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BSc in Chemical and Biological Engineering

ASSESSING THE POTENTIAL OF ALGAL BIOFILMS FOR NUTRIENT REMOVAL IN WASTEWATERS

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To Nilva and Antônio.

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ABSTRACT

This study seeks to assess the performance of lab-scale microalgal-based biofilm systems in regards to nutrient removal and biomass growth. This entails the utilization of two bioreactors (A and B), with a raceway pond configuration. In the case of B, a moving bed biofilm reactor (MBBR) format was achieved by introducing polypropylene carriers as a substrate for microbial growth.

The results revealed that B was able to accumulate 23 times more biomass than A. This higher biomass retention capacity is attributed to the presence of carriers that provided an extra surface for biofilm growth. Only 4% of the total biomass consisted of suspended cells in B. Additionally, the removal of total nitrogen (TN) and total phosphorus (TP) was similar in both reactors, indicating that the incremental biomass present B did not lead to an optimized bioremediation by the culture. TP mass balance revealed high retention of P in A and B which hindered effective TP assimilation. Moreover, complete removal of ammonium (N-NH_4^+) was achieved in B earlier than A due to the presence of Ammonium Oxidizing Bacteria (AOB). N-NH_4^+ , nitrate (N-NO_3^-) and chemical oxygen demand (COD) analysis demonstrated signs of nitrification, however, despite the adjustments made to the photoperiod, high dissolved oxygen (DO) levels prevented the system from transitioning into anoxic conditions required for denitrification and consequently, a higher TN removal.

Limitations to the study included accurate sampling of the biofilm, establishing a microbial population capable of efficiently achieving TN and COD removal and understanding the intricacies of P removal by the culture. Future research aims to tackle these limitations through indirect methods of biofilm quantification, adjustments to photoperiod and the nutrient composition of the growth medium.

Keywords: WWT, microalgae, suspended systems, moving bed biofilm reactor, nutrient removal, biomass growth.

RESUMO

Este estudo visa avaliar o desempenho de sistemas de biofilme de microalgas em escala laboratorial em relação à remoção de nutrientes e à produção de biomassa. Isto envolveu a utilização de dois birreatores (A e B), com a configuração de lagoa em circuito fechado. No caso de B, foram adicionados carriers de polipropileno como substrato para o crescimento microbiano garantindo à este reator uma configuração de reator de leito móvel.

Os resultados revelaram que o reator B foi capaz de acumular 23 vezes mais biomassa do que o reator A. Esta maior capacidade de retenção de biomassa no sistema B foi atribuída à presença de carriers, que forneceram uma superfície adicional para o crescimento do biofilme. Apenas 4% da biomassa total no sistema B consistia em células em suspensão. Ademais, a remoção de azoto total (AT) e fósforo total (FT) demonstrou tendências semelhantes em ambos os reatores, o que indica que o aumento da biomassa no sistema B não otimizou a biorremediação. O balanço de massa de FT revelou uma retenção significativa de F em ambos os reatores, refletindo uma assimilação ineficaz de FT. A remoção completa de azoto amoniacal (N-NH_4^+) ocorreu mais tardiamente em A do que em B devido à presença de bactérias nitrificantes (AOB). Análises de N-NH_4^+ , nitrato (N-NO_3^-) e carência química de oxigênio (CQO) demonstraram sinais de nitrificação. No entanto, apesar dos ajustes feitos no fotoperíodo, os altos níveis de oxigênio dissolvido (OD) impediram a transição do sistema para condições anaeróbicas necessárias para a desnitrificação e, conseqüentemente, uma maior remoção de AT.

As limitações do estudo incluíram desafios relacionados à amostragem precisa do biofilme, ao estabelecimento de uma população microbiana capaz de alcançar eficientemente a remoção de AT e COD e à compreensão das complexidades da remoção de P pela cultura. Pesquisas futuras visam abordar estas limitações por meio de métodos indiretos de quantificação de biofilme, ajustes no fotoperíodo e na composição de nutrientes do meio de crescimento.

Palavras-chave: Tratamento de águas residuais, microalgas, sistemas suspensos, reator de leito móvel, remoção de nutrientes, crescimento da biomassa

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ACRONYMS

WWTP	Wastewater Treatment Plant
MBS	Microalgae- Bacterial Systems
TN	Total Nitrogen
TP	Total Phosphorus
COD	Chemical Oxygen Demand
BOD	Biological Oxygen Demand
TSS	Total Suspended Solids
MBBR	Moving Bed Biofilm Reactor
HRAP	High Rate Algal Ponds
F/FS	Fertiliser to Feed Solution Ratio
N:P	Nitrogen to Phosphorus Ratio
FF	Filling Fraction
AOB	Ammonium Oxidising Bacteria
NOB	Nitrate Oxidising Bacteria
HRT	Hydraulic Residence Time
SRT	Solid Residence Time

1 INTRODUCTION

1.1 Framework and Relevance of the Study

Recovery and treatment of wastewaters (WW) are crucial for the preservation of ecosystems and public health protection. Over the last century, the increase of the world population has led to a decrease of clean water sources, establishing a need for more efficient wastewater treatment (WWT) engineering technologies. On the other hand, with the pursuit of more stringent effluent standards to enhance water quality, comes also a great concern about the environmental impact and the economic viability of the WWT systems in place.

1.1.1 Challenges and Limitations of Conventional WWT

Currently, the conventional system of WWT consists of 3 main stages (Kesari et al.,2021): 1) Primary treatment, 2) Secondary treatment and 3) Tertiary treatment as can be seen in figure 1.1.

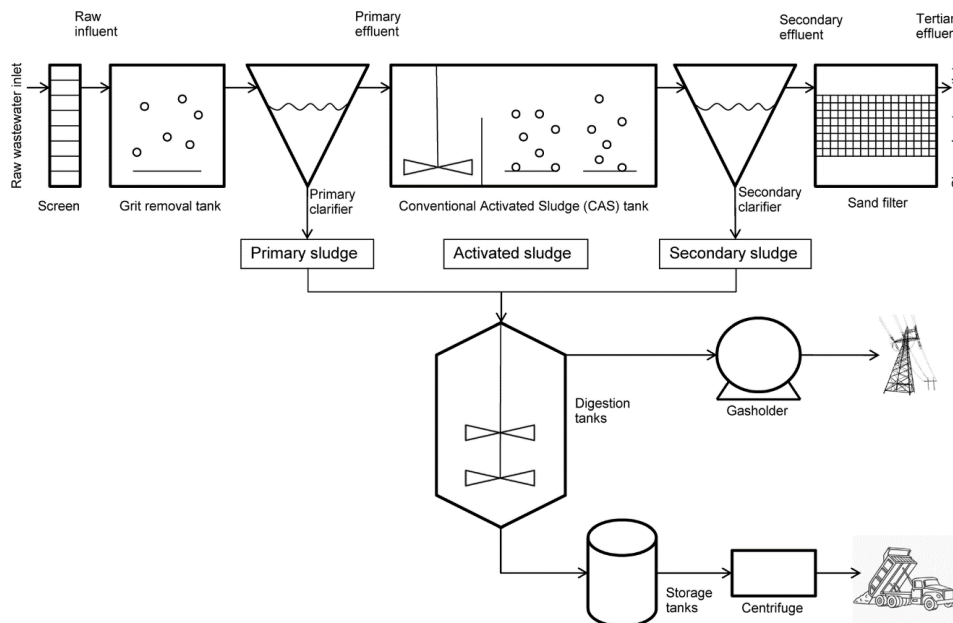


Figure 1.1- Typical WWT processes. Source: (Berthod et al., 2016)

Primary Treatment involves the screening and gravity-driven and/or chemical sedimentation of large solids and suspended particles present in raw wastewater. Primary treatment is followed by Secondary Treatment. In this stage, the dissolved organic matter present in the primary effluent is decom-

posed by microorganisms into CO₂, water, and energy for their growth. The resulting clear water undergoes additional processes, including nitrification, denitrification, chlorination, filtration, and disinfection, to ensure it is safe for discharge. Meanwhile, the solid waste generated during primary and secondary treatment (known as Primary and Secondary Sludge) is processed for various purposes, such as landfill disposal, use as fertilizers, building materials, and energy production. Lastly, Tertiary Treatment is crucial for achieving nearly 99% removal of all impurities from wastewater.

The energy demands of the Primary and Tertiary Treatment stages are typically lower compared to traditional Secondary Treatment. The high energy demand associated with Secondary Treatment can be attributed to the need for aeration to support the growth and proliferation of microorganisms. This aeration can account for a substantial portion of the plant's energy costs, ranging from 45% to 75% (Rosso, Stenstrom, & Larson, 2008). Furthermore, the overall emissions of greenhouse gases (GHG) such as N₂O and CO₂ is an increasing concern in this field.

In addition, sludge management also constitutes an obstacle for achieving an energy positive process in WWTPs. The increasing sludge production due to higher loads of WW coupled with the emergence of toxic pollutants have been increasing the costs of sludge treatment and hindering the commercial applications and disposal options. Secondary sludge also faces limited applications due to its low value biomass, and often is combined with primary sludge to increase the energy efficiency of the process.

1.1.2 Microalgal-Based Solutions for WWT

To address the limitations of conventional WWT processes, a potential strategy involves incorporating microalgae in biological processes either alone or in combination with other microorganisms such as bacteria. The utilization of microalgae in WWT has been gaining increasing attention from two fronts 1) Reducing aeration costs by utilizing microalgae as natural source of oxygen for heterotrophic microorganisms and 2) Enhancing nutrient removal through microalgae's capacity for bio assimilation of pollutants, in particular nitrogen and phosphorus. Additionally, microalgae cultivation can contribute to the reduction of GHG emissions, including CO₂ and N₂O as photosynthesis consumes CO₂ to produce O₂, and nitrogen removal process by microalgae does not release N₂O. Moreover, the resulting algal biomass is enriched in valuable compounds and can be harvested and used in various industries such as energy, food, cosmetic, pharmaceutical and others.

Numerous microalgae cultivation systems have been engineered throughout the years. According to the growth mode of the cells, these systems can be divided into two categories – Suspended Systems and Immobilized Systems.

In Suspended Systems, the cells grow freely in the aqueous medium whereas Immobilized Systems the cells grow as biofilm either bonded to each other or to a solid substrate through the production of EPS. Biofilm-based cultures offer advantages such as lower risk of washout, longer Solid Residence Time (SRT) and higher Area/Volume (A/V) ratio, which often translates into better process performance compared to Suspended Systems. From a downstream process perspective, these systems provide an easier and more cost-effective alternative for harvesting the resulting biomass.

Immobilized systems have been employed in WWT facilities for decades, utilizing bacterial populations to treat polluted influents. Moreover, Microalgal-Bacterial Systems (MBS) have been of increasing interest in the industry due to the synergistic interactions established between these two microorganisms.

The ever-changing nature of WW influents require a resilient population of microbes for its biological treatment, which places MBS as a preferable choice compared to monocultures of microalgae for this application. Therefore, combining immobilized cultivation methods in MBS holds significant potential to achieve better process performance and biomass recovery, while also providing a more sustainable and energy-efficient biological treatment.

1.2 Research goals and Hypothesis

This work aims to contribute to the efforts being made to create a more sustainable biological treatment in WWTPs, by understanding the intricacies of bioremediation and biomass production in microalgal-based biofilm systems.

In particular, the goal is to compare the performance of lab-scale microalgal- based biofilm systems to suspended systems in the context of nutrient removal and biomass productivity. To achieve this, two bioreactors (A and B) following the raceway pond configuration will be in place, however, B will comprise of polypropylene carriers that will serve as a substrate for microbial growth. A diverse population of microorganisms with prominent presence of microalgae sourced from nature will be growing in synthetic wastewater. With these two systems in place, the environmental factors limiting the development of the culture will be investigated as well as potential strategies to overcome those limitations.

The focus point is to understand the role of the increased surface area for biological growth offered by the carriers on process performance. By answering the following question: “Can the addition of carriers enhance the yield of bioremediation and biomass production in a suspended microalgal-based system?”, this study will provide insight into the feasibility of this method while also understanding the parameter aspects to have in mind when operating with this configuration in future projects. Figure 1.2 comprises an overview of the experimental work developed in this study.

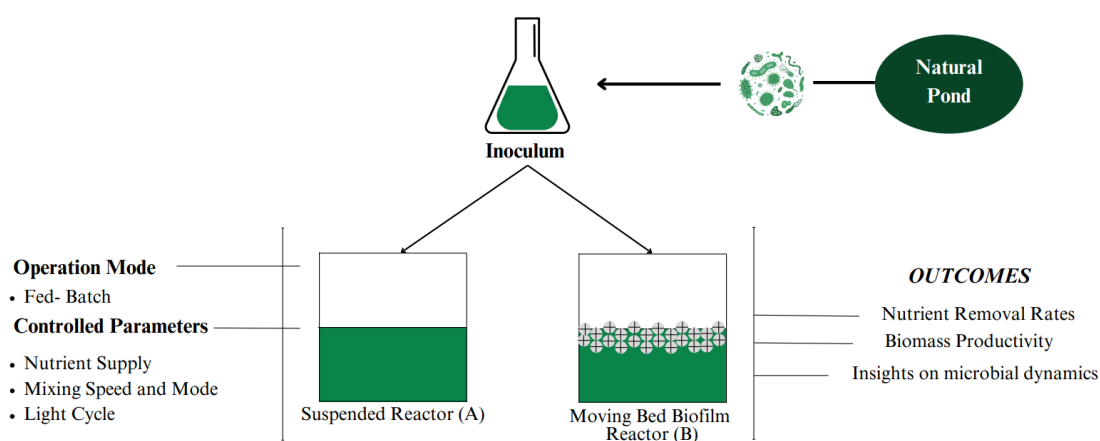


Figure 1.2 – Graphic Abstract of the present study

2 STATE OF THE ART

2.1 Current Role of Microalgae-based Systems in the WWT Industry

The large scale application of microalgal-based systems dates back to the early 20th century, when (Oswald et al., 1957) developed the high rate algal ponds (HRAP). These open raceway ponds distinguish themselves from other pond systems by their primary objective of maximizing algal biomass concentration to enhance their efficiency in WWT. Many countries including Israel, France, Morocco, the United Kingdom, Spain, Australia, China and New Zealand (Young, Taylor, & Fallowfield, 2017) have been employing these systems to treat a variety of wastewaters including municipal, agricultural and industrial (Craggs et al., 2015; de Godos et al., 2010; Godos et al., 2009).

HRAPs (Figure 2.1) are meticulously designed to facilitate the rapid proliferation of microalgae. This process plays a crucial role by supplying the oxygen necessary for driving the aerobic bacterial breakdown of organic compounds. Consequently, this breakdown generates the CO₂ essential for photosynthesis and subsequent nutrient absorption (Oswald, 1991). The presence of solar UV radiation, in conjunction with the heightened daytime oxygen levels and pH levels mediated by microalgae, naturally leads to pathogen disinfection within the WW (Davies-Colley, Craggs, & Nagels, 2003). The synergy between biological and solar treatments results in the WW achieving a level of treatment comparable to secondary and partial tertiary stages (Rawat et al., 2011).

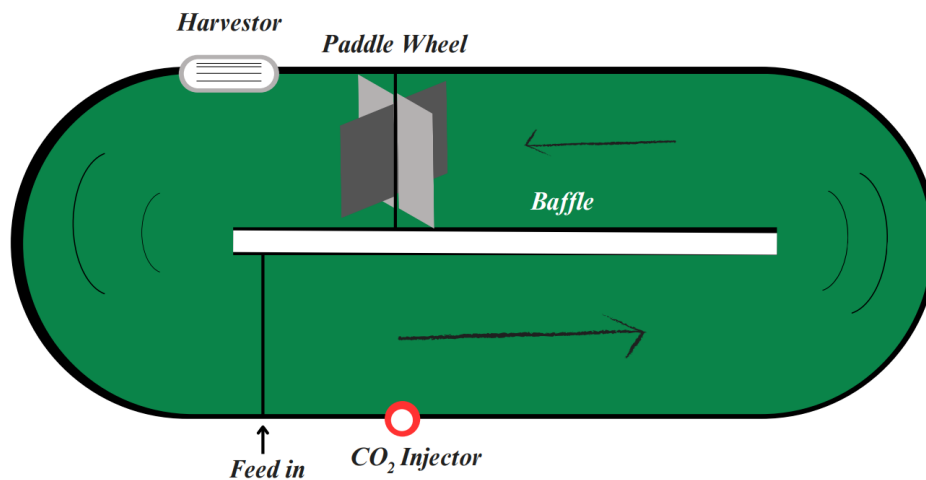


Figure 2.1 - Schematic diagram of open high rate algal pond with natural illumination. Adapted from (Ashok, Gupta, & Shriwastav, 2019)

In a review by Li et al., 2023, the global distribution of research articles on HRAPs indicates that Spain is the leading country with the highest number of publications (25,26%) in this field. This increase in research activity is attributed to the launch of a microalgae cultivation project in Spain called

'All-gas' a collaboration involving Aqualia, the world's third-largest water treatment company, along with other European companies such as BDI-Bio Energy International (Austria), Fraunhofer-UMSICHT (Germany), HyGear (The Netherlands), University of Southampton (UK), and Volkswagen (Germany) (Commission, 2011). This project received substantial funding from the EU Innovation and R&D Fund and had a duration of 4 years, ultimately being extended for 2 more years, ending in 2015 (International, 2017).

The project's results demonstrated that in one hectare of algae cultivation, approximately 1000 m³/day of municipal WW could be treated to meet EU Standards. Additionally, it was possible to achieve an algae biomass yield exceeding 90 tons/hectare/year. The Life Cycle Assessment (LCA) conducted by Fraunhofer-UMSICHT revealed that, when converted to biomethane, and considering water purification, the energy return on investment (EROI) was close to 2, which is higher than most plant biofuels (e.g., corn ethanol and palm oil diesel, typically around 1.3) (Aqualia, 2016).

Literature shows that the roots of microalgal-based technology is starting to run deep in the soil of the smaller scale WWT industry. Nevertheless, larger scale comparative studies have also been conducted in order to understand to which degree microalgae can attenuate the environmental impact of conventional WWTPs, while also obtaining efficient bioremediation process.

