control	n.d.	n.d.	n.d.
	1:5	400.1	164.7 ± 9.1	58.8 %
	1:10	200	n.d	100.0 %
	1:20	100	n.d	100.0 %
	1:40	50	n.d	100.0 %
NO_3^- (mg/L)	Control	124	5.5 ± 4.3	95.6 %
	1:5	101.1	186.5 ± 0.4	0 %
	1:10	50.5	9.9 ± 0.2	80.3 %
	1:20	25.3	7.9 ± 0.9	68.6 %
	1:40	12.6	7.5 ± 3.7	40.9 %
PO_4^{3-} (mg/L)	Control	9.5	0.1 ± 0.0	99.1 %
	1:5	5.6	70.5 ± 0.6	0 %
	1:10	2.8	n.d	100.0 %
	1:20	1.4	0.0 ± 0.0	97.1 %
	1:40	0.7	0.1 ± 0.0	80.5 %

5.2.2. Cultivation of *Chlorella* sp. under different wastewater dilutions

Chlorella sp. was cultivated in 2 L-tbcPBRs under outdoor conditions, with the same WW dilutions as described for *Scenedesmus* sp. (1:5, 1:10, 1:20, and 1:40). The maximum DW was observed in cultures grown with a WW dilution of 1:20 after 9 days of trial, where it was achieved a 2.5 ± 0.4 g/L of DW. The second best-performing treatment was the cultures grown with a WW dilution of 1:10, followed by the Control (NB^+), and lastly the WW dilution 1:5 (Figure 5.2). Cultures grown with a WW dilution

of 1:10 achieved a maximum DW of 2.4 ± 0.5 g/L after 10 days of growth, while cultures grown with a dilution of 1:5 collapsed after 8 days.

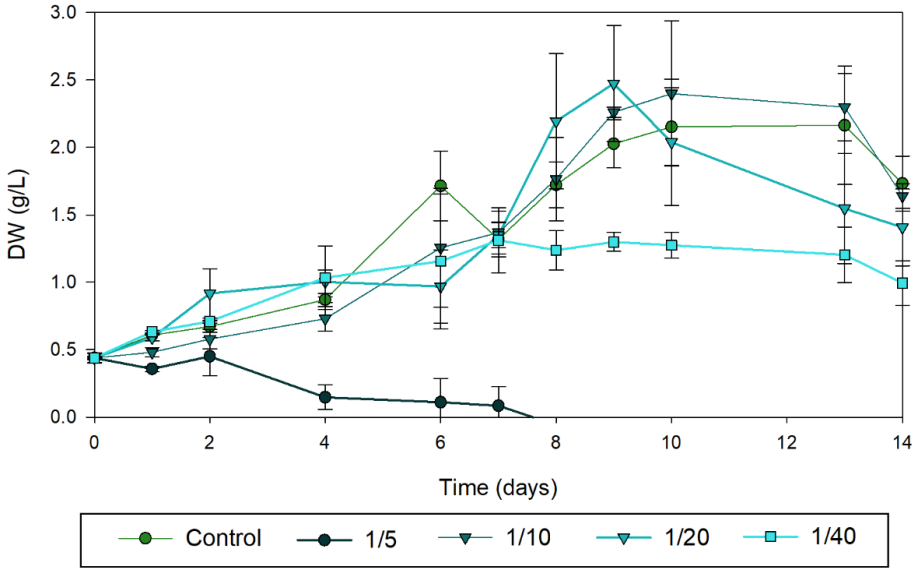


Figure 5.2: Growth performance, in dry weight (g/L), of *Chlorella* sp. grown in 2 L-tubular bubble column photobioreactors, under different dilution rates of wastewater, in triplicate.

At the beginning of the trial, nutrient concentration differed between treatments (Table 5.3). In cultures grown with different WW dilutions, NH_4^+ concentration ranged between 64.7 and 517.9 mg/L, while nitrates and phosphates varied between 9.9 and 79.3 mg/L, and 0.7 and 5.6 mg/L, respectively. For the control, NB^+ was used at a final NO_3^- concentration of 4 mM, containing 124.0 mg/L of nitrates and 9.5 mg/L of phosphates (Table 5.2). At the end of the trial, higher NH4+, NO3- and PO43 levels were only detected on cultures grown with WW diluted 1:5. On the remaining treatments, NH_4^+ was absent, whereas nitrates were detected at 6.8, 8.0 and 7.5 mg/L, and phosphates at 0.8, 0.8, and 0.4 mg/L in cultures cultivated with WW diluted at 1:10, 1:20, and 1:40, respectively.

Table 5.3: *Chlorella* sp. cultures' nutrient variation from day 0 (t_0, inoculation day) and day 14 (t_{14}, end of the trial). n.d - not detected

Nutrient	Culture	t_0	t_{14}	Consumption

NH_4^+ (mg/L)	Control	n.d.	n.d.	n.d.
	1:5	517.9	171.4	66.9 %
	1:10	259	n.d	100.0 %
	1:20	129.5	n.d	100.0 %
	1:40	64.7	n.d	100.0 %
NO_3^- (mg/L)	Control	124	8.9	92.8 %
	1:5	79.3	188.9	0 %
	1:10	39.7	6.8	82.8 %
	1:20	19.8	8	59.7 %
	1:40	9.9	7.5	24.3 %
PO_4^{3-} (mg/L)	Control	9.5	0.3	96.8 %
	1:5	5.6	10	0 %
	1:10	2.8	0.8	73.2 %
	1:20	1.4	0.8	44.9 %
	1:40	0.7	0.4	44.6 %

Both 1:10 and 1:20 WW dilutions proved to be useful for the cultivation of *Chlorella* sp. in the tbcPBRs. Even though a 1:10 dilution was apparently more efficient in nutrient removal, the 1:20 dilution of WW presented higher biomass production. Nonetheless, a 1:20 dilution of WW was selected for the next trial, using the same initial NH_4^+ concentration for both microalgae.

Higher growth performance was observed in Nam *et al.* (2016), where *Chlorella vulgaris* achieved 3.96 g/L when grown in swine WW diluted 8 times (300 mg/L ammonium) in 250 mL-baffled flasks. These different DW values obtained can be explained, since the author's cultures were grown indoors, with controlled conditions, while the cultures of the present work were all grown outdoors. A different approach used by Wang *et al*. (2021), growing *Chlorella vulgaris* in pre-treated swine WW, removed ammonium through ammonia stripping. Nonetheless, a maximum DW of 2.43 g/L on microalgae culture co-operating with bacteria was achieved, similar to the current study's maximum.

5.2.3. *Scenedesmus* sp. vs *Chlorella* sp. under the same wastewater dilution

After testing the growth of both species individually under outdoor conditions, a specific WW dilution rate was selected to simultaneously compare the growth of both species in a fed-batch regime. A dilution of 1:20 was selected since both microalgae showed higher biomass productivity at this WW dilution. All cultures were allowed to grow for 19 days. The best growth performance was observed for the control group of *Scenedesmus* sp., which achieved a maximum biomass concentration of 5.5 ± 1.3 g/L at day 17. *Scenedesmus* sp. grown in WW reached 2.6 ± 0.7 g/L, whereas *Chlorella* sp. grown in WW (2.3 ± 0.2 g/L) and control group of *Chlorella* sp. (2.2 ± 0.4 g/L) on the same day (Figure 5.3).

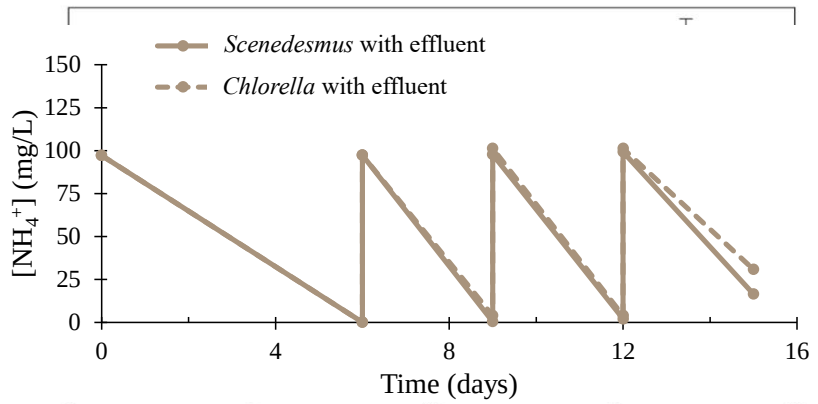


Figure 5.4: Ammonium variation during the trial with both strains grown in swine wastewater diluted 1:20 (v/v).

Figure 5.3: Growth performance, in dry weight (g/L), of *Scenedesmus* sp. and *Chlorella* sp. grown in 3-L tubular bubble column photobioreactors, with Nutribloom® Plus (control group) and 1:20 (v/v) dilution of swine wastewater. C_SCS – control *Scenedesmus* sp.; C_CHS – control *Chlorella* sp.; E_SCS – *Scenedesmus* sp. with effluent; E_CHS – *Chlorella* sp. with effluent

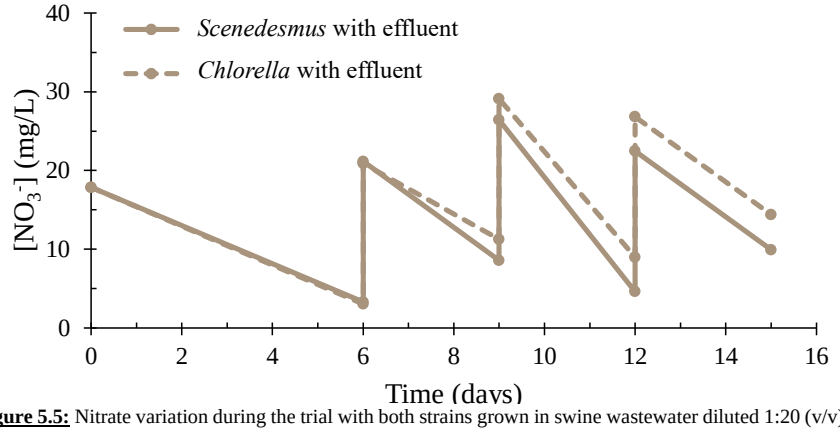


Figure 5.5: Nitrate variation during the trial with both strains grown in swine wastewater diluted 1:20 (v/v)

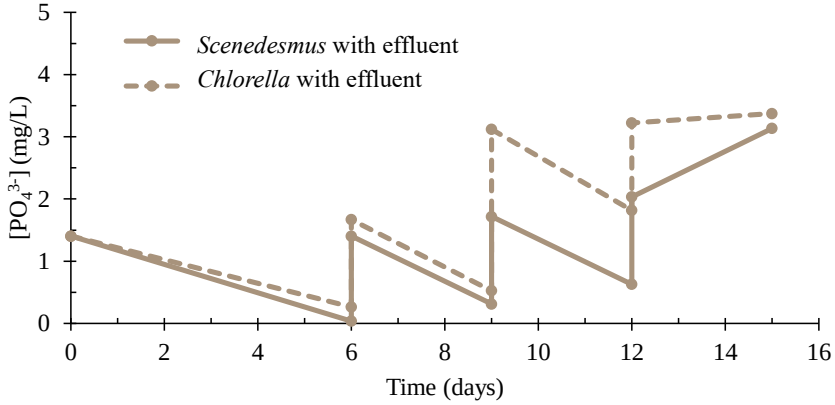


Figure 5.6: Phosphate variation during the trial with both strains grown in swine wastewater diluted 1:20 (v/v).