For example, Saúco et al., 2021 conducted a yearlong experiment employing a hybrid system for WWT alongside a conventional WWTP with a daily treatment capacity of 4000m³. In his study, various treatment methods were combined such as HRAP, dissolved air flotation, and constructed wetlands. The hybrid system achieved impressive removal rates: 96,6% for COD, 76,6% for TN, 89,8% for TP, and 99,9% for TSS. It also achieved strong disinfection rates of 4 and 5 log units for total coliforms and E. coli, respectively. One of the highlights of this study was the hybrid system's energy demand - only 0,1 kWh/m³, five times lower than the conventional activated sludge system.

Other studies have also been explored in the energy recovery front such as Hasport et al., 2022's assessment of a hybrid system model, revealing an energy requirement almost 350-fold lower than conventional systems, and Olsson et al., 2018, who studied the feasibility of substituting waste activated sludge for microalgal-bacterial substrate for anaerobic digestion and obtained similar methane yields for microalgal-bacteria substrate and waste activated sludge.

These studies indicate that efforts are being made not only by academia, but also by governmental institutions to incorporate microalgal-based solutions into the field of WWT. Meanwhile, there is also an increasing need to understand microalgae as a whole, including their metabolism and their response to environmental changes. The recent advancements in this subject will be investigated in the following sections.

2.2 Nutrient Requirements and Removal Mechanisms in Microalgal-Based Systems

The key nutritional elements that determine microalgal growth are carbon, nitrogen and phosphorus. In an attempt to achieve a general quantification of algal biomass composition in an elementary level, in 1934 Alfred Redfield studied the C, N and P content of marine phytoplankton across the oceans and landed on an approximate C:N:P ratio of 106:16:1 (Tyrrell, 2001). This fact is of immense help when it comes to the cultivation of microalgae, as it prevents their limited growth (Spaargaren, 1996). However, the various papers published over the years, using diverse nutrient compositions, provide evidence that this stoichiometry can vary based on different factors (Arrigo, 2005).

Furthermore, within the context of WWT and biomass composition, the objective is not solely to sustain a healthy microorganism population, but also to enhance the bioremediation and bioaccumulation processes, with a strong emphasis on sustainability. This section will delve into the recent developments in both microalgae monocultures and algal-bacterial cultures within the WWT domain.

2.2.1 Carbon

Photosynthetic microorganisms primarily assimilate carbon in the form of dissolved CO_2 in aquatic environments. Once CO_2 is dissolved in water, a weak acid-base buffer system is established according to the pH of the culture. Within the ideal pH for optimal microalgae growth (7-10) (Wehr, 2007), bicarbonate prevails as the dominant form of carbon. At lower pH levels, the preferred form is H_2CO_3 and at pH levels above 10, the CO_3^{2-} is the dominant form.

The supplementation of inorganic carbon is an essential part of microalgal cultivation. To produce one ton of algal biomass, a minimum of 1,83 tons of carbon dioxide is needed (Chisti, 2007). Roughly half of the expenses associated with biomass production can be attributed to carbon dioxide (Chisti, 2013). To tackle this, more sustainable alternatives have been studied. In (Ma et al., 2022)'s review, eighteen different studies were selected from a time span between 2018 and 2021, displaying microalgae's ability to utilize various carbon sources for their metabolism and reproduction. Among the carbon sources investigated were bicarbonate ions from sodium bicarbonate and urea, as well as organic sources such as acetate and glucose, alongside conventional CO_2 sparging.

2.2.1.1 Bicarbonate

The uptake efficiency of inorganic carbon sources during microalgae growth depends primarily on their solubility and utilization efficiency. In an aqueous environment, relying solely on CO_2 as the carbon source in the medium may not provide an adequate supply of energy-yielding substrates and essential nutrients. On the other hand, introducing excessive CO_2 gas can result in the loss of unused gas and a decrease in the pH of the culture as stated previously. The addition of NaHCO_3 has the potential to address these issues, thanks to its good solubility, ease of storage, and environmentally friendly properties (Yeh, Chang, & chen, 2010).

Umetani et al., 2021 showed that photosynthetic activity was well supported under 20 mM NaHCO_3 inorganic carbon regime, and that under a mixed regime of 20 mM NaHCO_3 and 5% CO_2 , nutrient removal was enhanced. This could be related to the fact that the introduction of NaHCO_3 facilitates the absorption of CO_2 by microalgae, all the while preserving the culture pH above 7 which could also facilitate the uptake of other nutrients (Qi et al., 2019).

When cultivating microalgae, it is common to find sodium bicarbonate in the medium recipes, either as the primary source of carbon, or in conjunction with sparged CO_2 . However, despite its benefits, sodium bicarbonate can increase salinity and pH, which can ultimately affect the healthy development of the culture. For that reason, other sources of bicarbonate have been entering the spotlight such as urea.

The bicarbonate ions released during the enzymatic breakdown of urea by microalgae can be utilized as a carbon source. Krausfeldt et al., 2019 observed that the carbon in urea can be uptaken by cultures of cyanobacteria under low concentrations of dissolved CO_2 . Most recently, Rosa et al., 2023 reported that cell growth and lipid accumulation were enhanced by urea supplementation in the culture of *Chlamydomonas reinhardtii* under mixotrophic conditions. Urea has been previously utilized as a

source of nitrogen and despite its exploration as a carbon source being in the beginning stages, it shows promising results for a more cost-effective cultivation process.

2.2.1.2 Organic Sources

The growth of microalgae in the presence of organic carbon sources is associated with heterotrophic and/or mixotrophic metabolism. Microalgae utilize these organic molecules as sources of both carbon and energy (Chojnacka & Marquez-Rocha, 2004; Perez-Garcia et al., 2011). In certain instances, microalgae cultures exhibit more favorable responses to this approach compared to their reactions to inorganic carbon sources, as was observed by Mondal et al., 2016. In his study, different organic and inorganic carbon sources were used to cultivate two microalgal species, *Chlorella sp. BTA 9031* and *Chlamydomonas sp. BTA 9032*.

Based on the results displayed in figure 2.2, the higher biomass concentration values were achieved by using cheese whey permeate and molasses compared to the more refined carbon sources (A and B) such as bicarbonate and CO₂. Nonetheless, this study also shows that carbon uptake can vary within species as well as the form of organic carbon available.

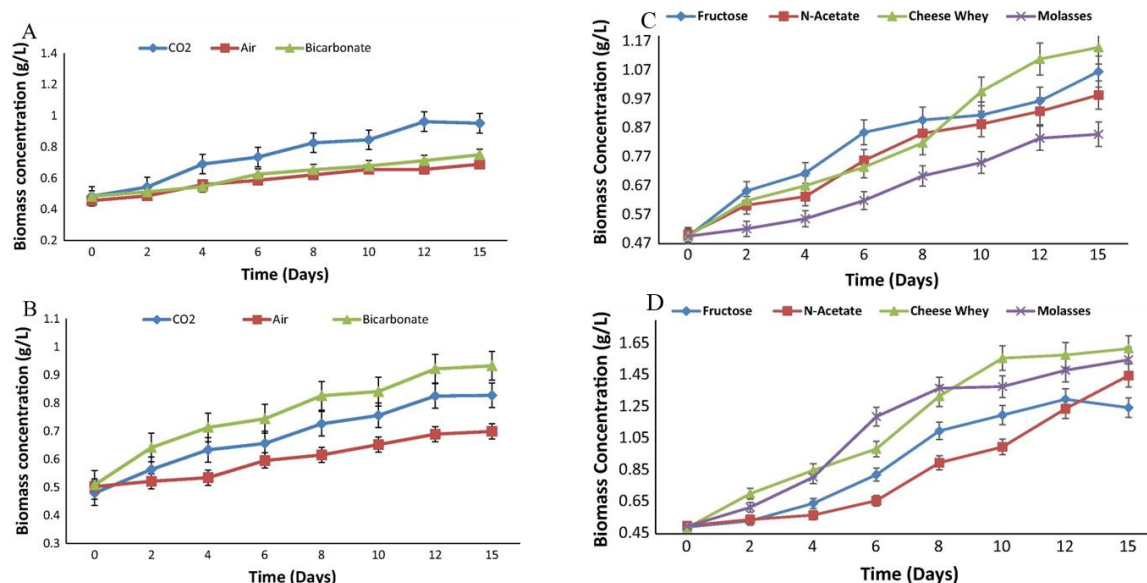


Figure 2.2 – Effect of different Carbon Sources in *Chlamydomonas sp. BTA 9032* (A and C) and *Chlorella sp. BTA 9031* (B and D) from (Mondal et al., 2016)

Cultivating microalgae in environments rich in organic matter carries a significant risk of contamination by rapidly proliferating heterotrophic species. In certain scenarios, this contamination can result in microbial competition and negatively impact the efficiency of processes, especially when the goal is to bioremediate polluted influents. Furthermore, when the primary objective of the microalgal culture is to boost microalgal growth, this contamination can cause substantial harm (Chojnacka & Zielińska, 2012).

2.2.2 Nitrogen

In microalgal biomass, nitrogen ranks second in abundance following carbon. Nitrogen plays a crucial role in fundamental biochemical compounds within the biomass, including nucleic acids (DNA, RNA),

amino acids and pigments. Typically, nitrogen can be assimilated in the forms of NO_3^- , NO_2^- , NH_4^+ , as well as in organic forms such as urea. Microalgae exhibit a preference for assimilating the most reduced forms of nitrogen, with the order of preference being $\text{NH}_4^+ > \text{NO}_2^- > \text{NO}_3^- > \text{Urea}$ (Markou, Vandamme, & Muylaert, 2014).

NO_3^- , NO_2^- and NH_4^+ are compounds that are dominant in wastewaters. Pereira et al., 2016 investigated the potential of *Chlorella vulgaris* for nitrogen removal from landfill leachates, which typically contain high ammoniacal-nitrogen concentrations, high chemical oxygen demands, and low phosphorus concentrations. Since phosphorus is required to achieve microalgae growth, different N:P ratios were evaluated. The NH_4^+ and NO_3^- removal achieved in the experiment can be seen in Figure 2.3.

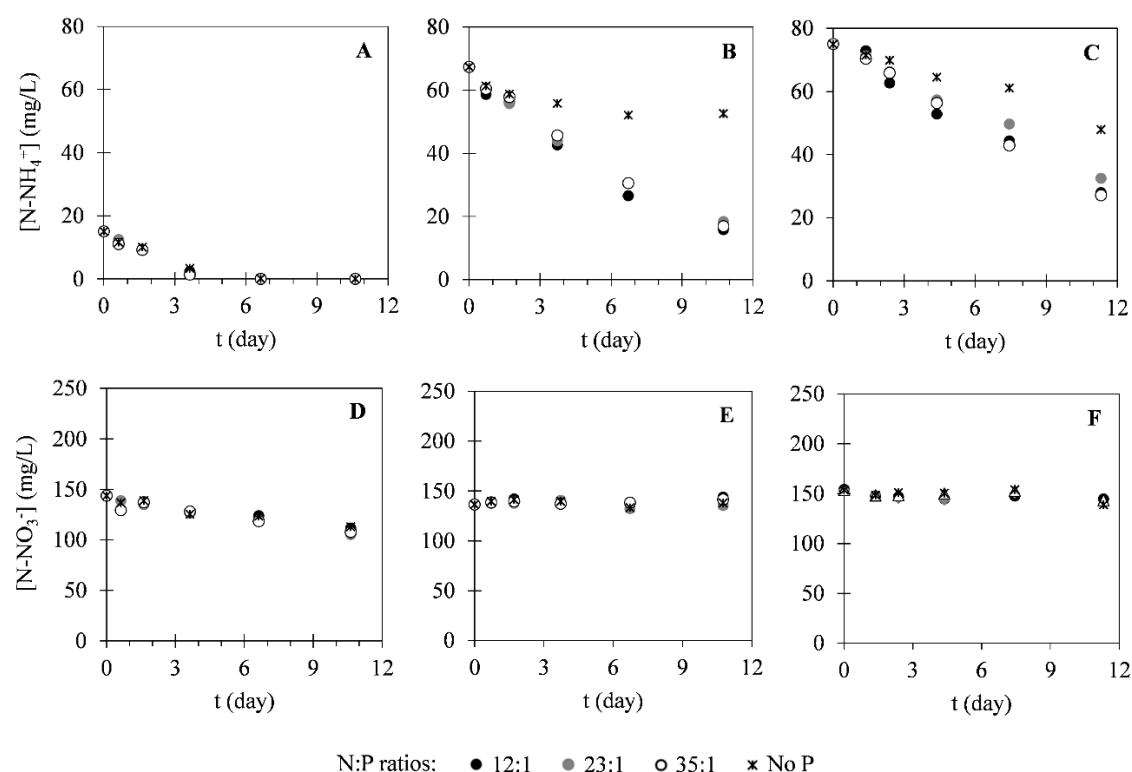


Figure 2.3 - Temporal variation of N-NH_4^+ (A–C) and N-NO_3^- (D–F) concentrations determined in assays I (A and D), II (B and E) and III (C and F) under different N:P ratios. from Pereira et al., 2016

As the results suggest, over the course of the experiment the ammonium concentration decreased, however, the nitrate remained relatively constant. In the discussion section of this paper, the authors attribute this to NH_4^+ being the preferential species to be assimilated by microalgae, as has been explored by literature. On the other hand, the presence of phosphorus was reported to have a positive effect on the ammonium removal, which highlights the importance of establishing an ideal N:P ratio to prevent nutrient limitation in the culture.

The ability of microalgae to remove nitrate and nitrite from wastewaters has been studied. The review by Taziki et al., 2015 suggests that a variety of factors need to be considered when aiming for the bioremediation of these compounds. For instance, strains like *Neochloris oleoabundans*, *Dunaliella tertiolecta* and *Chlorella vulgaris* show high nitrate uptake capacities however these strains have to be paired with the right parameters in order to achieve the desired results. These parameters include light intensity, temperature, initial concentrations of nitrate and cell immobilization.

An easier alternative is outsourcing the removal of nitrates and nitrites by attributing this task to microorganisms that are already known to remove these compounds in WWTPs – nitrifiers and denitrifiers.

Through the Nitrification-Denitrification pathway, total nitrogen removal is achieved, however, nitrification can be inhibited by light, and denitrification only occurs in the absence of oxygen, which becomes a problem when microalgal-bacterial systems are operating under autotrophic conditions. To overcome this obstacle the process can be controlled through the management of the light dark cycle, the culture density and the algae- denitrifier inoculum ratio (Fallahi et al., 2021).

The competition for ammonium is also a factor to take into account when aiming to achieve efficient total nitrogen removal in MBSs. The review by González-Camejo et al., 2022 aims to identify factors that influence this competition. In 11 papers analyzed, higher nitrification rates were consistent with lower microalgae performance. Despite there being reports of microalgae negatively interfering with nitrifiers (Choi et al., 2010; Sepehri, Sarrafzadeh, & Avateffazeli, 2020; Wu et al., 2020), and even symbiotic relationships emerging from the co-culture of these two microorganisms (Bankston, Wang, & Higgins, 2020; Vargas et al., 2016), the parameters behind this competition, or lack thereof, are still unclear. The influence of factors such as temperature, light, nitrogen concentration, pH, dilution rate and EPS production were studied based on the existing literature. The results can be found in the following table:

Table 2.1 – Factors Affecting Nitrogen Source Competition in Microalgae-Bacterial Consortia. (González-Camejo et al., 2022)

Parameter	Microalgae	Bacteria	Ref
Temperature	- The optimal temperature range for microalgae is around 20°C. - Microalgal cultures are highly sensitive to temperature fluctuations above their optimal growth range	High temperatures (20-30°C) favors the growth of AOB	(González-Camejo et al., 2022; González-Camejo et al., 2019)
Light	Light availability favors microalgal growth in mixed cultures	NOB are photosensitive while AOB can endure light exposure. Therefore, under an illuminated system, partial nitrification rates are higher and can lead to the accumulation of nitrites in the system	(Galès et al., 2019; Guerrero & Jones, 1996; Lu et al., 2020; Meng et al., 2019)
NH₄/NH₃	- Microalgae growth shows significant decline in NH₄⁺ concentrations below 10 mgN/L. - Increased concentrations of NH₄ can also result dominant presence of NH₃ which negatively impacts microalgae growth	- Under limiting concentrations of NH₄⁺, AOB shows more resistance than microalgae as the half saturation constant of AOB (0,5 mg N/L) - AOB and NOB growth is also affected by the presence of NH₃	(Mohsenpour et al., 2021; Pachés et al., 2018; Rossi et al., 2020; Weon, Lee, & Koopman, 2004)
NO₂/HNO₃	Abundance of nitrite negatively affects microalgae as the presence of HNO₃ has detrimental effects on microalgae activity	HNO₃ has inhibitory effects on both AOB and NOB growth	(Blackburne et al., 2007; Yang et al., 2004)
pH	Optimal pH range for microalgae is 7-10	Optimal pH for NOB and AOB is 7,4 and 7,8	(Claros et al., 2013; Eze et al., 2018)
SRT	If factors such as temperature, light, pH and nutrient availability are in favor of microalgal growth, then longer STRs will also favor microalgal growth	In algal-bacterial cultures, shorter STR benefit fastest growing organisms in the medium	(Gonzalez-Camejo et al., 2020; Winkler et al., 2017)
EPS	Heterotrophic and mixotrophic microalgae can benefit from the presence of EPS in the medium	- EPS production favors heterotrophic bacteria growth over photoautotrophic microalgae. - It can reduce nitrifying bacteria due to oxygen depletion	(Fallahi et al., 2021; Kwon et al., 2019)

The manipulation of these parameters can be done according to the end goal of the process. For instance when ammonium influent concentration is high (e.g centrate), nitrification is the desired pathway. The presence of microalgae in this scenario can help reduce the aeration costs. On the other hand, when the aim is to promote biomass productivity (e.g to increase sludge value for energy production),

the system should be engineered towards achieving a balance between these two groups of microorganisms.