The ammonium present in the cultures with swine WW in both cultures displayed variations of 100 mg/L every 3 days, from day 6 to day 12, starting to increase on day 12 (Figure 5.4). This variation is related to microalgal uptake and the increase of the pH during the trial since this system did not have any pH control.

The nitrate variation (Figure 5.5) maintained stable during the trial. The phosphorus concentration (Figure 5.6) increased after 12 days of trial, where no uptake was visible in both strains. These results, complemented with bibliographic research, suggest that the control of the pH is crucial for efficient nutrient uptake, since phosphates started to increase after 12 days of trial, while it should have started to become the limiting nutrient (Zheng *et al*., 2019).

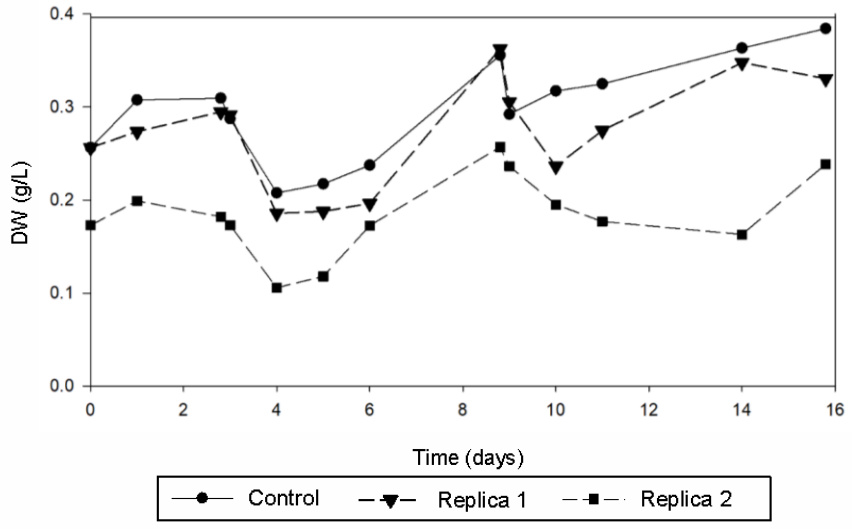
These results highlighted the need for pH control using CO_2 injection, which was addressed in the following production systems.

5.3 Pilot Raceways

5.3.1. *Scenedesmus* sp. cultivation

After the tbcPBR trials, *Scenedesmus* sp. was cultivated in two 600 L-pilot RW (Replica 1 and Replica 2) with a 1:40 dilution of WW and NB^+ (Control) in a fed-batch operation approach for 10 days. In order to avoid culture collapse, a higher dilution was employed (1:40, v/v), using a fed-batch regime, where new WW was added every 3 days. Using a dilution of 1:20 (v/v) resulted in the crash of the culture in a preliminary trial (data not shown), leading to a higher dilution in this system in the following trials.

The best growth performance was obtained on day 15 where the maximum DW of 0.4 g/L was obtained in the control culture, followed by WW Replica 2 and WW Replica 1, achieving 0.3 and 0.2 g/L (Figure 5.7). As expected, a higher biomass concentration was observed in tbcPBRs than in the pilot RW, 5.5 g/L and 0.4 g/L, respectively.



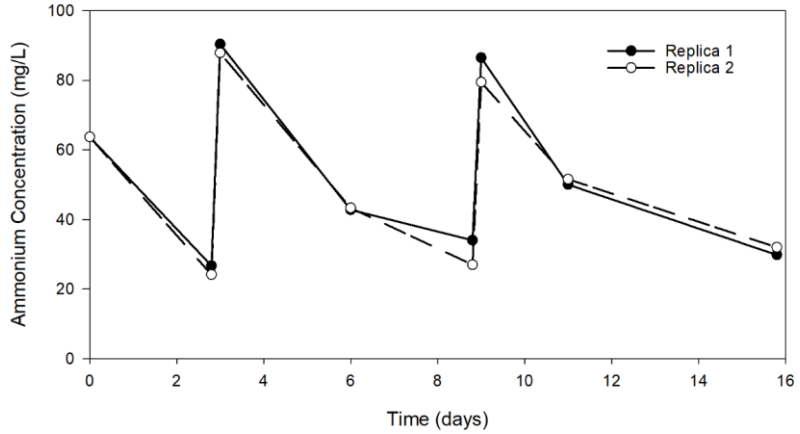


Figure 5.8: Ammonium variation along the assay, where the increase corresponds to an addition of wastewater.

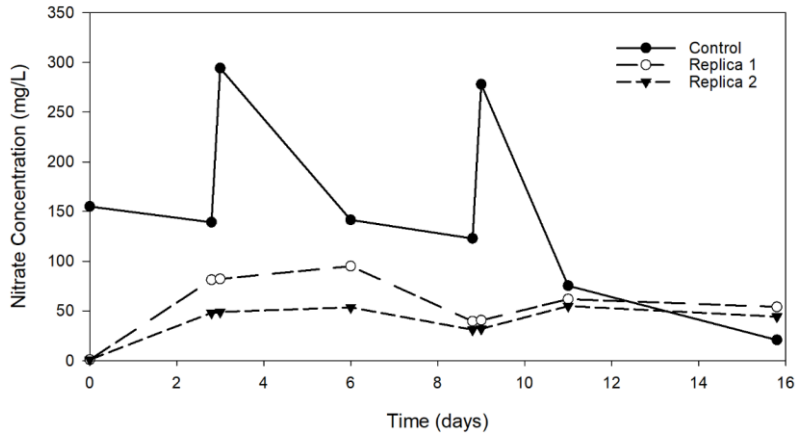


Figure 5.9: Nitrate variation along the assay, where the increase of nitrate concentration does not only correspond to the addition of wastewater.

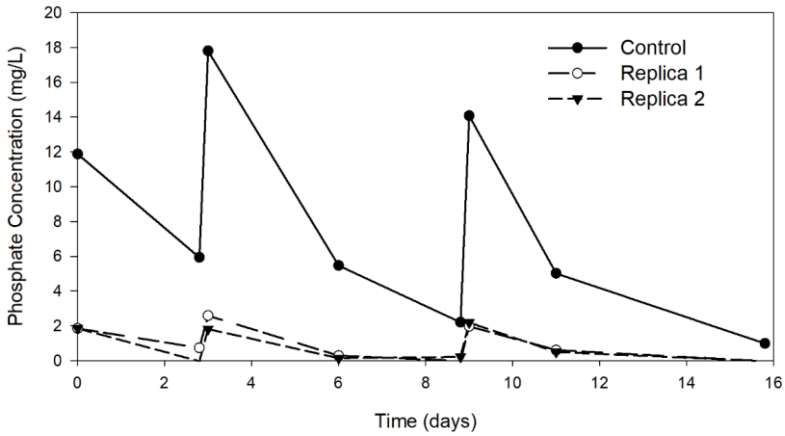


Figure 5.10: Phosphate variation along the assay, where the increase corresponds to an addition of wastewater.

In terms of nutrient consumption, it can be observed that in both ammonium (Figure 5.8) and phosphate concentrations (Figure 5.10) there was a clear decrease during the trial. However, phosphates were always at low concentrations, being the limiting nutrient in the cultures grown in WW.

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Regarding nitrate concentration (Figure 5.9), an interesting result was observed over the assay, where the nitrates levels increased to values higher than 90 mg/L. This result suggests the presence of nitrifying bacteria in the WW, responsible for converting ammonium into nitrates (Rajta *et al*., 2020).

5.3.2. Chlorella sp. cultivation

Chlorella sp. cultures were grown in the same conditions as described for the cultures of *Scenedesmus* sp., having two pilot RWs growing in 1:40 dilution of WW (Replica 1 and Replica 2) and one RW growing with NB^{+} as a culture medium (control).

The maximum DW was observed in the control cultures after 9 days of growth, achieving 0.9 g/L, followed by WW Replica 2 and WW Replica 1 on day 8 with 0.8 and 0.7 g/L, respectively (Fig. 5.12). Compared to the tbcPBRs, lower biomass production was observed in the pilot-scale RW. Open systems present an increased light path (20 cm), decreasing light penetration (self-shading) (Hosseini *et al*., 2015). In an open system, there are more disadvantages, such as higher contamination risks, low light distribution, and pH variation depending on the column height (Xiaogang *et al*., 2020; Bhatia *et al*., 2021). On the other hand, open ponds display low capital and operational costs, significantly decreasing the overall cost of biomass production (Jerney and Spilling, 2018; Li *et al*., 2019).

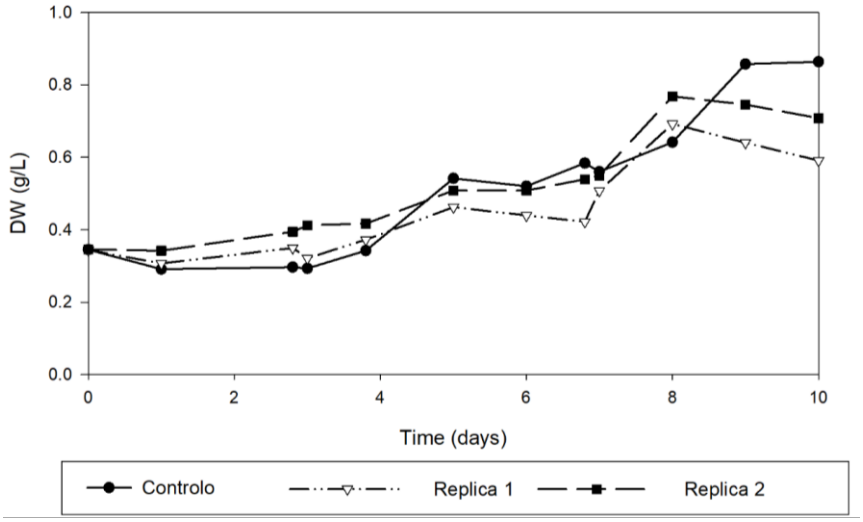

Figure 5.11: Growth performance, in dry weight (g/L), of *Chlorella* sp. in 600 L-pilot raceways, with Nutribloom® Plus (control group) and under the same wastewater dilution (1:40, v/v).

Comparing *Chlorella* sp. with *Scenedesmus* sp. grown in pilot-scale RW, *Chlorella* sp. grown in WW diluted 1:40 achieved a higher DW (0.7 – 0.8 g/L) compared with *Scenedesmus* sp. grown in WW with the same dilution (0.2 – 0.3 g/L). Therefore, *Chlorella* sp. was selected to be used in an industrial RW.

In terms of nutrient variation, the addition of more swine WW was dependent on the ammonium presence in the cultures (Figure 5.12), avoiding levels higher than 100 mg/L of ammonium to prevent culture collapse. Accordingly, WW was added three times (45 L) during the duration of the trial.