Some successful results from literature include Luo, Le-Clech, & Henderson, 2020's that observed an improvement in TN concentrations of over 250% by adding activated sludge to monoculture containing different species of green algae. Another study by Tang et al., 2022 achieved a TN removal efficiency of $(98,5 \pm 1,2)\%$ in *Chlorella vulgaris*-*B* and *licheniformis* consortium. In the case of Dhaouefi et al., 2018, with an HRT of 10 days, the MBS was able to remove $(87 \pm 11)\%$ of TN from textile wastewater.

These findings prove that it is possible to establish an equilibrium between microalgae and bacteria cultures that ensures efficient rates of TN removal. This offers promising prospects for the widespread implementation of MBSs in the secondary treatment of influents with high nitrogen content.

2.2.3 Phosphorus

Phosphorus is the third most important nutrient for microalgae growth and metabolism. Phosphorus is present in molecules such as RNA, DNA and adenosine triphosphate (ATP) and membrane structures. Microalgae can assimilate phosphorus in the form of polyphosphate or orthophosphate for the synthesis of these molecules. In addition, phosphorus removal can occur under alkaline environments leading to the precipitation of phosphate salts.

In conventional WWT, biological phosphorus removal is achieved during tertiary treatment, by making use of the ability that certain bacteria (known as Phosphate Accumulating Organisms, PAOs) have of accumulating this element in the presence of oxygen. However, in the absence of oxygen, PAOs release polyphosphate back into their surrounding environment. The rate at which they uptake phosphate surpasses the rate of release, for that reason, biological phosphorus removal is carried out in anaerobic-aerobic cycles in many WWTPs (Jeon et al., 2001).

Similarly to PAOs, the release of phosphorus post assimilation also occurs in algal systems, however, this is usually associated with biomass degradation after cell death. This phenomenon is observed in open systems such as ponds and lagoons and was studied by Powell et al., 2011. These outcomes highlight the necessity of biomass harvesting to achieve effective phosphorus removal in microalgal-based systems.

In general, significant phosphorus removal by monocultures of microalgae has been reported in literature as can be seen in Table 2.2.

Table 2.2- TP removal efficiencies reported over the years

Species	WW type	TP removal efficiency	Source
<i>Chlorella sp.</i>	Centrate, municipal WW	80% (215,1 mg/L)	(Y. Li et al., 2011)
<i>Chlorella sp.</i>	Animal wastewater	51,9–57,9% TP (0,7–4,4 mg/L)	(Chen et al., 2012)
<i>Chlorella</i> , <i>Cryptomonas</i> , <i>Scenedesmus</i>	Municipal wastewater	93% TP (4,22 mg/L)	(Novoveská et al., 2016)
<i>Chlorella vulgaris</i>	Dairy manures	92% TP (180 mg/L)	(Wang et al., 2010)

Chlorella sp. has demonstrated significant phosphorus accumulation abilities, making it a popular choice for the bioremediation of phosphorus-rich waters. Nevertheless, some intriguing findings have emerged from co-cultivating microalgae with PAOs. For example, Ji et al., 2020 stated that extended reaction durations (3–7 days), inadequate phosphorus removal rates (less than 50%), and high aeration demands (3 L/min) emerged as the primary challenges observed in documented microalgal-bacterial granular sludge processes. Leading from here, their study investigated the EBPR by microalgal-bacterial granular sludge processes and within 6 hours, consisting of a 2-hour dark phase and a 4-hour light phase, approximately 86% of phosphorus was removed from the system. During the dark phase, there was a slight release of phosphorus, followed by significant phosphorus uptake during the light phase, accompanied by the accumulation of polyphosphoric within microalgal cells. This uptake and release followed a similar trend to the one observed in conventional EBPR, which could indicate that this behavior can be attributed to PAOs. In Wang et al., 2016's study, this exchange of phosphorus between bacteria and microalgae was observed, and it was suggested that bacteria can serve as a phosphorus reservoir for microalgae.

Based on the results from the literature, microalgae can function as an additional entity, consuming the phosphorus released by PAOs while also reducing aeration costs.

2.3 Algal Biofilms

Biofilm-centered solutions have emerged in the field of WWT as a promising technology, offering a multitude of advantages compared to traditional activated sludge systems. These benefits encompass a rich microbial diversity, a concentrated and active biomass, shortened HRT, reduced sludge generation, and heightened efficacy in removing pollutants (Y. Zhao et al., 2019). In contemporary WWT practices, diverse biofilm-based bioreactor systems, including Membrane Biofilm Reactors (MBR), Trickling Filters (TF), Rotating Biological Contactors (RBC), and Moving Bed Biofilm Reactors (MBBRs), have found extensive application for the treatment of highly contaminated wastewater.

2.3.1 Emergence of biofilms as a cost- effective alternative to suspended systems

The earliest report found of algal biofilms for WWT was by Torpey et al., 1971. In this study, algal growth was induced by the presence of light in an attempt to enhance nitrogen and phosphorus removal from the effluent of primary tanks at a municipal WWT plant. A higher harvestability of the filamentous algae that grew as a biofilm, in contrast to the planktonic algae typically found in oxidation ponds was also acknowledge in this study.

Not only is the harvestability of the resulting biomass easier when employing biofilm growth, but it is also cost-effective. Nowadays, after growth, specialized harvesting and dewatering operations such as sedimentation, flocculation, flotation, centrifugation, and/or filtration are needed to concentrate the biomass (Davis et al., 2011). For example, Grima et al., 2003 reported that water removal contributed up to 30% of total capital and operating costs, and Davis et al., 2011 reported that harvesting alone contributes to 21% of capital costs in an open pond system. An economic assessment conducted by Ennaceri et al., 2023 evaluated the costs for biofilm-based cultivation systems as 8-10 times lower than those of suspended systems. The parameter that presented the highest savings was operational expenditure, which includes power, nutrient supplementation, water, labor, maintenance/insurance, taxes, bio-carrier for the biofilm system, and electro flocculation for suspended culture.

In addition to lower production costs, biomass productivity is highly enhanced in immobilised systems. Gross, Mascarenhas, & Wen, 2015 implemented two systems with Rotating Algal Biofilms (RAB) (Figure 2.4) for biomass production. The results showed that, on average, the raceway-based RAB and the trough-based RAB outperformed the control raceway pond by 309% and 697%, respectively. Recent studies suggest that higher biomass productivity in biofilm-based systems can be related to increased surface area offered for additional microbial growth (Mousavian et al., 2023).

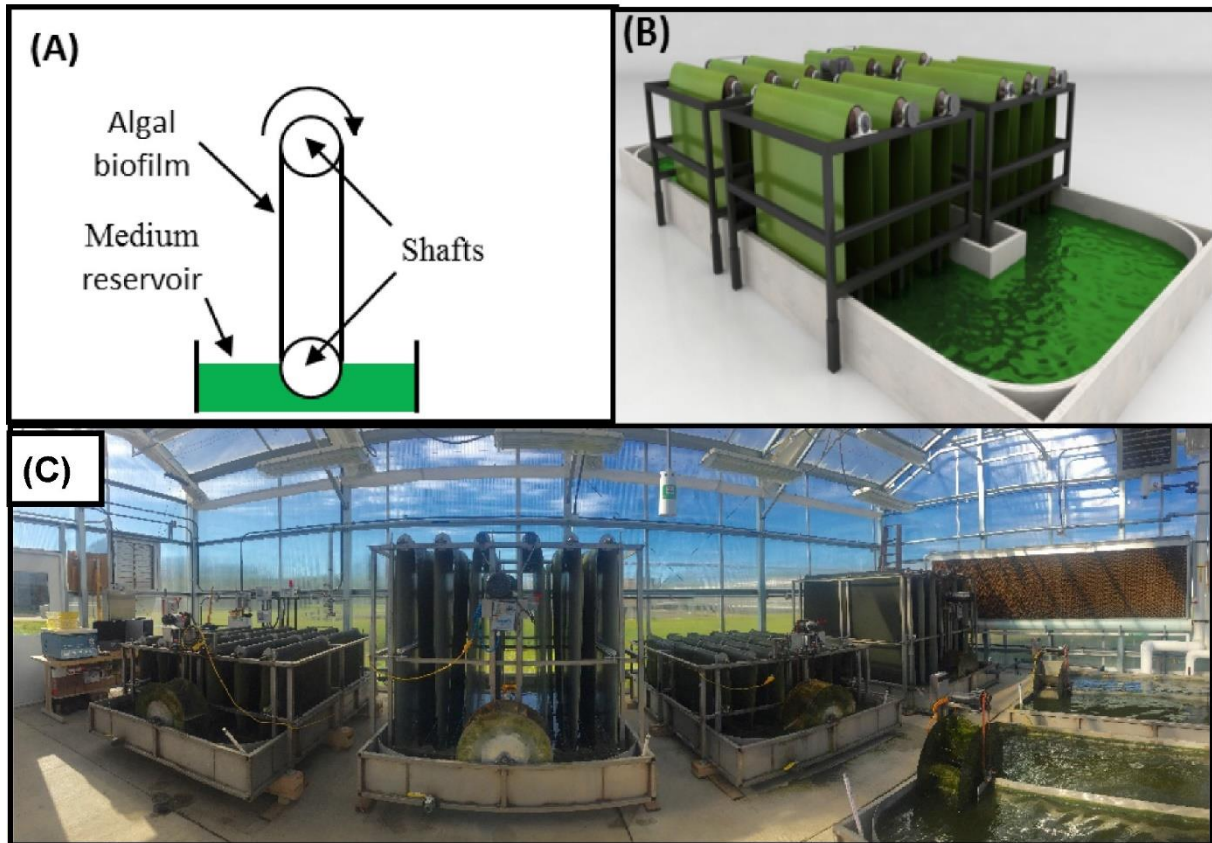


Figure 2.4- (A) Diagrams illustrating the design of the RAB reactor; (B) Integration of the RAB system into an open pond raceway; (C) Deployment of the pilot-scale RAB reactor at the Metropolitan Water Reclamation District's O'Brien facility in Skokie, Illinois, for wastewater treatment. Source: Gross, Mascarenhas, & Wen, 2015

2.3.2 Nutrient Removal in biofilm-based systems

In the context of the research question in this present study, it is hypothesized that the addition of a substrate for biofilm growth has the potential to enhance nutrient removal in microalgal-based systems. This hypothesis is grounded in the fact that N and P removal by microalgae primarily occurs through biomass assimilation, and in addition, biomass productivity has been shown to be positively influenced by reactor configurations that support biofilm growth. In order to understand if this correlation is plausible, it is important to take into account the current advancements in nutrient removal by microalgal-based biofilms.

- **Nutrient Removal Profile:** Boelee et al., 2011 observed that nutrient removal in algal biofilms follow the same growth pattern of the biofilm itself – an increase in removal as biofilm growth peaked followed by a drop in nutrient uptake during biofilm as sloughing occurred.
- **Nitrogen Removal Pathways:** Posadas et al., 2013 achieved a nitrogen removal of $(70 \pm 8)\%$ attained mainly through stripping and biomass assimilation. However, nitrification, denitrification and anammox are nitrogen removal pathways that can be observed in algal biofilms. For example, Zhang et al., 2022 was able to construct an integrated algal/partial nitrification/anammox biofilm system (IAPNABS) through light cultivation without the supplementation of oxygen. Moreover, upon observing significant nitrification rates on algal biofilm systems, Babu, 2011 hypothesized that algal biofilms acted as a protective barrier, effectively preventing the washout of nitrifying bacteria, which in turn, display lower growth rates than microalgae.
- **Phosphorus Removal:** Xu et al., 2020 identified EPS as a site for phosphorus accumulation in addition the expected intracellular assimilation as polyP. Furthermore, P entrapment in EPS was stated to be influenced by pH and the redox gradients that exist within the EPS. These gradients are a results of oxygen penetration into the aggregates that determine the P retention capacity. Given that the immobilization of phosphorus by EPS is more favorable under oxidized and alkaline conditions, it is primarily in the outer layers where the majority of the extracellular entrapment occurs.
- **Enhanced Nutrient Removal – Comparative Study:** X. Zhao et al., 2018 assessed the performance of pilot-scale RAB reactors with two different heights (0,9 meters and 1,8 meters) in treating supernatant derived from sludge sedimentation at a local plant. The RAB reactor exhibited significantly greater efficiency in removing TP and Total Kjeldahl Nitrogen (TKN) compared to a control open pond, notably achieving a four-fold increase in TP removal capacity per unit footprint area when compared to the open pond.

These findings indicate that the structural considerations of algal biofilms offer additional removal pathways for nitrogen and phosphorus, however, there is a shortage in comparative studies between suspended and immobilized systems. Having more insights in this subject can aid in obtaining better knowledge on the differences and similarities between these two systems, resulting in engineering processes that are more sustainable, energy efficient and equally or even more effective than the existing alternatives in the industry.

3 MATERIALS AND METHODS

Throughout the experiment, initial protocols were developed based on literature. However, as the experiment progressed, some adjustments had to be made in order to lead the systems to optimal performance. Therefore, this section will compile a detailed explanation of these initial parameters as well as the observations that led to the change and adjustments of these same parameters.

3.1 Inoculum Preparation

Before initiating the reactors, it was first necessary to prepare the inoculum. For a more cost- efficient process, the inoculum was prepared in the lab. Note that the aim was not to obtain a non-axenic culture of microalgae as bacteria is needed for biofilm formation, instead the focus was on creating the ideal conditions for a robust population of microorganisms to emerge with a prominent presence of microalgae. To achieve this, Fresh Water Samples (FWS) were collected from 2 different sites (FWS₁ and FWS₂) and added to 3 different Mediums (M₁, M₂ and M₃) that were prepared attending to the nutrient requirements for microalgae growth. Factors such as photoperiod cycle, agitation and temperature were taken into account to design the ideal conditions for the desired outcome. It took a total of three protocols to achieve the intended inoculum. These protocols will be explained next.

3.1.1 Protocol 1 - 100 mL solutions (FWS₁, FWS₂, M₁ and M₂)

Protocol 1 consisted on the preparation of 100 mL solutions using different volume combinations of freshwater sample and medium. 2 trials were conducted during the protocol.

1st Trial (FWS₁, M₁)

In the first trial, the freshwater sample used was collected from the Upper Lake located in front of the College of Engineering & Architecture at University College Dublin. The growth medium was prepared following the Synthetic WW recipe by Osachoff et al., 2014. The N/P ratio was adjusted based on the study conducted by Su, Mennerich, & Urban, 2011. On this study an algal-bacterial biomass productivity of 1,82E-03 g/L.h was achieved along with a nitrogen removal of 93%-100% and a phosphorus removal of 55%-73%. The recipe for this medium can be found in (Appendix 7.1). The medium preparation was followed by a sterilisation step that consisted on placing the stock solution in an Astell steam autoclave at 121°C for 15 minutes.

The 100 mL solutions had the following volume combinations and can be found in Figure 3.1

- a) 100 mL FWS₁
- b) 90 mL FWS₁ + 10 mL M₁

c) 50 mL FWS₁ + 50 mL M₁



Figure 3.1 – 1st Trial – Protocol 1 Shake Flasks a), b) and c)

After the preparation of the different 100 mL solutions, a cotton plug was added to the shake flask as this allows for the diffusion of gases while preventing dust and external bacteria to enter the system (Swale, 1982). Once ready, the shakflasks were taken to the Orbital Incubator Shaker Stuart Scientific SI50 (Figure 3.2). This incubator was illuminated by YASBED LED Grow Light Strips set for a photo-period of 12 hours, an agitation speed of 150 RPM and a temperature of 20 °C.



Figure 3.2 – 1st Trial- Protocol 1 Shake Flasks a), b) and c) in the incubator

After 2 days the formation of granules at the bottom of samples B and C were observed. This could be attributed to self-immobilization by the bacterial production of exopolymers (Weber et al., 2007) (Figure 3.3).

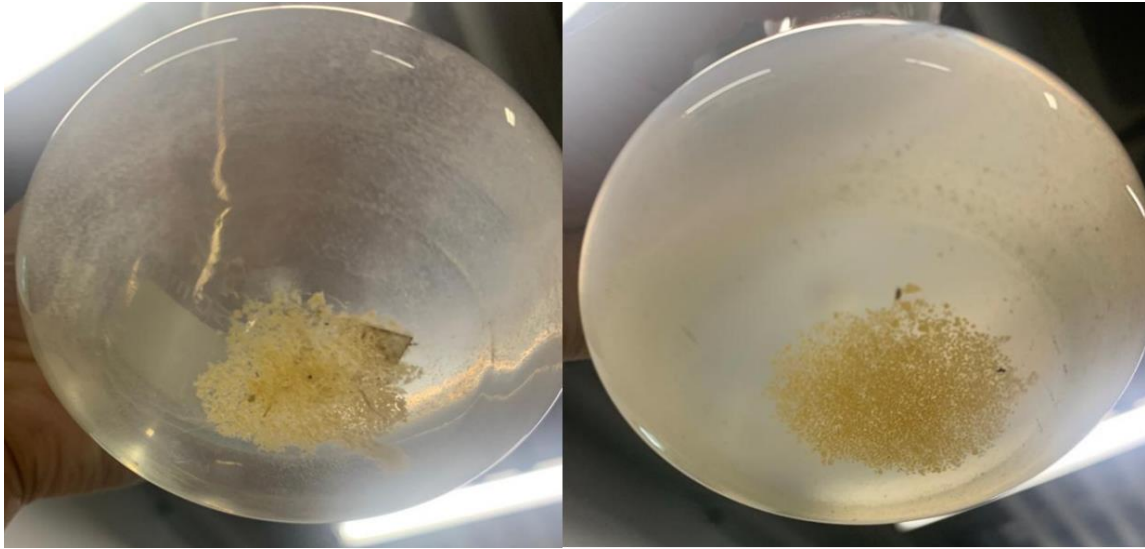


Figure 3.3- 1st Trial- Protocol 1 Shake Flasks b) and c) after 2 days in the incubator.

The lack of signs of microalgae growth and the prominence of heterotrophic organisms in the culture led to the adoption of a different approach – to change the medium to a more carbon limited recipe.

2nd Trial (FWS₁, FWS₂ M₂)

The synthetic WW media was replaced by a Knop's media recipe (Appendix 7.1) reinforced with a source of inorganic carbon (M₂) to allow the growth of photosynthetic organisms in the culture. Similarly to M₁, this one also underwent sterilisation in the autoclave at 121°C for 15 minutes.