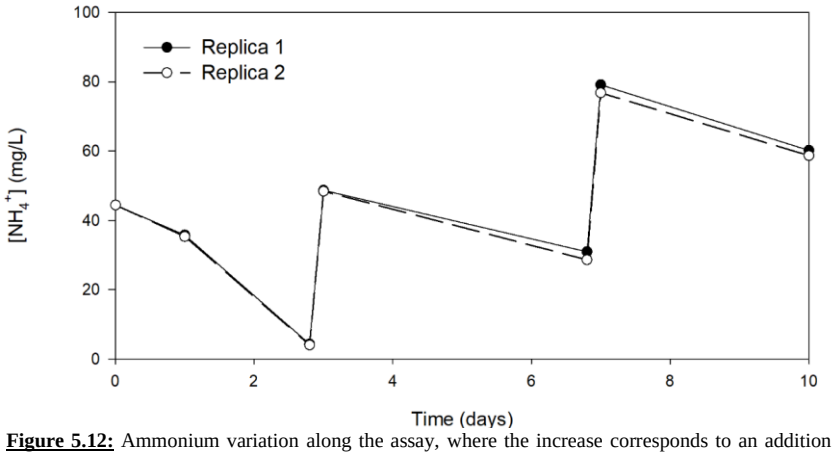

Figure 5.12: Ammonium variation along the assay, where the increase corresponds to an addition of wastewater.

Figure 5.13 shows a total uptake of nitrate in cultures grown in WW diluted 1:40 (v/v, Replica 1 and Replica 2) on day 3, showing reduced nutrient uptake in the following days. This event might be related to the preference of microorganisms to uptake nitrogen in the reduced form present in the medium (ammonia). Therefore, until all ammonia in the system was uptaken, cultures consumed low nitrate concentrations (Chen and Wang, 2020).

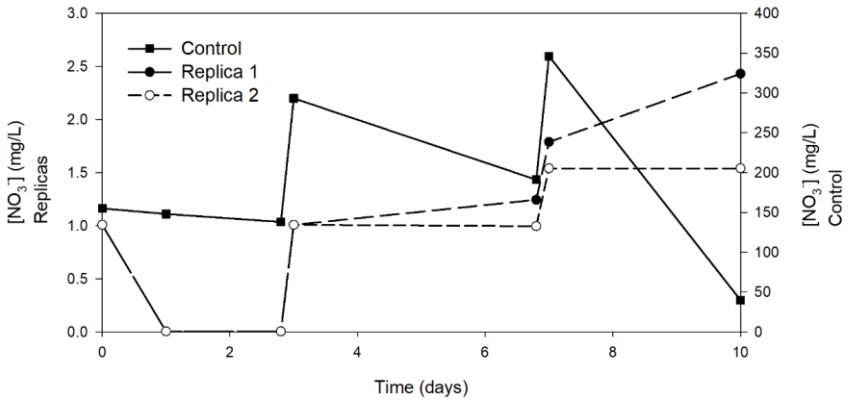

Figure 5.13: Nitrate variation along the assay, where the increase corresponds not only to wastewater administration, but also to the possibility of the presence of nitrifying bacteria.

Finally, the phosphorus variation (Figure 5.14) presented low values during the trial, being the limiting nutrient in the system, suggesting that increasing this nutrient would probably increase the biomass dry weight (Su, 2021; Yaakob *et al*., 2021).

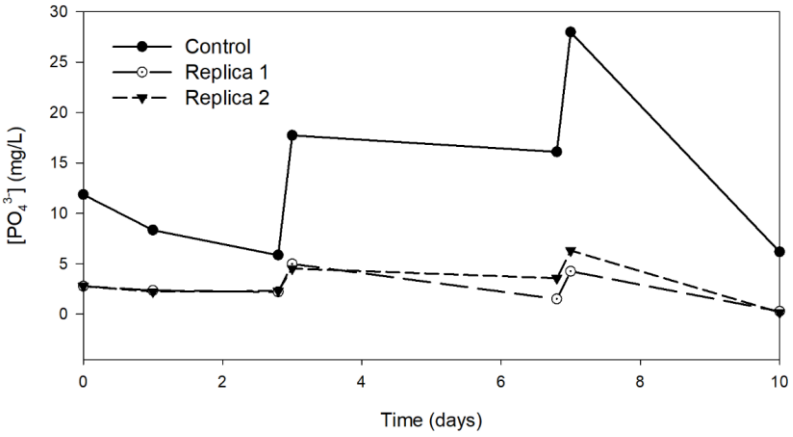

Figure 5.14: Phosphate variation along the assay, where the increase corresponds to an addition of wastewater

Overall, it is very difficult to compare the present data with other works, since most works use pre-treatments such as filtration of the WW, autoclavation, among others, while others use the raw swine WW in laboratory conditions (Cheng *et al*., 2020; López-Pacheco *et al*., 2021).

5.4 Industrial Raceway using *Chlorella* sp.

Chlorella sp. was cultivated in a 110 m^3-industrial RW with swine WW diluted 1:22 (v/v). The culture grew using a batch approach for 10 days, followed by a semi-continuous regime for more 17 days (Figure 5.15).