An additional source of fresh water was implemented in the system (FWS₂). This sample was collected from a domestic pond located in the Dublin Region. With this, three different 100 mL solutions were prepared (Figure 3.4) with the following compositions:

- d) 100 mL of FWS₂
- e) 50 mL FWS₁ + 50 mL M₂
- f) 50 mL FWS₂ + 50 mL M₂.

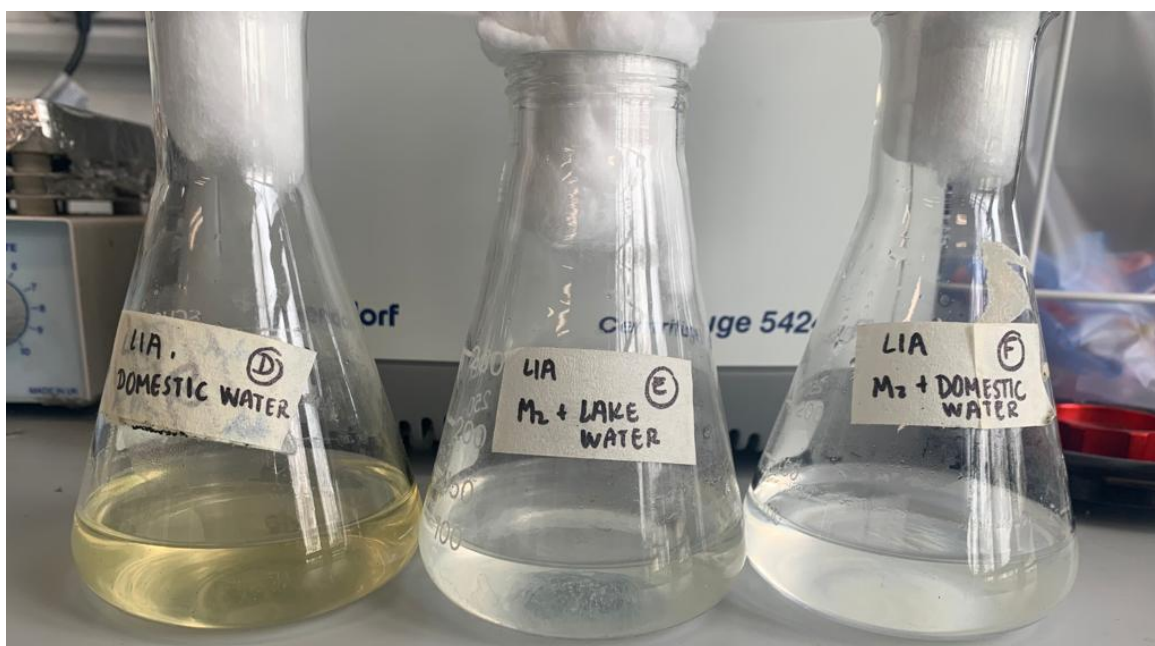


Figure 3.4- 2nd Trial – Protocol 1 Shake Flasks d), e) and f)

On day 2 signs of granules started to emerge in e) and f) (Figure 3.5).

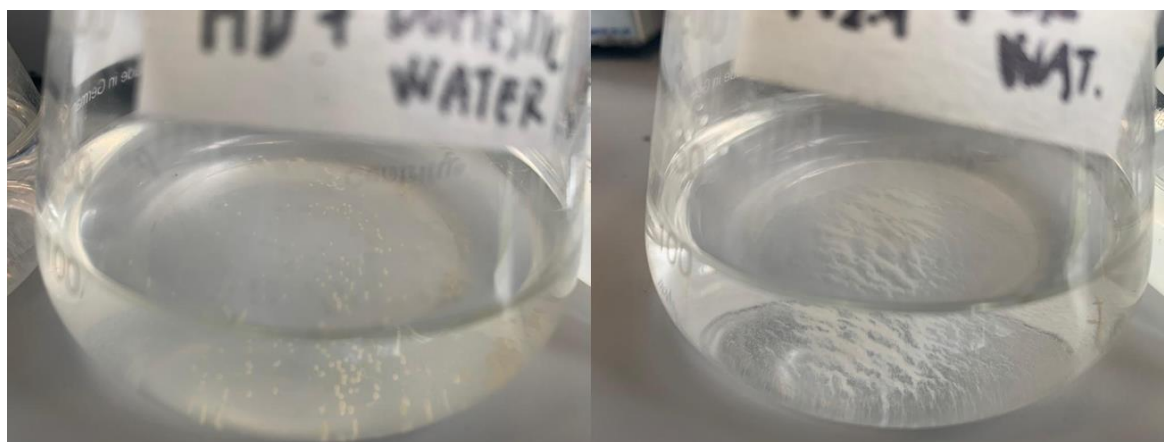


Figure 3.5 – 2nd Trial- Protocol 1 Shake Flasks e) and f) after 2 days in the incubator.

As for the pure FWSs, no visible changes were observed. When handling a natural microbial culture, it is expected that over time the population will evolve (Hoang et al., 2023). For this reason, these 6 cultures were kept in the incubator in case microalgal activity emerged, while other strategies for the inoculum preparation were explored.

3.1.2 Protocol 2 - Auto- Flocculation Method (FWS₁, FWS₂, M₁ and M₂)

For this protocol FWS₂ was used as this sample presented a visible green tone (Figure 3.4) (shake flask d)), which could indicate the presence of photosynthetic organisms. The premise of the protocol was to isolate microalgae present in the sample by inducing auto-flocculation. (Matter et al., 2019) observed

that algae cells suspended in the liquid naturally aggregate to create sizable clusters, leading to their settling due to gravity. Once this was achieved, these clusters would be put in contact with an ideal growth medium in a test tube before ultimately being transferred to a shake flask for higher quantities of inoculum to be produced. The scheme of this protocol is illustrated in figure 3.6.

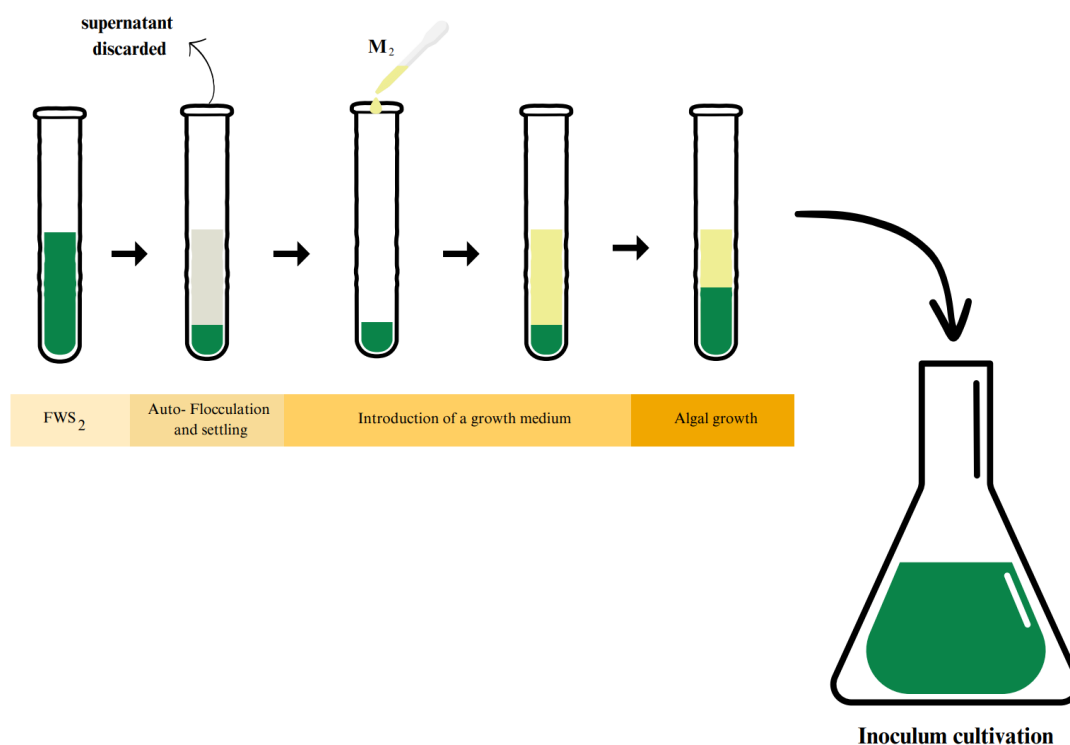


Figure 3.6 – Schematic Diagram of Protocol 2 – Auto-Flocculation Method

With this, 15 mL of FWS₂ was added to a test tube and placed in an incubator with the same settings as the shake flasks (a 12-hour photoperiod, agitation speed of 150 RPM and 20 °C). After 5 days, the supernatant was replaced by M₂ and the culture was left to grow in the sample tubes. Every 2 days the supernatant would be discarded and the tube would be refilled with M₂. 7 days after M₂ was first introduced to the culture, it was time to transfer the culture to a shake flask and make the volume up to 100 mL.

The observations made during this time span (Figure 3.7) reveal that it was indeed possible to obtain signs of photosynthetic activity in the medium as the aggregates precipitated in the bottom of the test tube and presented a green color.

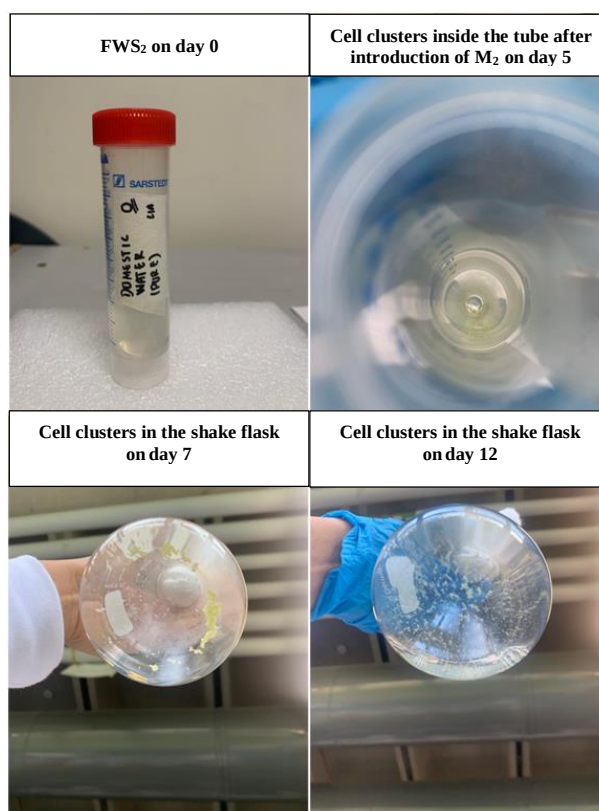


Figure 3.7- Protocol 2 – Cell clusters formation over the course of 12 days

Meanwhile, a third strategy was being also explored as this experiment was taking place in order to assess if it was possible to achieve a more simple and efficient protocol for the inoculum preparation.

3.1.3 Protocol 3 - Microalgae Cultivation In Duran Bottle (FWS₂ and M₃)

In this protocol FWS₂ and a Fertiliser Medium (M₃) were used. For the preparation of the stock of M₃ a commercial fertiliser, Strawberry Fertiliser by Vitax Organic, containing the essential nutrients and trace elements for algal growth was diluted in distilled water at a ratio of 1/500. The solution was supplemented with a source of inorganic carbon for photosynthesis. The recipe can be found in Appendix 7.2. Similarly to M₁ and M₂, the M₃ stock solution was sterilised in the autoclave.

For the preparation of the culture, 100 mL of FWS₂ was added to a 500 mL Duran Bottle (DB) and made up with M₃. The DB was placed at the lab bench, in front of a window exposed to natural light at room temperature. No agitation was implemented. Pictures taken reveal visible microalgae growth (Figure 3.8) within only 7 days. For that reason this was the protocol adopted for the preparation of the inoculum.

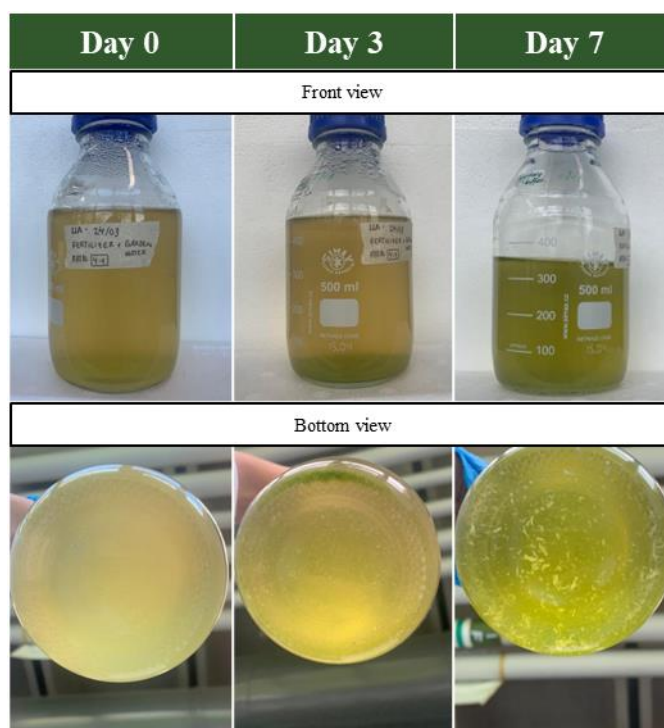


Figure 3.8- Protocol 3- Culture development over the course of 7 days

The content of this DB was then used as a seed culture to prepare 3L of the inoculum that would start the reactors.

First 300 mL of the inoculum was taken from the DB and equally distributed to 3 different 1L DBs. 2,7L of M_3 was prepared and equally distributed through the 3 different 1L DB. The culture was placed on the lab bench. The remaining 200 mL from the seed inoculum was made up with an additional 800 mL of M_3 and placed on the lab bench to serve as a spare inoculum. This process is illustrated in figure 3.9.

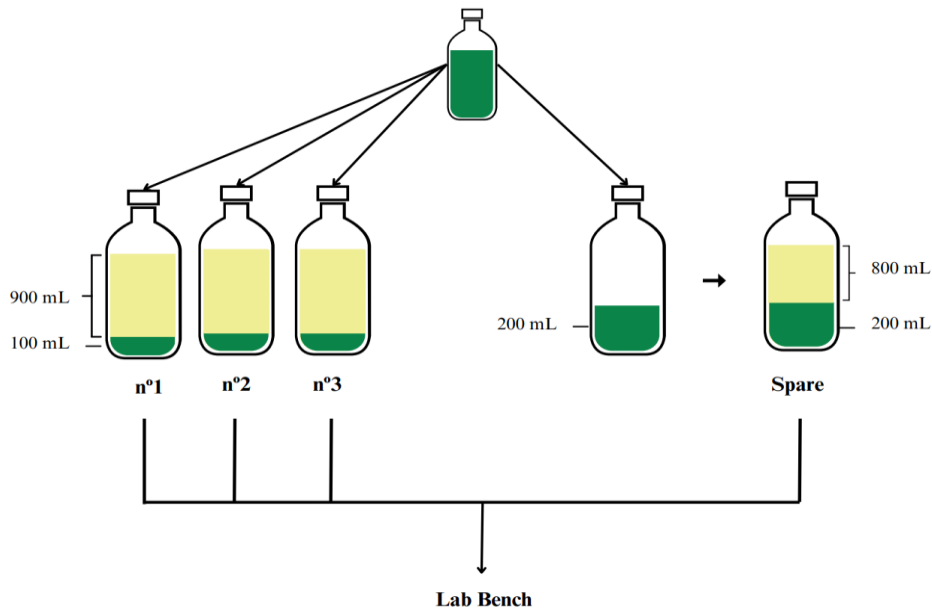


Figure 3.9 - Schematic Illustration of the inoculum preparation

Note that during this time, the previous solutions in the shake flasks displayed no evidence of algal growth, and therefore were discarded.

3.2 Raceway Pond Experiment

3.2.1 Experimental Design and Set-Up

The experimental configuration used in this study (Figure 3.10) consists of two identical bioreactors (BRs): a Suspended System Reactor (SR) and Moving Bed Biofilm Reactor (MBBR) (*polypropylene*, 20 L each, 22,3 cm × 34,7 cm × 25,8 cm). Each BR was equipped with an inlet stream sourced at the Feed Tank (FT), 25L. Two silicon tubes pass through a Peristaltic Pump (P-PUMP, *Millipore XX 80 002 30 Peristaltic pump*) to feed each BR under a fed batch regime controlled by a digital timer. Inside both BRs a baffle was placed to ensure the circulation of the water. For the mixing, two sets of paddle wheels (4 blades) operated connected to a speed controller. An overflow weir was added to SR and MBBR that allows for both reactors to maintain a constant working volume (V) of 6,2 L. The excess effluent was collected in two distinct collection tanks (CTKs). Artificial LED light (*Valoya Oy AP67*, Helsinki, Finland), was plugged into a timer for the control of the light cycle. The carriers chosen as a substrate for biofilm formation were K1 media by *Evolution Aqua* (Figure 3.11).

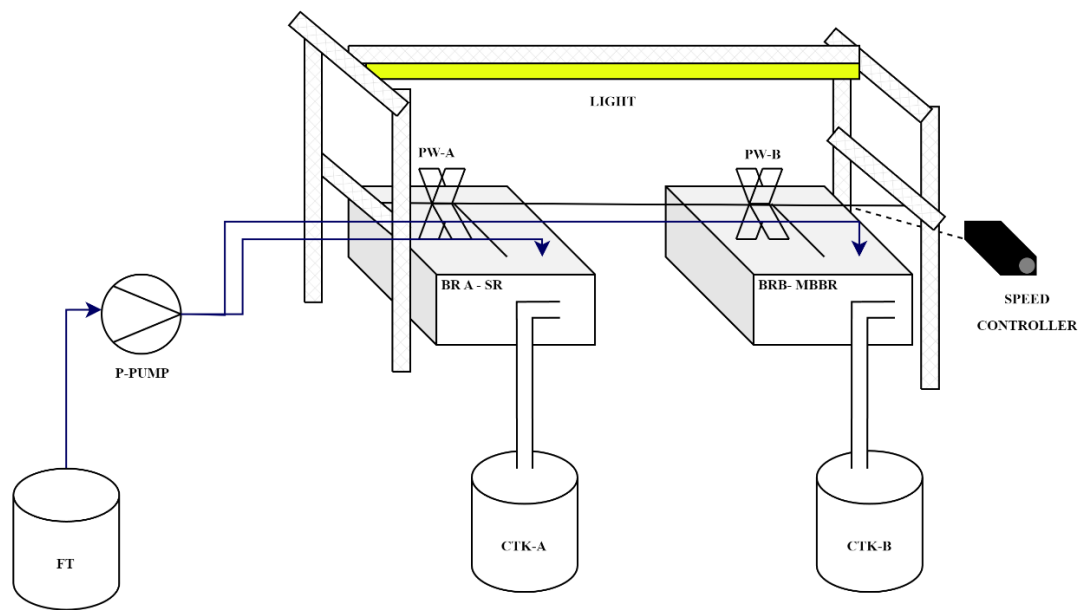


Figure 3.10- Raceway Pond Experiment- Design and Set Up: FT - feed tank; P-Pump – peristaltic pump; PW-A/B – Paddle wheel A/B; BR A – SR – Bioreactor A – Suspended reactor; BR B – MBBR – Bioreactor B- Moving Bed Biofilm Reactor; CTK-A/B – Collection tank A/B



Figure 3.11- K1 media box (left), K1 media carriers (right)

3.2.2 Reactor Initiation Protocol

3.2.2.1 Reactor Composition

The working volume of each reactor is 6,2 L. It was determined that the inoculum medium ratio would be 1/5, with this, 1,24 L of inoculum was added to each reactor and the remaining volume was made up with 4,96 L of M₃.

The amount of carriers that would make up a 100 mL beaker was counted in order to estimate the volume of each carrier. It was determined that 90 carriers equate to approximately 100 mL. On the other hand, based on literature, it is known that Filling Fractions (FF) below 70% are recommended to allow the carriers to move freely in suspension (Rusten et al., 2006). Furthermore, it has been reported that organic matter is removed at FF of 50%-60%, whereas nutrient removal is more effective under lower FF within the range of 30%-40% (Madan, Madan, & Hussain, 2022).

With this, a total of 19 beakers were added to reactor B (1,9 L), making the FF ratio of approximately 30%. The set-up of both bioreactors can be seen in figure 3.12.

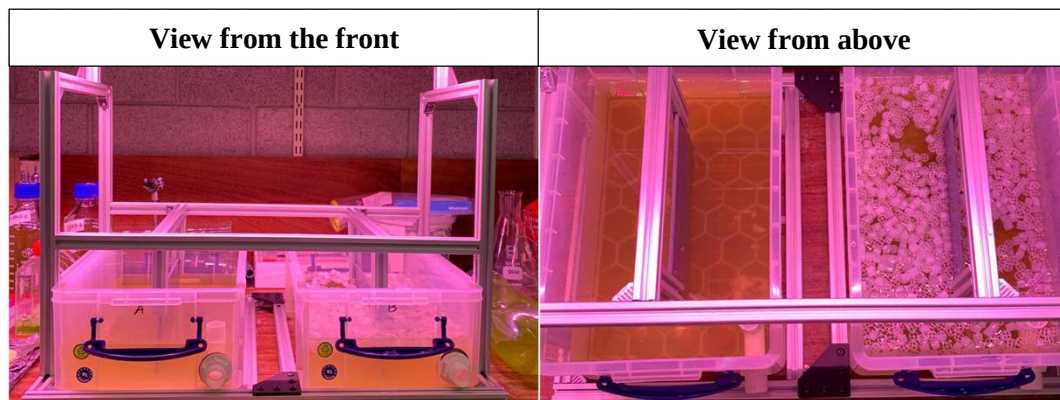


Figure.3.12 – Experimental set up - Reactor A and B from different perspectives

3.2.2.2 Pump Operation Mode

An HRT of 2 days was set. With the working volume, V , of each reactor being 6,2 L, the flowrate was determined according to the following equation:

$$Q = \frac{V}{HRT} \Rightarrow Q = 3,1 \text{ L} \cdot \text{day}^{-1}$$

This flowrate is equivalent to 2,153 mL/min. Under that value, continuous feeding was unfeasible due to the pump's inability to provide a flow rate at such a low level. For that reason, a fed batch feeding mode had to be implemented. The principle of this would be that the pump (figure 3.13, right) would be connected to a timer (figure 3.13, left) that would control the feeding cycles of the reactors. The first scenario considered was to feed both reactors every hour for 1 minute. That would mean that each cycle would pump 0,1291(6) L of solution to the reactors at a flowrate of 2,153 mL.s⁻¹.cycle⁻¹. Knowing that under that flowrate it would take 4,64 seconds to fill up a volume of 10 mL, the test of the pump was conducted with a graduated cylinder and the setting was established. However, the digital timer used allowed for the programming of up to only 10 cycles. With that, the final solution was to program the timer for 8 cycles, 3 minutes each, where a volume of 0,1292(6) × 3 = 387,54 mL of feed solution would be pumped to each reactor every cycle.



Figure 3.13 - Digital timer used for the pump (Left) and Peristaltic Pump (Right)

3.2.2.3 Medium Preparation

The feed tank used in this experiment has a capacity of 25 L, however, it was determined that the feed would not be used to its full capacity unless necessary. With this, every Monday and Wednesday, 15 L of feed solution would be prepared as over a period of 48 hours, 12,4 L of feed solution is fed to the reactors. On Fridays, 20 L of feed solution would be prepared as over a period of 72 hours 18,6 L would be fed to the reactors. Finally, in the occasion of there being a long weekend 24,8L of feed solution would be necessary to feed the reactors over a period of 96 hours, therefore 25 L of feed solution would be prepared on these days. The remaining volumes of feed would either be discarded or tested for nutrient composition.

The initial feed solution was prepared following the same recipe of M₃, however, tap water was used instead of deionized water and the sterilization step was not included in the protocol. These measures were adopted for convenience and in an effort to bring the experiment closer to the everchanging nature of the influents of conventional WWTPs. Over the course of the experiment, the composition of the feed solution changed as will be explored in the following section.

3.2.3 Environmental Conditions and Experimental Framework

To determine suitable growth conditions and develop a better understanding of the culture, a series of adjustments were made to the reactors throughout the experiment. These changes resulted in 3 distinct moments: 1) Stage 1 – Reactor Stabilisation, 2) Absence of Mixing and 3) Stage 2 – Reactor Assessment. Table 3.1 illustrates the duration of each phase as well as the parameters that were adjusted.

Table 3.1 - Duration of the moments of the experiment as well as the factors that were adjusted in each moment

Stages	Duration	Factors adjusted
Stage 1	From day 0 to day 59 (59 days)	Feed composition Photoperiod Mixing speed
No mixing	From day 59 to day 64 (5 days)	During this time mixing was interrupted
Stage 2	From day 64 to day 106 (47 days)	Mixing device Photoperiod

3.2.3.1 Stage 1 - Reactor Stabilisation

Figure 3.14 displays in detail the timeline of the adjustments made during stage 1 in the reactors.

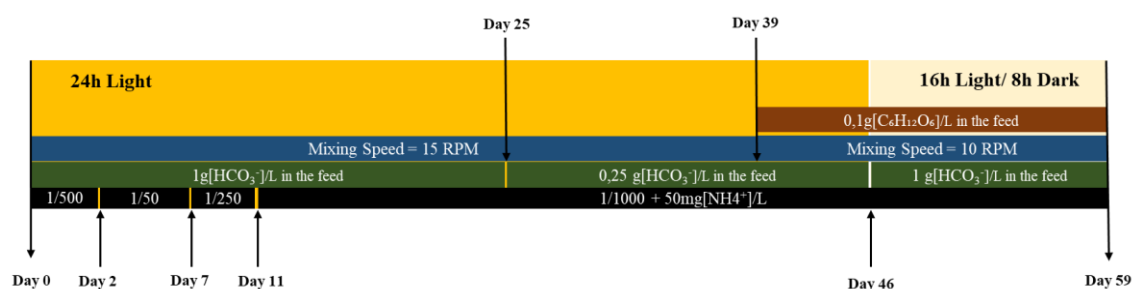


Figure 3.14 - Adjustments made in the systems during the first stage of the experiment. The black bars represent the Fertiliser to Feed Solution ratio

3.2.3.1.1 Feed Solution

Fertiliser and F/FS ratio

The main source of nutrients in the feed solution was the fertiliser. The fertiliser used during the inoculum was *Vitax Organic Strawberry Feed Liquid Fertiliser*. Despite containing the essential nutrients for algal growth, this fertiliser did not specify the forms of nitrogen present in its composition. Therefore, from day 7, *Vitax Organic Strawberry Feed Liquid Fertiliser* was replaced by *miracle-gro all-purpose concentrated liquid plant*. The total nitrogen content of this fertiliser is of 7% where 3,5% is Urea Nitrogen, 1,7% is Nitric Nitrogen and 1,8% is Ammonium Nitrogen.

The initial Fertiliser to Feed Solution Ratio (F/FS) was of 1/500. It is known that increasing the F/FS ratio will lead to an increase in the nutrient content of the feed solution. However, it will also lead to a darker medium. In hopes of removing any obstacle impacting light penetration in the culture, the F/FS ratio was set to 1/1000. By making this adjustment it was also possible to obtain a clearer view of the culture's development e.g. sites of attached growth in the corners of the reactor, which would not be visible otherwise. This progression can be observed in figure 3.15.



Figure 3.15- Reactor A and Reactor B at 3 different days, under three different F/FS ratios: A-1/50, B-1/250, C-1/1000.

To make up for the decrease of nutrients, in specific, N-NH_4 , and to ensure the N:P ratio did not limit biological growth, 50 mg/L of ammonium chloride, NH_4Cl , was added to the feed solution on day 11. This led to a final concentration of N-NH_4 to be 74 mg/L. In autotrophic experimental conditions, microalgae have shown high adaptation in mediums with 50 mg/L of ammonium and a decrease in biomass from 200 mg/L (Meier et al., 2022). This places the ammonium concentration used in this experiment within the range of high adaptability and optimal biomass productivity.

Bicarbonate Concentration

Bicarbonate, HCO_3^- was used in this experiment as a source of inorganic carbon, at a concentration of 1 g/L in the initial feed solution.

As the experiment progressed, between days 18 and 25, an increase in the pH above the ideal levels (7-9) (Wehr, 2007) was observed which will later be addressed in the results section. This occurrence is expected as photosynthesis involves the capture of H^+ ions, ultimately leading to the increase of pH of the culture (Zerveas et al., 2021).

To investigate if a decrease in HCO_3^- could lead to a decrease in the overall pH of the culture, the concentration of HCO_3^- in the feed solution was set to 0,25 g/L. Mokashi et al., 2016 reported that under this value, *C. Vulgaris* culture experienced lower biomass productivity. Therefore, this adjustment to the system could put the culture at risk of carbon limitation. For that reason, the concentration of bicarbonate was set back to 1 g/L on day 46.

Glucose

Glucose was introduced on day 39. During this stage, the system was believed to be under carbon limitation due to the decrease of bicarbonate on day 25, which can have a direct impact in biomass productivity as previously stated. In addition, it was found that when cultivated autotrophically using glucose as the main carbon source, microalgae achieved the highest biomass accumulation (Gim et al., 2014). Glucose supplementation is also linked to visible biofilm formation (Agarwal & Jain, 2013) and enhanced bacterial activity in mixed cultures (Haider et al., 2023).

3.2.3.1.2 Mixing Speed

Having a mixed system ensures uniform nutrient distribution and an even treatment across the reactor. However, throughout this stage of the experiment, it was suspected that the mixing structure was interfering with the biofilm's lifecycle, possibly preventing biofilm maturation.

Shear forces have been a contributor to constant biofilm thickness in MBBRs (Wang et al., 2020). On the other hand, abrasion due to collision between particles can damage the surface of the biofilm (Gillett, Waugh, & Lawrence, 2016). Therefore, as much as biofilm formation can benefit from mixing, it is necessary to ensure that the mixing in place is not negatively affecting the biofilm attachment and maturation in the first place.

The collisions that take place in the system are 1) carriers + carriers, 2) carriers + reactor walls and 3) carriers + blades from the mixing device. By decreasing the mixing speed, the rate at which these collisions occur will also decrease, leaving more time for biofilm to be formed. For that reason, a mixing speed of 10 RPM was set on day 46.

3.2.3.1.3 Photoperiod

Initially, the cultures were exposed to constant light supplementation, to continue to induce microalgal growth. Since photosynthesis is one of the contributors of increasing pH in the medium, the possibility of interrupting the uptake of H^+ ions was explored by implementing a period of darkness. Introducing a window where both microalgae and the remaining heterotrophs release CO_2 to the environment was hypothesized to be a natural way of controlling the rising pH of the culture.

3.2.3.2 No mixing

Interrupting the mixing all together was a consequence of experimental error, as the mixing controller was not able to provide constant signal under the speed that was set. This interruption prevents the system from achieving a homogenous state with equal nutrient distribution, creating dead zones ultimately affecting the reactor's performance. Despite this, visible biofilm growth was observed during this stage (Figure 3.16). This could be a result of the direct contact between the feed and the carriers, as the incoming solution was provided to the reactor from above. In addition, the interruption of mixing also eliminates collisions of all nature which could also have contributed to this observation.

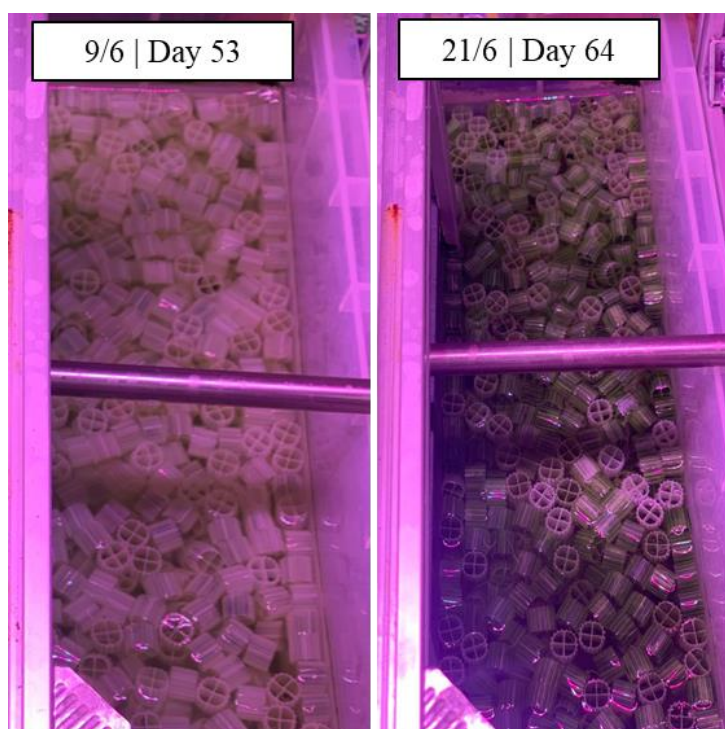


Figure 3.16 - Visible Biofilm growth under no mixing. A - MBBR on day 53; B- MBBR on day 64

3.2.3.3 Stage 2 - Reactor Assessment

Figure 3.17 displays in detail the timeline of the adjustments made during stage 2 in the reactors.

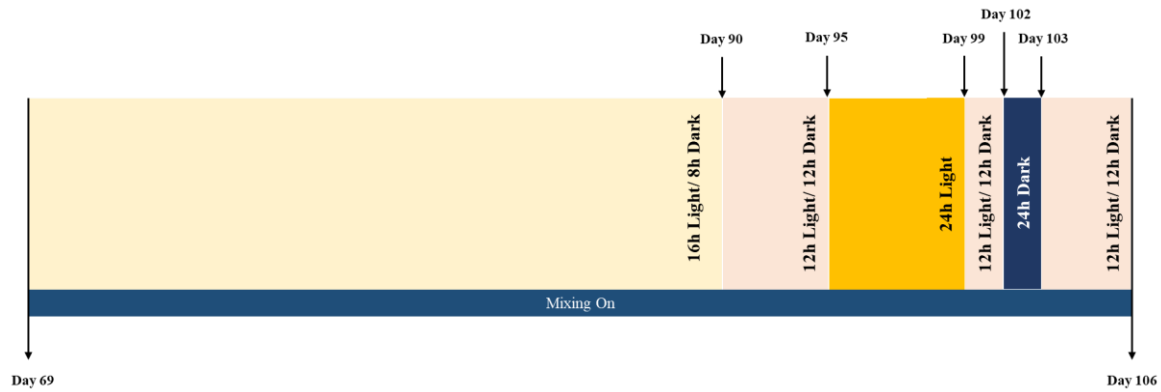


Figure 3.17- Adjustments made in the systems during the second stage of the experiment.

3.2.3.3.1 Mixing Device

On day 69 a new mixing device (Fluval Sea CP3 Circulation Pump) was implemented in the system. The configuration of this new mixing device allowed for the collision carrier + blades to be erased.

3.2.3.3.2 Photoperiod

During this phase, the focus was on optimizing the nutrient removal capabilities of the culture through adjustments in the photoperiod. Extending the dark period served as a strategic move to provide micro-organisms with additional time to consume the dissolved oxygen present in the medium. This extended dark phase, in turn, fosters an anoxic environment, which is highly conducive to the proliferation of anaerobic bacteria necessary to perform denitrification and anammox, thus intensifying the total nitrogen removal in the reactors.

Furthermore, clear indications of bacterial growth and denitrification within the feed tank were observed, a topic will be explained further into the next chapter. By deliberately creating and maintaining an anoxic environment during the dark period, the introduction of anaerobic bacteria into the system is facilitated via the incoming feed solution, which arrives to the reactors every three hours. This approach eliminates the need for these anaerobic bacteria to emerge naturally from the culture.

With this in mind, a 12- hour photoperiod was established, however, on day 95, the pump that connects the feed solution to the reactors malfunctioned, resulting in higher flowrates and lower HRTs. In order to reestablish the biomass concentration in the reactors, the system was put under constant light supplementation for one week. The 12-hour photoperiod was reimplemented in the system followed by a period of 24 hours of darkness to assess the capacity of the culture to reach anoxic conditions. The system was set back to 12-hour period for the last 3 days of the experiment.