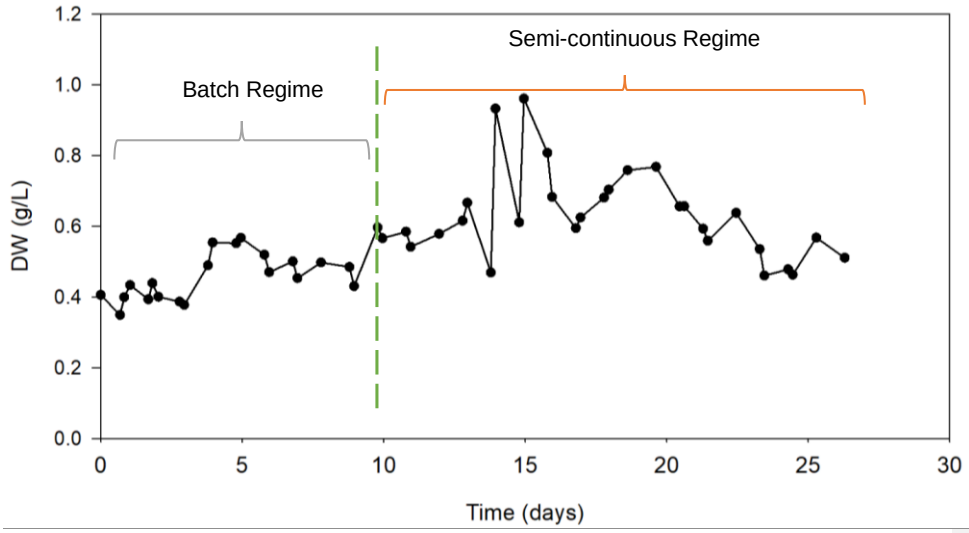

Figure 5.15: Growth curve of *Chlorella* sp. grown in swine wastewater in a 110 m^3-industrial raceway (RW). Dry weight (g/L) measurements were performed until the day of the trial (t27). n=1; During the first 10 days the RW was operated in a batch regime and afterwards using a semi-continuous regime for more 17 days.

The industrial-scale RW culture DW ranged between 0.41 and 0.96 g/L throughout the trial. The maximum DW was registered on day 15, when the culture reached 0.96 g/L. Following this day, all DWs values were lower, suggesting an increasing number of contaminants on the culture. Consequently, during the following days, increased stress was observed, evidenced by the formation of agglomerates, which

possibly led to an incorrect measurement of the DW (Alam *et al*., 2014; Cheng *et al*., 2020). By day 20, the presence of several contaminants, particularly vorticellas, rotifers and flagellates were observed in the culture (Figure 5.16). The contamination level and the limiting light due to the culture concentration and light path (0.20 m), observed from day 20 onward, probably contributed to the absence of growth shown in Figure 5.17.

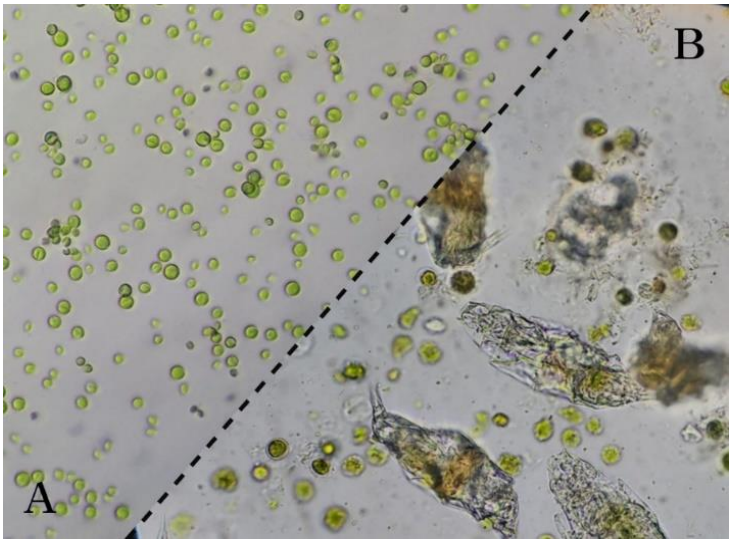

Figure 5.16: *Chlorella* sp. grown in the industrial raceway in 1:22 (v/v) wastewater. (A) inoculation day; (B) last operation day, highly contaminated with rotifers and vorticellas.

During the batch growth, the maximum NH_4^+ concentration registered was 59.6 mg/L (day 1), decreasing steadily to 16.7 mg/L (72.0 %) until the end of the batch regime (Figure 5.18). After day 20, *Chlorella* sp. was unable to uptake NH_4^+, increasing the toxicity (97.63 mg/L of ammonium) in the culture. The DW results (Figure 5.16) show that the high NH_4^+ levels might also have contributed to growth inhibition (Park *et al*., 2010; Akizuki *et al*., 2019).

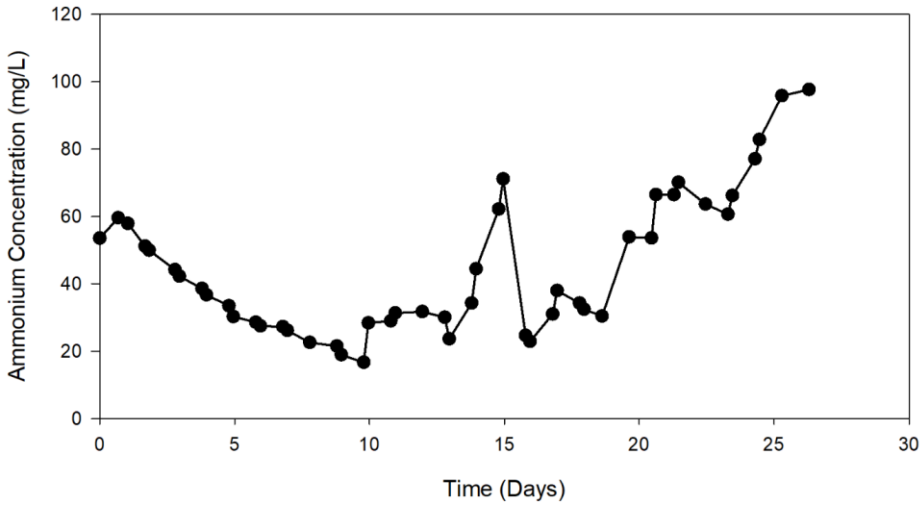

Figure 5.17: Variation of ammonium along the assay, where the increase corresponds to an addition of wastewater.