3.2.4 Sampling Collection and Primary Results

The sampling days for the reactors were on Mondays, Wednesdays and Fridays, with the exception of weeks where there would be long weekends, in this case the sampling days were on Tuesday and Thursday. The samples were taken in the morning and the results would be tracked in a google sheets.

There were two sites of sampling 1) The suspended liquor and 2) The carriers. The data provided by the sampling was the biomass concentration in the reactors, the biofilm concentration on reactor B, the nutrient concentration (Total Nitrogen, Ammonium Nitrogen, Nitrogen as Nitrate, Total Phosphorus and Chemical Oxygen Demand) and the pH.

3.2.4.1 Sampling of the liquor

The sampling process started by pipetting 30 mL of suspended liquor from both reactors into a beaker. The samples would undergo vacuum filtration. The filter would be set aside for the biomass dry weight to be assessed while the filtrate would be taken for pH measurements and nutrient tests. The process is illustrated in Figure 3.16.

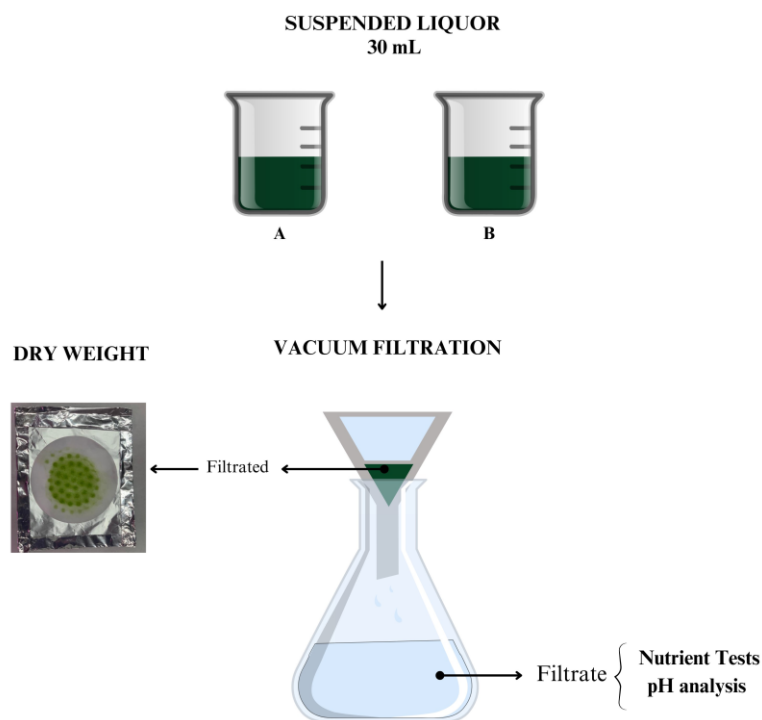


Figure 3.18 – Sampling of the suspended liquor and Vacuum Filtration Process

Under the absence of mixing, intense settling was observed. In order to quantify the total amount of biomass present in the reactor, the liquor started to be mixed with a spoon before the being sampled.

An important point to note is that under constant mixing, the system is homogeneous and the samples taken from the reactor are equivalent to the effluent stream. However, once mixing is interrupted, this approximation cannot be drawn since the liquor is homogeneous. This will have an impact on how the data is analyzed later on.

The feed was also tested to assess the nutrient concentration and underwent a similar procedure as the one illustrated above. However, the biomass was not quantified, only the filtrate was used for the testing.

3.2.4.1.1 Filtrated - Biomass Dry Weight

To determine biomass dry weight, 30 mL of the suspended liquor was vacuum filtrated on pre-dried and pre-weighted filters (Whatman filters, 700 nm) and later placed in the drying oven (OP Series Oven, 24 hours, 100°C). After being dried for the second time, the dried filters were weighed again and the biomass concentration of the reactor was calculated by the following equation:

$$Biomass (g/L) = \frac{m_{F2} - m_{F1}}{V_{sample}}$$

Where,

m_{F1} - Mass of the fresh pre-dried filters, in g

m_{F2} - Mass of the filters after undergoing vacuum filtration and being dried, in g

V_{sample} - Volume of the sample, in L

3.2.4.1.2 Filtrate - pH and Nutrient Analysis

The pH of the filtrate was assessed using a Mettler Toledo pH-meter (Columbus, OH, USA) that had been calibrated with buffer solutions (pH 4,01 ± 0,02, 7,00 ± 0,02, and 10,00 ± 0,02; slope 99,8%; Mettler Toledo). Subsequently, the prepared samples were transferred into 10 mL beakers, and pH measurements were conducted at 20 °C.

Nutrient concentrations of the filtrate were analysed using Hach Lange cuvette tests LCK303 (N-NH₄, 2- 47 mg/L), LCK302 (N-NH₄, 47-130 mg/L), LCK314 (COD, 15- 150 mg/L), LCK350 (Total Phosphorus Protocol, 2-20 mg/L), LCK238 (TN, 5- 40 mg/L), LCK338 (TN, 20-100 mg/L) and LCK339 (N-NO₃, 0,23- 13,50 mg/L).

3.2.4.2 Sampling of the biofilm

The total biomass concentration in reactor B was given by the sum of the biomass concentration of the suspended liquor and the biomass as biofilm concentration. The quantification of the biofilm was achieved through 2 different methods in this experiment.

3.2.4.2.1 Method 1 - Carrier Dry Weight

To determine the biofilm growth, 20 fresh carriers were dried in the oven for 24 hours at 100 °C. Their weight was taken 5 times and the mean was calculated. The average weight was determined as the standard weight of fresh carriers. The results can be found in Table 3.2.

Table 3.2 Weight of 20 carriers before and after being dried

No. carriers	K1 before being dried (g)	K1 after being dried (g)
20	2,9288	2,9283

Every sampling day, 20 carriers were taken from reactor B, placed on a kitchen sieve over paper towel and let dry for 10 minutes. Afterwards, the carriers were placed on tin foil, labelled and taken to the drying oven for 24 hours at 100°C. Once dried, the carriers would be weighted and the difference in mass between these carriers and the 20 dried carriers would be measured and represented as m_{DB} (mass of dried carriers)

The conversion from g to g/L would be done based on the knowledge that 90 carriers are equivalent to 100 mL. With this, the Volume of 1 Carrier V_{1-K1} , is determined by:

$$V_{1-K1}(L) = \frac{100 \times 10^{-3}}{90} = 0,001(1)$$

With a sample of the size of $n = 20$ carriers, the volume of each sample can be calculated $V_{20-K1}(L) = 0,001(1) \times 20 = 0,022$.

By dividing the mass obtained by 0,0022 the Concentration of the Biofilm, c_{DB} would be determined.

3.2.4.2.2 Method 2 - Carrier Dry Weight with Biofilm Isolation

The fact that the carriers had different weights and the variability of the sample was unknown, a different method was employed to quantify the biofilm in the second stage of the experiment.

Similarly to the previous method, 20 carriers were removed from the reactor and placed on a kitchen sieve over paper towel and let dry for 10 minutes. The carriers were later placed in a test tube and filled with tap water to the 30 mL mark. The tube was shaken for 1 minute to break down the biofilm. The carriers were later placed on tin foil and taken to the oven to be dried (24 hours, 100 °C). This was done to quantify the amount of biofilm that did not detach from the carriers. The volume of the remaining solution was reassessed and recorded. Afterwards, the dry weight of this same solution was taken, following the procedure conducted to determine the dry weight of the suspended liquor.

The dry weight of the carriers would be measured as it was done in the *Method 1- Carrier Dry Weight*. This was only implemented to quantify the biofilm that would not break off from the carriers.

With this method, the total biofilm would be given by the following expression:

$$X_b (g/L) = c_{DB} + \frac{m_{F1} - m_{F2}}{V_{sample}}$$

Where,

X_b - Biomass as biofilm, in g

c_{DB} - Concentration of the dried biofilm

m_{F1} - Mass of the fresh pre-dried filters, in g

m_{F2} - Mass of the filters after undergoing vacuum filtration and being dried, in g

V_{sample} - Volume of the sample, in L

3.2.5 Experimental Assessment and Secondary Results

With the data collected from the sampling protocol, was used to obtain secondary results i.e, results that provide a comprehensive overview of the observations that were made throughout the experiment.

Nutrient Removal Rate

The nutrient removal rate represents the rate at which the microorganisms consume or transform the nutrients from the incoming feed. This parameter is calculated by the following expression:

$$S_{Removal\ Rate}(g/m^3 \cdot day) = \frac{[S]_{in} - [S]_{out}}{V_{reactor}} \times Q$$

Where,

S- Nutrient in question

$[S]_{in}$ – Concentration of the nutrient in question in the influent of the reactor, g/m³

$[S]_{out}$ – Concentration of the nutrient in question in the effluent of the reactor, g/m³

$V_{reactor}$ – Reactor Volume, m³

Q- Flowrate, L/day

It is worth noting that the nutrient removal rate is indirectly proportional to the concentration of the nutrient in the reactor. In addition, under the absence of mixing, the samples taken from the reactor are no longer equivalent to the samples taken from the effluent as previously mentioned, therefore, the nutrient removal rates cannot be calculated with the data from collected during this period.

Nutrient Loading Rate

Similarly the loading rate represents the rate at which the nutrients are fed to the reactor and is given by

$$S_{Loading\ Rate}(g/m^3 \cdot day) = \frac{[S]_{in}}{V_{reactor}} \times Q$$

Graphical comparison of the loading rate and the removal rate when plotted against time provides a visual representation of the reactors' performance as will be studied in the results section.

Specific Removal Rate

Specific removal rate represents the capacity that the cells present in the culture have to uptake the nutrients:

$$SRR (g[S]/g[biomass].day) = \frac{[S]_{removal\ rate}}{X}$$

Where,

$[S]_{removal\ rate}$ – The removal rate of a given substrate

X - The concentration of biomass in the system.

Biomass Growth Rate

The growth rate was given by the following expression:

$$X_{growth\ rate}(g/L.day) = \frac{dX}{dt}$$

Where,

dX – The difference between the final and initial biomass concentration

dt – The time in which the biomass concentration variation takes place

Average and Standard Deviation

The analysis of the results was also benefited from the determination of average and standard deviation. Calculating the average allowed for a better understanding of the reactors' dynamics throughout the different stages. Coupling that information with the standard deviation of the parameters in question, provided insights on the stability of the systems over the course of the experiment. The formulas used were the following:

$$\bar{X} = \frac{\sum_{i=1}^n x_i}{n} \qquad \sigma = \sqrt{\frac{\sum (x - \bar{x})^2}{n-1}}$$

Where,

$\bar{x}$ - Mean average

x - Observation

σ - Standard Deviation

n – Number of observation

RESULTS AND DISCUSSION

4.1 pH Progression

4.1.1 pH over the course of the experiment

In this study, pH was considered as a system variable rather than a control parameter. Therefore, the analysis of the pH evolution will be conducted with the aim of gaining a better understanding of the culture's dynamics, rather than solely determining optimal productivity ranges.

Figure 4.1 and table 4.1 display the pH progression in both systems over the course of the experiment and the standard deviation of this same parameter in the same time period respectively.

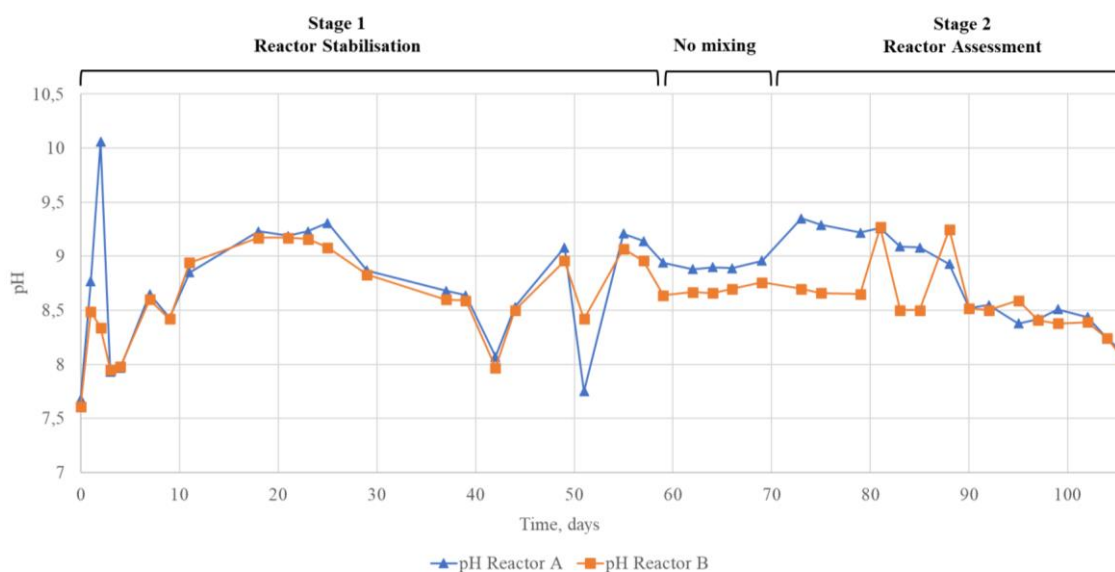


Figure 4.1 - pH progression in reactors A and B over the course of the experiment

Table 4.1 -Standard Deviation of pH values on reactors A and B over the course of the experiment(SD - Standard Deviation)

Reactor	Stage 1		No mixing		Stage 2	
	A	B	A	B	A	B
SD of pH from day 0 to day 106	0,58	0,44	0,03	0,04	0,42	0,32

Based on the Standard Deviation (SD) and the pH progression, both systems achieved highest stability under the absence of mixing. This was also a period where the systems did not undergo any environmental adjustments. It is worth noting that the pH values recorded during this time equate to the average pH of the culture and it is not representative of the systems as a whole. This is due to the fact that lack of mixing results in uneven ionic distribution.

The SD analysis indicates that B was slightly more stable than A, however, the high SD values as well as the inconsistent profile of the curves during Stage 1 and 2 can be attributed to the ongoing environmental changes to which the cultures were being submitted during those stages. In order to better understand how the correlation between the experimental factors and the behaviour of the culture, a more thorough analysis will be conducted next.

4.1.2 pH during Stage 1

Figure 4.2 depicts the pH progression in Reactor A and B during the 1st Stage of the experiment.



Figure 4.2 - pH progression in Reactor A and B during the 1st Stage. F/FS - Fertiliser/Feed Solution; Black bars represent the different F/FS ratios implemented

In the first 11 days, an overall increasing pH trend in both reactors is observed. However, there are noticeable drops on days 3 and 9 in both reactors. These drops may be attributed to the reactors' response to adjustments in F/FS ratios made on days 2 and 7. Alternatively, they could be a consequence of microbial interactions as microorganisms adapt to their new environment.

Ratzke & Gore, 2018 highlighted that different species have varying pH preferences, and the proton turnover resulting from biochemical reactions was acknowledged. This grants microbes the capacity to influence the pH of their surroundings. This influence on pH may become more pronounced when significant changes are done to the growth medium as observed during this time span.

From day 11 until day 25, the pH remained similar in both reactors, and stabilized between 9 and 9.5, which is outside the range for ideal microalgae growth (Wehr, 2007). Similar pH levels were previously reported by (J. Li et al., 2018) when culturing *C. Vulgaris* using NaHCO_3 as the solely source of carbon.

The attempt to decrease the pH by decreasing the concentration of bicarbonate led to a slight drop in the pH of the system. According to literature, carbon limitation can have affect chlorophyll content of certain species of microalgae and in turn, hinder photosynthetic activity (Fleischer, 1935) which is the main contributor to the rise in pH levels.

After day 39, the pH in both cultures suffered an accentuated decrease. It is believed that this drop can be linked to the proliferation of heterotrophs that consume OH^- ions to perform reactions such as nitrification (Wilczak, 1996) and respiration (Heyman et al., 2020). However, the trend rapidly shifted from day 42 to day 44 which could indicate that microalgae started to use glucose as a source of carbon to perform photosynthesis.

The pH results from day 49 reveal that shortening the photoperiod and increasing the bicarbonate concentration to 1g/L lead to a more alkaline environment. Under these conditions, the cultures experienced a sudden drop on day 51. However, the pH levels were reestablished on day 55, and until day 59, both systems evolved towards lower pH values. These fluctuations can be related to the microorganisms' metabolisms. For example, on day 51, heterotrophic activity was more prominent on A than on B and between days 55 and 59 the opposite was observed. The profile of the curves reveal a shift between autotrophic and heterotrophic metabolisms from days 46 to 55 with the last 3 data points revealing a gradual increase of heterotrophic behavior in both systems.

The microorganisms' metabolisms are the main contributors to the pH fluctuations in this system. Despite not having a detailed qualitative nor quantitative information regarding the microbial population, the culturing conditions adopted in this experiment are known to promote or prevent the growth of certain species. With this, Figure 4.3 compiles the five main biochemical reactions that are likely to be performed by the microbial population in the system, along with their impact on pH.

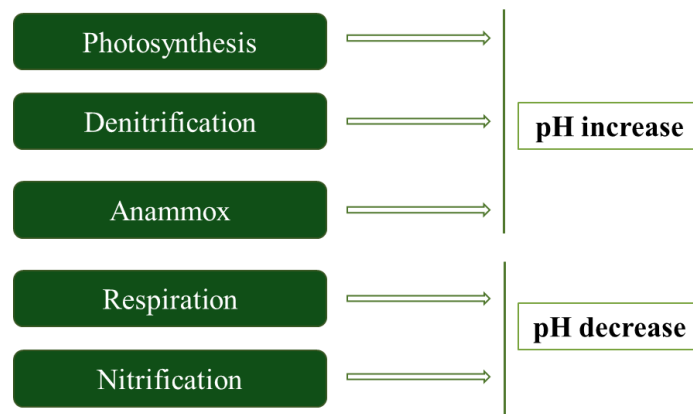


Figure 4.3- Biological reactions and their impact on the pH progression of a mixed culture. Sources: Photosynthesis (Wu et al., 2022); Denitrification –(riffat, 2013); Anammox (Ren et al., 2021); Respiration (Heyman et al., 2020); Nitrification (Wilczak, 1996)

It is worth noting that reactions such as denitrification and anammox only take place under anoxic conditions (Dissolved Oxygen < 0,2 mg/L (Van Der Lee et al., 2017)) which can only be reached if the rate of consumption of oxygen is higher than the rate of production. Therefore, constant periods of light exposure and photosynthesis rules out the possibility of there being any anoxic reactions in the reactors.