The nitrate concentration went from 93.2 mg/L to 80.2 mg/L (13.9 %) from day 0 to day 10, and from 93.5 mg/L to 61.4 mg/L (34.3 %) between day 10 to day 27 (Figure 5.19). At the beginning of the trial, two peaks of high NO_3^- were observed (t1 and t2, Figure 5.19), which probably resulted from sampling errors. However, after that, the NO_3^- concentration remained between 75 and 150 mg/L. Afterwards, during the semi-continuous regime, the NO_3^- concentration started to decrease gradually, which can be explained due to the presence of protozoa that can uptake NO_3^-, such as vorticellas, flagellates, ciliates, and zooplankton, such as rotifers (Figure 5.18) (Manan *et al*., 2016).

During the batch process, it would be expected that the nitrates levels would increase during this period in case of the presence of nitrifying bacteria. However, obtained results suggest that *Chlorella* sp. and denitrifying bacteria might be responsible for assimilating some part of the nitrates (Rajta *et al*., 2020; Manasa and Mehta, 2021).

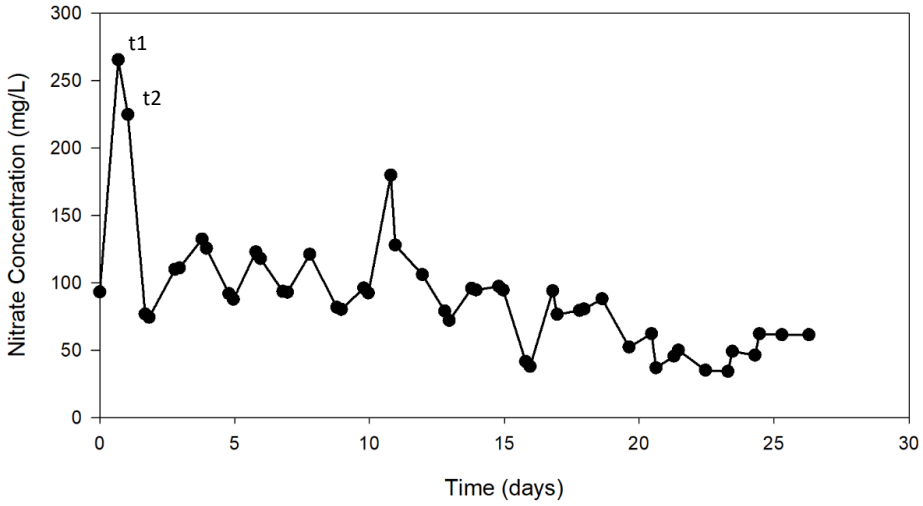

Figure 5.18: Variation of nitrate along the assay, the increase corresponds not only to an addition of WW, but to also to possible to bacteria oxidation of ammonia.

The PO_4^{3-} concentration started at 5.3 mg/L, decreasing steadily to values below the detection limit of the kit used until day 8, maintaining these low values throughout the rest of the trial (Figure 5.19). These results suggest that although a semi-continuous growth system was employed, with the frequent addition of P to the culture, the P levels were probably a limiting factor for microalgae growth. At the end of the trial, a lack of P uptake efficiency on day 24, resulting in a slight increase of PO_4^{3-} concentrations, was observed, suggesting possible cell death and cell disruption of *Chlorella* cells (Deng *et al*., 2019).

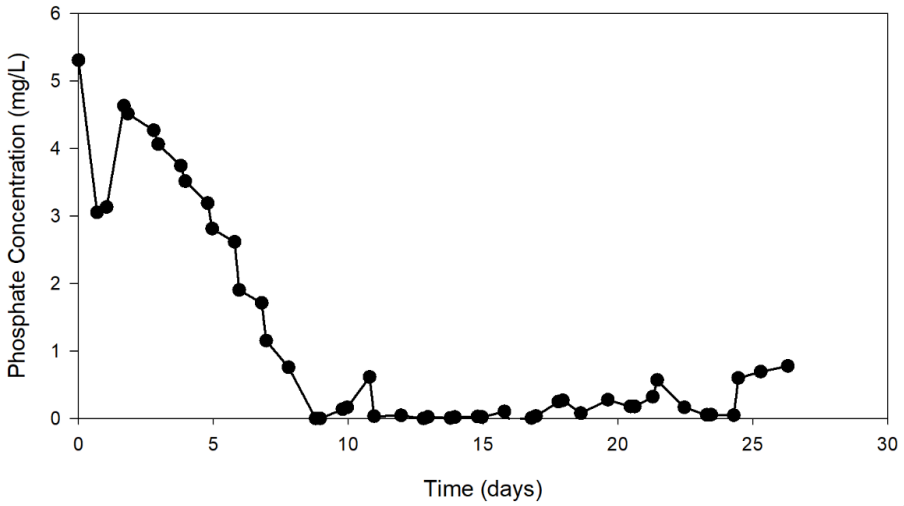

Figure 5.19: Variation of phosphates concentration along the assay, the increase corresponds to addition of more wastewater. After day 24, the increase of phosphate concentration is correlated to cell death.

No literature data was found on industrial studies of WW treatment using microalgae as a phycoremediation tool. Until today, most studies use laboratory or pilot scales' data to reflect industrial scale (Hasan *et al*., 2014; Nam *et al*., 2017; Chen *et al*., 2020). Unfortunately, that information can be misleading and hardly comparable with the data gathered in the present work. For example, the usage of filtered and autoclaved WW is achievable on small scales, however, at an industrial scale, where thousands of litres should be treated, the abovementioned methods are unfeasible.

5.5 Biochemical Analysis

The biochemical composition of *Chlorella* sp. grown in swine WW in the industrial scale raceway pond is shown in Figure 5.20. The total content of proteins, lipids, carbohydrates and ashes registered was 36.31 ± 1.27 %, 5.58 ± 1.25 %, 48.34 ± 1.58 % and 10.83 ± 0.37 %, respectively.

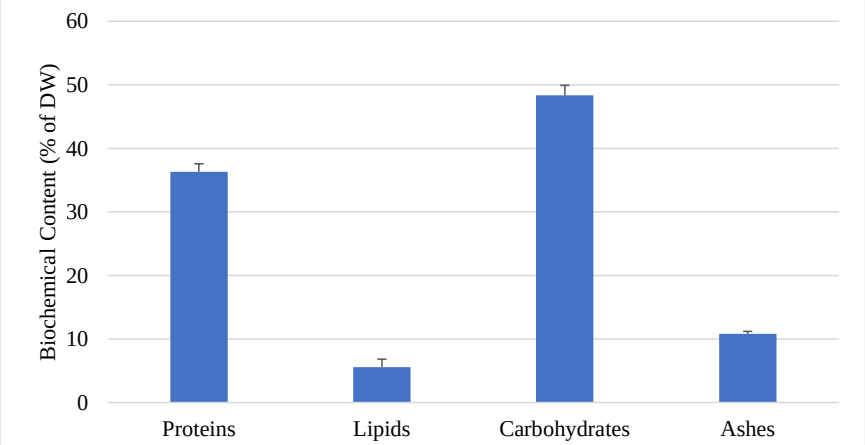

Figure 5.20: Quantification of total protein, lipid, ash, and carbohydrate contents (% of DW) from *Chlorella* sp. cultivated with swine wastewater in a 110 m^3-industrial raceway. Values are expressed as mean ± standard deviation (n=3).

Comparing these results with those obtained by Zhu *et al*. (2013) and Gupta *et al.* (2017), the protein content of *Chlorella zofingiensis* and *Chlorella sorokiniana* grown in WW was lower (19.82 and 25.5 % per DW, respectively). The microalgal protein content in the current study could be related to the N present in the culture (Wang *et al*., 2015). Therefore, the higher concentration of NH_4^+ in the WW effluents could have led to higher protein contents (Silveira *et al*., 2021).

On the other hand, the lipid content of *Chlorella* sp. grown in the industrial RW with swine WW was lower than 19.8 % of DW reported by Gupta *et al.* (2017) for *Chlorella sorokiniana* grown in WW. However, the values described in this study are similar to the values obtained by Dinnebier *et al*. (2021) using *Chlorella sorokiniana* grown in WW, which registered a lipid content of 3.1 % of DW. The low lipid content obtained in this work indicates that the cultures were growing actively since microalgae cultures commonly display low lipid contents under favourable culture conditions.

Regarding carbohydrates, the results obtained are comparable to those obtained in *Chlorella vulgaris* JSC-6 (Wang *et al*., 2015) and *Chlorella* sp. (Michelon *et al*., 2019), 46.6 % and 41 % of DW, respectively. Since the *Chlorella* genus is considered carbohydrate-rich (Ho *et al*., 2013; Wang *et al*., 2015; Souza *et al*., 2020), N-abundant swine WW such as the one used in this trial, might also be responsible for the increased carbohydrate content (Wang *et al*., 2015; Kaur *et al*., 2021).

Finally, the ash content was lower when compared to Silveira *et al*. (2021) that obtained 31 to 39 % of DW, using a 15 L-PBR. This lower value can be explained by the length of the industrial RW (80 meters on each side), having a higher possibility of residue precipitation in the industrial RW, and thus not being present in the biomass sample.

Considering the overall biochemical composition, the high carbohydrate content suggests that the biomass produced in the industrial scale RW using swine WW could be used to produce biofuels, namely for bioethanol or biogas production. Additionally, due to the relevant protein content, produced biomass also holds potential for the agriculture sector, as a source of biofertilizers and/or biostimulants. Although other applications could be suggested for the produced biomass, since cultures were grown in swine WW, the biomass upgrade is highly restricted due to the potential presence of pathogens and contaminants.

6. Conclusion

The treatment of wastewater using microalgae is a subject of high interest. Nevertheless, some caution must be taken, such as the usage of CO_2 to regulate pH, to assure the reduced formation of NH_3 and/or N_2 and also, in some cases, the high-water usage to dilute WW.

The present work provided knowledge about how microalgae can be applied as a phycoremediation tool for the treatment of swine WW and prepare methodically for each trial to prevent false results from external factors. *Chlorella* sp. and *Scenedesmus* sp. showed to be promising good candidates for this purpose, as they were successfully used in a pilot-scale PBR and 600 L-RWs, using swine WW as a nutrient source. It was also possible to conclude that *Chlorella* sp. can grow in a 110 m^3-industrial raceway using swine WW. This is particularly important since most previous works only focused on laboratory and pilot-scale systems, using synthetic or autoclaved swine WW.

It was possible to conclude that microalgae are a good treatment tool for swine WW treatment, since the main objectives of this work were attained: i) the selected species *Chlorella* sp. and *Scenedesmus* sp. were able to remove N and P present in the swine WW; ii) *Chlorella* sp. have shown higher biomass productivity than *Scenedesmus* sp., and; iii) *Chlorella* sp. was able to bioremediate 17 m^3 of swine WW in an industrial RW in 20 days.

However, in order to improve the recycling of water and nutrients present in WWs, additional studies are needed. These studies must focus on the percentage of initial inoculum and the use of polycultures. Nonetheless, this study is another step toward implementing circular economy processes since WW bioremediation is already confirmed.

The continuous improvement of industrial-scale processes to ensure an efficient recirculation of N and P compounds using microalgae holds a high potential to lower the impact of unsafety discharge of these WWs and production of microalgal biomass as well as future job opportunities in the biotechnology sector.

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