4.1.3 pH under No Mixing and during Stage 2

Figure 4.4 depicts the pH progression in Reactor A and B under the absence of mixing and during the 2nd Stage of the experiment.

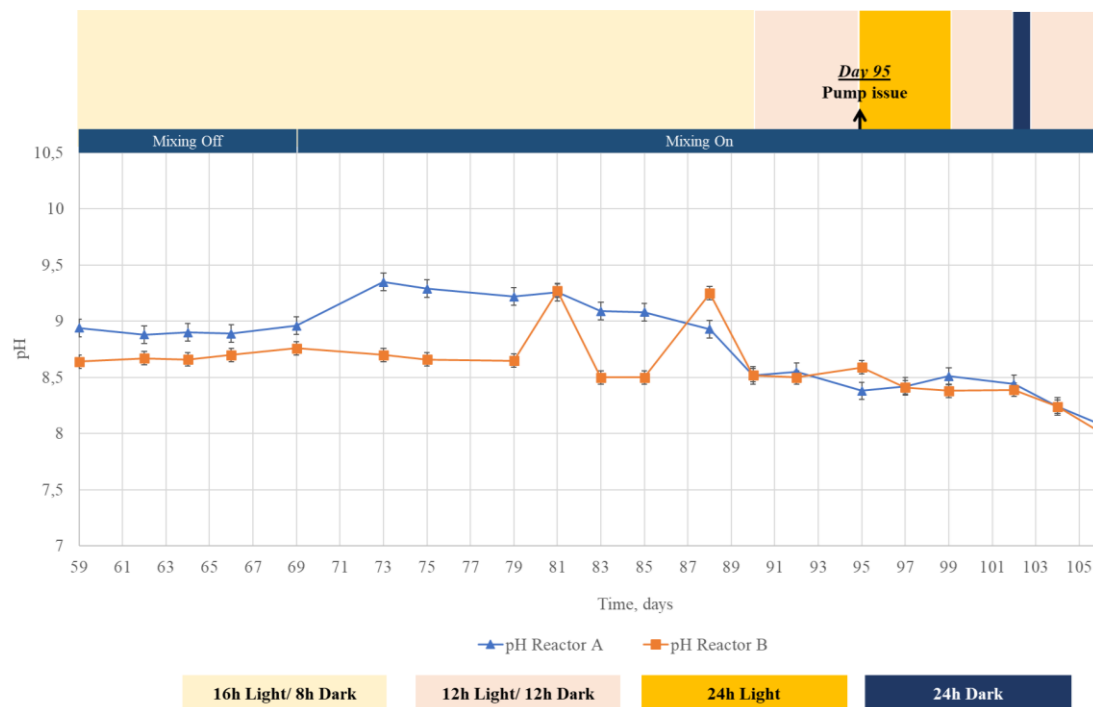


Figure 4.4- pH progression in reactors A and B in the absence of mixing and during the 2nd stage of the experiment.

Under no mixing, the pH remained relatively constant in both reactors. The progression over this period suggests that the culture in A has reached an equilibrium at higher pH ranges than B, indicating higher rates of photosynthesis in this reactor.

The introduction of mixing on day 69 led the pH in A to increase above ideal levels once again, however, this was followed by a gradual decline until day 90 (from 9,35 to 8,52). As no environmental changes are occurring during this time span, this declining trend can be attributed higher rates of nitrification or respiration, suggesting a growing population of heterotrophs in the culture. The progression of the curves in A and B during this period reveals a divergence of microbial communities in both reactors which had not been previously observed up until now.

In the case of B, pH levels remained largely unaffected by the implementation of mixing and remained relatively stable during this time span (day 59- day 90) with the exception of days 81 and 88, when sudden increases were observed. The deviations observed in this time span may be attributed to isolated disturbances that occurred in the system as under this period, no controlled parameter was changed and the progression does not exhibit a trend that can justify these fluctuations. Excluding these points, the pH in B ranged between 8,50 and 8,76, the consistency observed during this period indicate the establishment of an ionic equilibrium.

The declining trend in pH observed in A from day 59 up until day 90 was disrupted by the introduction of a 12- hour photoperiod. Initially, the pH experienced a slight increase from 8,52 to 8,55 before subsequently dropping to 8,38. As for B, the fluctuations remained within the range recorded earlier in this stage.

Both systems reacted oppositely to the week of constant light supplementation, with A registering a slight rise in pH associated with increasing photosynthetic rates, whereas in B, nitrification and/or respiration are believed to have induced the drop in pH observed in this time span.

After a 24 hour of no light supplementation, the sampling started to take place during the dark period. The low pH values registered on days 104 and 106 in both A and B can be attributed to the fact that under a dark environment, only heterotrophic metabolism is taking place, resulting in higher concentrations of CO₂ in the medium.

4.2 Biomass Concentration in the Reactors

4.2.1 Biomass over the course of the experiment

Figure 4.5 and table 4.2 display the biomass concentration progression in both systems over the course of the experiment and the standard deviation of this same parameter in the same time period respectively.

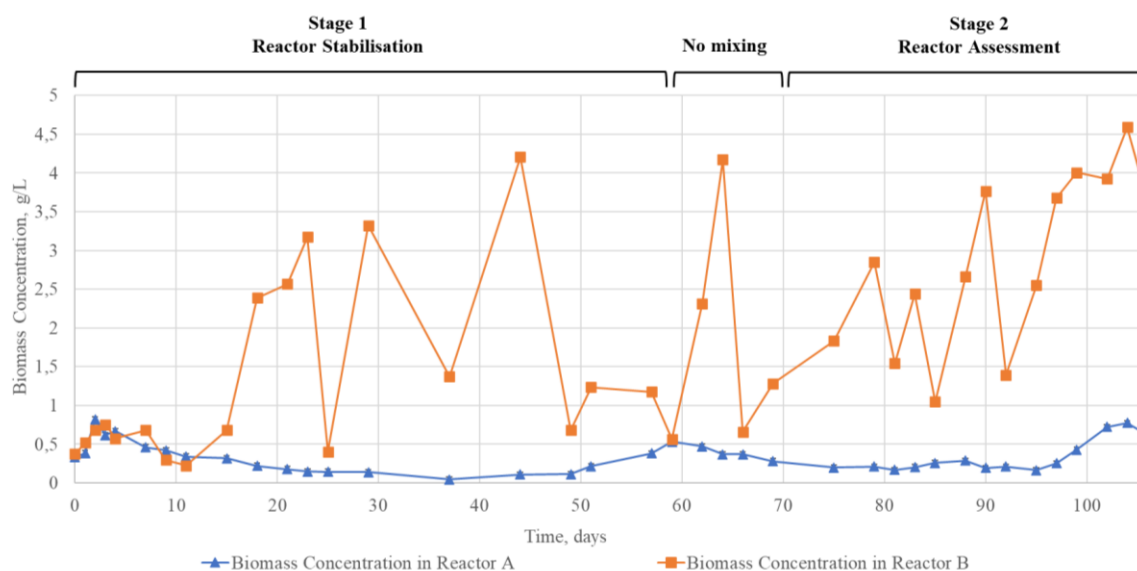


Figure 4.5- Biomass Concentration in Reactors A and B over the course of the experiment

Table 4.2- Standard Deviation of Biomass Concentration on reactors A and B over the course of the experiment

	Stage 1		No mixing		Stage 2	
Reactor	A	B	A	B	A	B
S.D. of Biomass Concentration from day 0 to day 106	0,21	1,14	0,07	1,33	0,21	1,07

Based on the SD, system B is depicted as the most unstable system throughout all three moments of the experiment, which is backed up by the inconsistent profile displayed in figure 4.5. The reason for this can be attributed to the variability of the carriers along with the different and independent forms of biomass growth (attached and suspended) that coexist in this system. This diverse nature can result in fluctuations motivated by different factors which will be later explored in this section.

For A the system appeared to be more stable under no mixing, and from a statistical perspective, the system displays the same level of stability during stages 1 and 2.

4.2.2 Biomass during Stage 1

Figure 4.6 depicts the biomass concentration progression in Reactor A and B during the 1st Stage of the experiment.

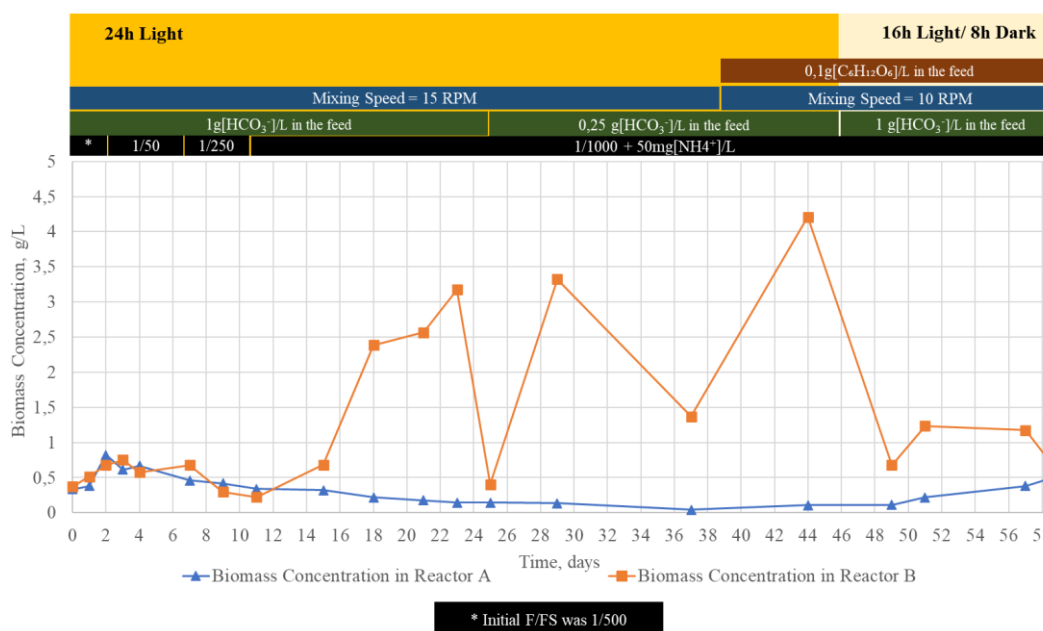


Figure 4.6- Biomass Concentration in Reactors A and B during the 1st Stage of the Experiment

Initially, the biomass concentration in A increased reaching a peak on day 2. The 1/50 F/S ratio appears to have negatively impacted the culture. The ammonium concentration of the feed solution with a 1/50 F/S ratio of 300 mg/L, which is above the ideal concentration for microalgal growth (Meier et al., 2022), this could have led to lower growth rates in the culture. However, diluting the medium to ideal range of ammonium concentration for algal growth (74 mg/L) (Li et al., 2019) did not make a difference in the declining trend registered up until day 11. The lowest biomass concentration in A was observed on day 37.

Day 37 depicts an inflection point in the progression of system A. However, the introduction of glucose may be directly associated with the increase in biomass observed in the system at the following sampling point (day 44). This could be associated with mixotrophic metabolism, i.e, when both autotrophic and heterotrophic metabolism is performed by microalgae. This occurs when light and an organic source of carbon is provided to the culture (Abiusi et al., 2022). In addition, this metabolism has been known to increase biomass productivity in microalgal-based cultures (Abiusi et al., 2020).

The culture's growth seems to have been further enhanced after the reintroduction of 1 g/L of bicarbonate in the feeding solution (day 46), which is reflected in the increased slope of the biomass concentration profile from day 49 until day 59. This is consistent with the observations made by Mokashi

et al., 2016. In his study, optimal biomass productivity of *C.Vulgaris* was registered under 1 g/L of HCO_3^- while 0,25 g/L was shown to be a limiting concentration of inorganic source for the culture.

On the other hand, in the first 11 days, B followed a similar progression than A, mainly because during this time, no biofilm was able to be detected on the carriers through the dry weight measurements. It wasn't until day 15 that an increasing trend in biomass growth was detected in this system.

To understand the origins of the biomass fluctuations observed during this phase, the graph in Figure 4.7 offers the biomass distribution in reactor B along with the progression of the biomass in reactor A.

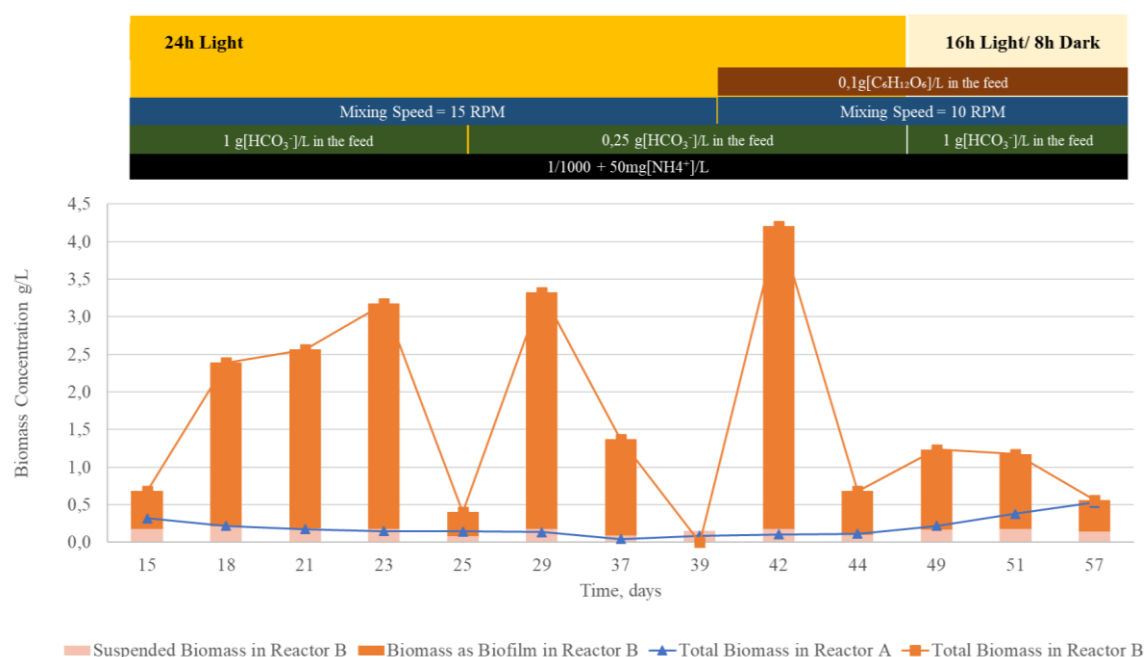


Figure 4.7 - Biomass Concentration in reactors A and B during the 1st Stage of the experiment

The suspended biomass in reactor B follows a progression similar to what was previously observed in A. This demonstrates that the addition of carriers to a suspended system, allows for a significant increase in the total amount of biomass in the biofilm reactor.

Regarding the progression of the total biofilm concentration in this system, initially, a gradual growth can be observed between days 15 and 23. A drop of 89% is observed on day 25. Since no environmental changes were made, it is possible that this drop could have been caused by experimental errors associated with the sampling methods adopted for biofilm quantification, or due to sloughing of the biofilm.

However, 4 days later, the biofilm returns to the concentrations recorded prior to the drop on day 25. A second decline is observed on day 37, equivalent to a loss of 59% of biofilm, possibly related to nutrient limitations to which the system was subjected between days 25 and 39.

On day 39, there were no records of biofilm in the system, similar to the drop on day 25; this fact may be related, once again, to the errors associated with the carriers' sampling method or sloughing.

On day 42, the highest biomass concentration was recorded in reactor B, 96% in the form of biofilm - 23 times higher than the concentration of free suspended cells. It is believed that these values are a consequence of the glucose supplementation to the system, which promotes bacterial growth for biofilm formation. Glucose supplementation has been closely linked to enhanced biofilm formation across literature, for example, (Moreira et al., 2013) that observed a proportional correlation between glucose concentrations and biofilm amount (as OD_{570 nm} values). (Namdarimonfared et al., 2023) has obtained success by using glucose to induce biofilm growth. In addition, glucose has also been found to be integrated into EPS of algal-bacterial biofilms (Qian et al., 2023).

The second largest drop in biofilm, was recorded between day 42 and day 44 - 86%, followed by a slight increase from day 44 to day 49. From there, a gradual decline is observed until day 57.

The average concentration of biomass as biofilm under mixing speed of 15 rpm and 10 rpm are 1,82 g/L and 1,42 g/L respectively, i.e, the higher attached biomass concentrations were registered under higher mixing speeds. These results call into question the assumption that was made in the previous chapter, stating that agitation speed was the primary limiting factor in cell adhesion, as the frequency of the collisions were preventing the permanent attachment on the carriers, ultimately hindering biofilm maturation. According to literature, high sheer stress can indeed cause biofilm sloughing, however, it can also lead to more compact and denser biofilms (Tsagkari et al., 2022; Paul et al., 2012). Therefore, the reduction of the mixing speed did not prevent sloughing from occurring, but rather it led to more loosely bonded biofilms.

Considering the differences between the biomass as biofilm and the suspended biomass observed in reactor B, this system can be divided into two subsystems:

- Subsystem B1: Corresponds to the suspended biomass. It exhibits a behavior similar to reactor A in Stage 1 of the experiment.
- Subsystem B2: Corresponds to the fraction of biomass that grows on the carriers as biofilm.

During this stage, monitoring the development of the biofilm posed challenges due to issues related to the biofilm quantification method and the time intervals between sampling. Variability in the weight of individual carriers led to situations where, when measuring the dry weight, the final weight of samples with biofilm appeared lighter than that of fresh samples. Consequently, this resulted in negative biomass values for the carriers, even when biofilm was visibly present. With this, some samples had to be excluded from the analysis. Moreover, these factors also impacted the confidence in the results presented in the graph during this time.

4.2.3 Biomass in the absence of mixing and during Stage 2

Figure 4.8 depicts the biomass concentration progression in Reactor A and B during the 2nd Stage of the experiment.

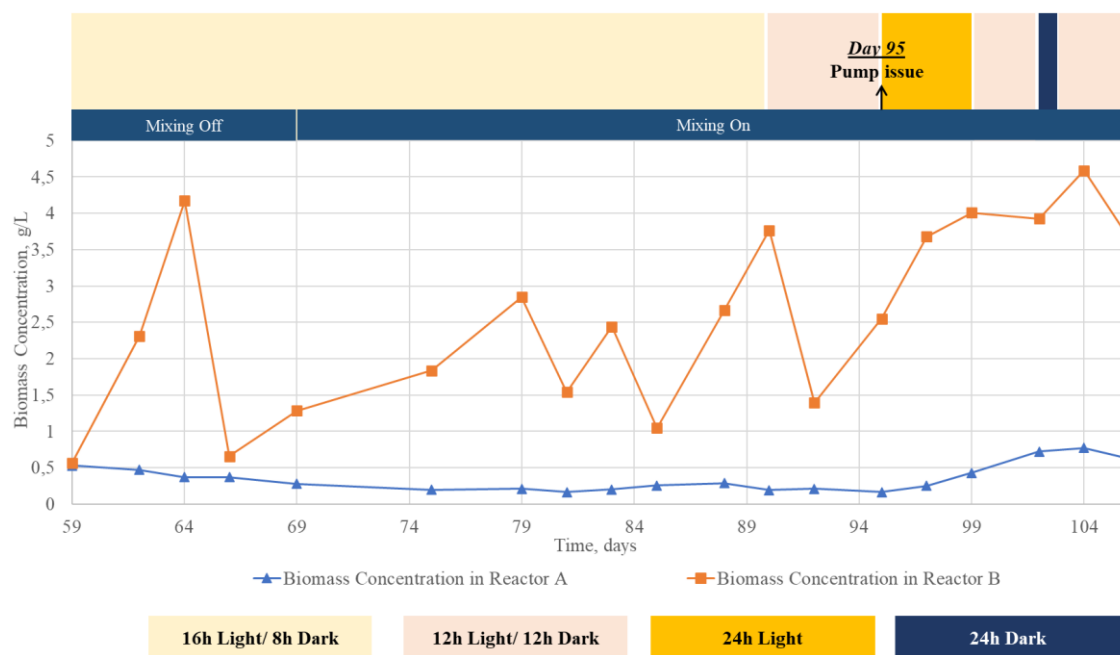


Figure 4.8 - Biomass Concentration progression in reactors A and B during the 2nd stage of the experiment – Reactor Assessment

Under the absence of mixing, system A recorded a biomass concentration decline, following a trend opposite to that observed in the final phase of Stage 1. This decline was gradual until day 75, and can be attributed to the formation of dead zones in the reactor due to the lack of mixing.

Between days 75 and 95 the biomass in A stabilized with values ranging between 0,163 g/L and 0,287 g/L and a standard deviation of 0,03 g/L and the progression shows that shortening the photoperiod did not have an impact on the biomass concentration. Furthermore, the pH analysis displayed an steady decline during this time interval.

Continuous light supplementation between days 95 and 99 led to an increase in biomass concentration in A. The implementation of a 12-hour photoperiod does not seem to have interfered with the growth trend of this culture, however, the sample collected on day 106 showed a slight decrease in biomass concentration, which may be a consequence of the prolonged exposure to darkness that the system was subjected to between days 102 and 103.

Regarding day 95, although the culture medium appeared to be more translucent than usual, and the pump test provided a flow rate of 0,285 L/min (2,21 times higher than the intended flow rate), these factors do not appear to have significantly affected the amount of biomass in system A. This could be attributed to the high settleability of the microorganisms. On average, 77% of the biomass in A was settled at the bottom of the reactor.

The fluctuations observed in B can be found in Figure 4.9.

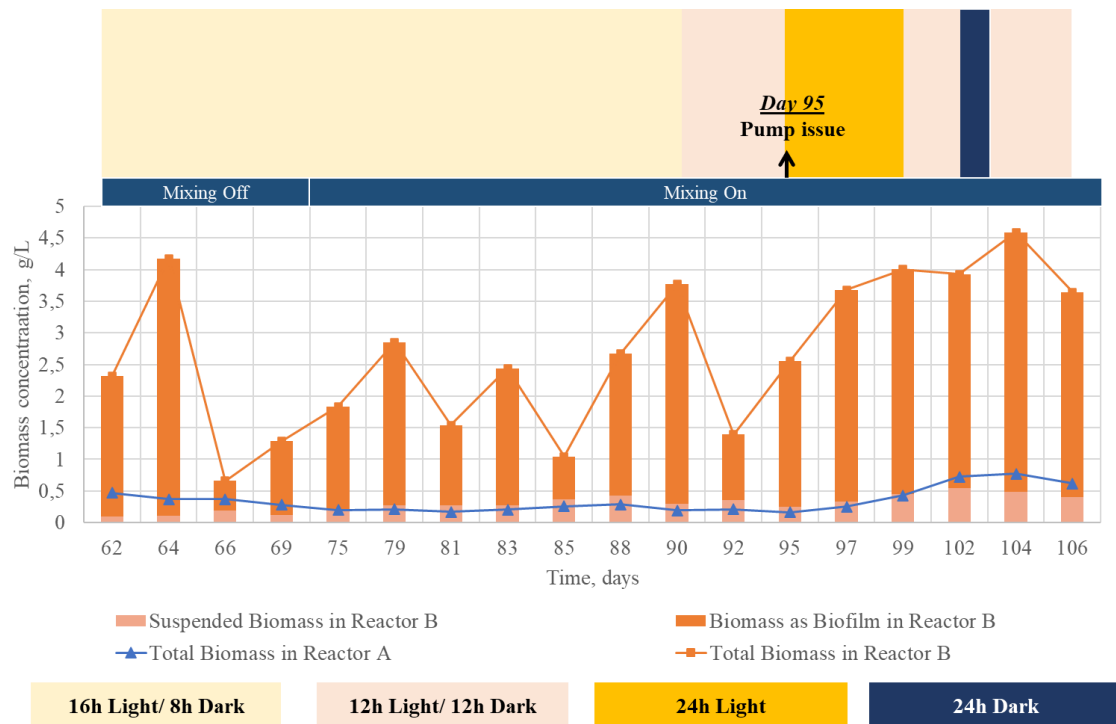


Figure 4.9- Biomass distribution in Reactor B

Similarly to what occurred in Stage 1, Subsystem B1 (Suspended biomass in B) - is of the same order of magnitude as A and exhibits a profile similar to that system in this time span. Additionally, Subsystem B2 (biomass as biofilm) accounts for an average of 87% of the biomass present in B during this stage.

The fluctuations observed during the absence of mixing can be attributed to the uneven distribution of biomass on the carriers, a consequence of nutrient scarcity in certain zones due to poor nutrient distribution. During this period, carrier samples were not representative of the entire system.

On the other hand, the fluctuations observed from day 73 onwards can be related to the life cycle of the biofilms, where increases can be attributed to development and maturation, while periods of decline may be linked to cell disruption (Sauer et al., 2022). It is important to mention that this theory assumes that all carriers are in the same phase of the biofilm cycle.

According to Petrova & Sauer, 2016, disruption of biofilm can occur through dispersion or sloughing. In case of dispersion, the cells escape the biofilm and enter the medium solution in suspension, which, in the culture in question, could result in washout. On the other hand, if sloughing occurs, then cell clusters undergo the same process as the free cells, however, there is a higher chance of the biomass being recycled as the higher density of these structures would allow them to precipitate in the system. The progression provided by the graph does not indicate a biomass equilibrium that would result from the sloughing + precipitation path for biofilm disruption, therefore, it is possible to conclude that the nature of the colonies present in this study allow for an individual dispersal of the cells and ultimately, their removal through washout.

With the biofilm concentration profile depicted in Figure 4.9, it is possible to estimate the level of biofilm development by comparing the consecutive sampling points. For instance, the biofilm on day

75 is less mature than the one on day 79, and on day 81, the drop indicates the beginning of a new cycle. With this distinction, 4 different cycles can be identified, and an estimation of the growth phases can be done (Table 4.3).

Table 4.3 – Assessment of the biofilm lifecycles observed in the 2nd Stage

Cycle	Earliest coloni- zation day	Lowest biofilm concentration	Maturation day	Max. biofilm concentration	Approx. duration of the cycle	Biofilm growth
-	-	g/L	-	g/L	day	g/L.day
1 st	Day 75	1,64	Day 79	2,57	4	0,23
2 nd	Day 81	1,27	Day 83	2,17	2	0,45
3 rd	Day 85	0,68	Day 90	3,48	5	0,56
4 th	Day 92	1,04	Day 104	4,10	12	0,26

The longest cycle was observed during a period marked by a series of adjustments made to the light (between day 90 and 106). In addition, the highest biofilm accumulation was also registered within this interval.

Kuhl et al., 1996 demonstrated that light limitation promotes the creation of inactive photosynthetic layers dominated by bacteria, EPS and other non-photosynthetic materials. Another study by Ylla et al., 2009 observed that light exposure is linked to algal-bacterial biofilm growth and that organic compounds released by photosynthetic microorganisms were readily accessible for bacterial heterotrophs.

The changes made in the photoperiods were not premeditated in this present study, however, when comparing the progression of the biofilms under a constant photoperiod of 16 hours and the progression after day 90, it is possible to conclude that the light supplementation can indeed have an impact on the stability of the biofilm structure.

The fluctuations observed in the first 3 cycles can be attributed to the disruption of the biofilm. Paula, Hwang, & Koo, 2020 found that in the presence of glucose and fructose, the cells colonizing the substrate exhibited growth patterns characterized by interwoven chains, resulting in filamentous mesh-like structures, and surprisingly, the production of EPS in the formation of these microcolonies was undetectable, resulting in biofilms that lacked structural integrity and stability. Another study conducted by Miyatake et al., 2014 identified a “self-destructive” behaviour in bacterial-microalgal biofilms, where carbon consumption by the microorganisms is responsible for the decomposition of more complex and high-molecular EPS into low molecular compounds.

There has been extensive research on how biofilm formation is affected by environmental factors (Hultberg et al., 2014; Salgar-Chaparro et al., 2020; Schultze et al., 2021), however, the nature of the interactions between the microorganisms populating the substrate also plays an important role in the overall stability of the biofilm and consequently the duration of its lifecycle.

The observations made over the course of this experiment show promising results regarding biomass productivity in MBBRs. Furthermore, the use of a wild microbial culture widens the possible biological interactions, however, the lack of qualitative and quantitative data about the species present in the medium prolong the experimental time needed to achieve a thorough understanding of the culture.

So far, one thing is certain, adding carriers to a medium populated with a mixed culture of microalgae, bacteria and other microorganisms, will in fact intensify the biomass production in that system. The remaining question is whether or not this increase in biomass production will be translated into higher rates of bioremediation.

4.3 Feed Nutrient Removal

During this stage of the experiment, bacterial activity was observed in the feed tank (Figure 4.10). Nutrient tests were conducted to understand the influence these microorganisms had on the removal of nutrients in the systems.



Figure 4.10– Microbial growth in the feed tank

The experimental procedure was designed so that every testing day, a new feed solution would be prepared. The HRT of the feed solution in the feed tank would range between 48 hours (to feed the cultures between Monday and Wednesday and Wednesday and Friday) and 72 hours (to feed the cultures over the weekend - between Friday and Monday). With this, the concentration of Chemical Oxygen Demand (COD), Total Nitrogen (TN), Nitrogen as Nitrate (N-NO_3^-) and Ammonium Nitrogen (N-NH_4^+) were measured, and after 72 hours the remaining feed solution was tested again. The results can be observed on Table 4.4.

Due to lack of tests, the Total Phosphorus (TP) concentration after 72 hours was not measured, therefore, the removal of TP in the feed tank remains unknown.

Table 4.4 – Nutrient Removal in the feed tank after a HRT of 72 hours

Parameter	Initial Feed Concentration	Nutrient concentration after 72h	Delta, Δ	Amount removed/produced
-	mg/L	mg/L	mg/L	%
TN	132,30	108,60	-23,70	(-) 18%
N-NO₃⁻	19,90	0,67	-19,23	(-) 97%
N-NH₄⁺	74,00	51,30	-22,70	(-) 31%
Other species of Nitrogen	38,40	56,63	18,23	(+) 32%
COD	111,00	120,30	9,30	(+) 8%

It is important to keep in mind that these tests were conducted over the maximum feed solution HRT to overestimate nutrient removal that can be occurring in the reactor. Furthermore, these variations occur over time, meaning that the concentration of nutrients being fed to the reactor declines over time as well. Since a continuous detailed study of the feed tank was not performed throughout the experiment, the nutrient removal profiles will be analysed based off the initial concentration of the nutrients without considerations of the removal that is occurring in the feed tank. Nevertheless, this information will be useful to better understand the dynamic of the reactors.

The increase in COD in feed solution could be attributed to the microbial population inhabiting the feed system, in particular in the feed tubes. This biological accumulation can lead to the release of organic compounds to the solution as it passes through the feed tubes (Figure 4.11).

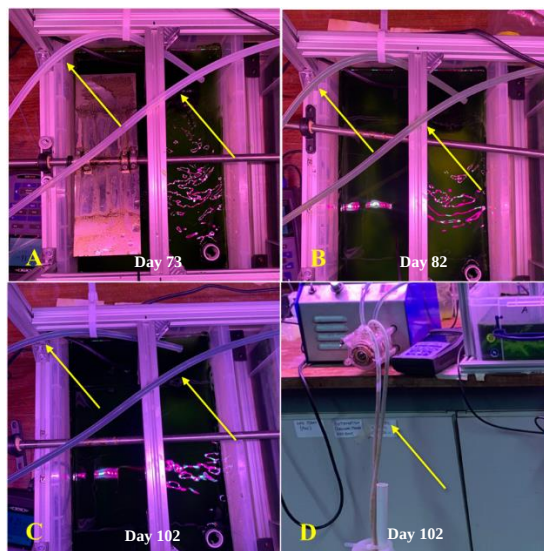


Figure 4.11– Accumulation of microorganisms in the feed tube. A – Day that the tubes were replaced; B – Biomass growth on the tubes after 9 days; C - Biomass growth on the tubes after 20 days; D - Biomass growth on the tubes after 20 days. Growth coming from the feed tank.

As can be observed on table 4.4, 97% of the nitrate of the feed was removed over the weekend. This is equivalent to a delta of 19,23 mg/L. In addition, it is known that heterotrophic growth leads the feed tank to reach anoxic conditions (Table 4.5). This indicates that the removal of nitrates could be performed by denitrifiers that are converting nitrates into nitrites and finally nitrogen gas.

Table 4.5 – Variation of Dissolved Oxygen (DO) levels in the feed tank over a HRT of 72 hours

DO on the day the feed was prepared	DO after 72 hours of the feed being prepared
mg/L	mg/L
7,98	0,12

On the other hand, 31% of ammonium nitrogen is removed by the system. This is equivalent to a reduction of 22,70 mg/L of ammonium nitrogen. According to literature (Riffat, 2013), under aerobic conditions ammonium is converted into nitrite and finally nitrate through nitrification, and under anoxic conditions ammonium is converted into nitrite and nitrogen gas. Ammonium is also assimilated by microorganisms for cell growth and maintenance.

Initially, the “other species of nitrogen” in the feed solution consisted of ureic nitrogen. In order to be processed by microorganisms, ureic nitrogen first needs to be transformed into ammonium, however, since urea is an organic molecule, it does not dissociate in water nor does it decompose unless energy is added to the system to induce hydrolysis, it is assumed that ureic nitrogen does not undergo any transformation in the feed tank. With this, looking at the concentration after 72 hours, this section’s concentration increased by 18,23 mg/L. It is possible that this additional nitrogen comes from conversion of 18,23 mg/L of nitrate into nitrite and the remaining 1 mg/L left could represent N₂ that resulted from total denitrification.

In addition, by observing the total nitrogen variation, 23,70 mg/L of TN was removed by the system, which could indicate that the 22,70 mg/L of ammonium is being assimilated by bacteria and the remaining 1 mg/L left is the nitrogen gas that escaped the system after complete denitrification.

Considering this, before studying the nutrient removal it is important to keep in mind that:

- The results of ammonium nitrogen removal rate will consist of an overestimate of the actual removal rate conducted by the culture as the loading rates considered for the calculations neglected the biological activity taking place in the feed tank;
- The profile of the nitrate removal rate will not in fact represent the removal of this nutrient. Instead, it will offer insights into the reactions occurring in the reactor and the microorganisms responsible for these reactions. This, in turn, contributes to a better understanding of the microbial composition of the culture.

4.4 Total Nitrogen, Ammonium Nitrogen and Nitrogen as Nitrate

The results from TN and N-NH_4^+ span from day 21 to 106, while the results of N-NO_3^- will only be analyzed in stage 2. The analysis of nutrient removal rates will not include the period of absence of mixing.

4.4.1 Total Nitrogen and Ammonium Nitrogen during Stage 1

During Stage 1, the extended photoperiods registered under these conditions make it unlikely that the culture reaches anoxic conditions. Therefore, reactions such as denitrification and anammox will be ruled out of this analysis.

With this, it can be assumed that the only pathway of nitrogen removal during this stage will be through biological assimilation by the culture. From this, 3 possible scenarios can emerge in the evolution profile of the graphs plot

ed ahead:

- 1) TN Removal Rates $\approx$ N-NH4 Removal Rates – Nitrogen is being removed through biological assimilation
- 2) TN Removal Rates < N-NH4 Removal Rates –
 - a. If TN removal decreases and N-NH4 removal increases, it means that the culture is not assimilating N-NH4, instead, nitrification is taking place.
 - b. If TN removal increases and N-NH4 removal increases, NH4 assimilation is taking place.
- 3) TN Removal Rates > N-NH4 Removal Rates – This is a reflection of the nitrogen removal occurring in the feed solution.

4.4.1.1 Total Nitrogen and Ammonium Nitrogen Removal in Reactor A

Figure 4.12 depicts the Total Nitrogen and Ammonium Nitrogen Loading and Removal Rates in Reactor A during the 1st Stage of the experiment.

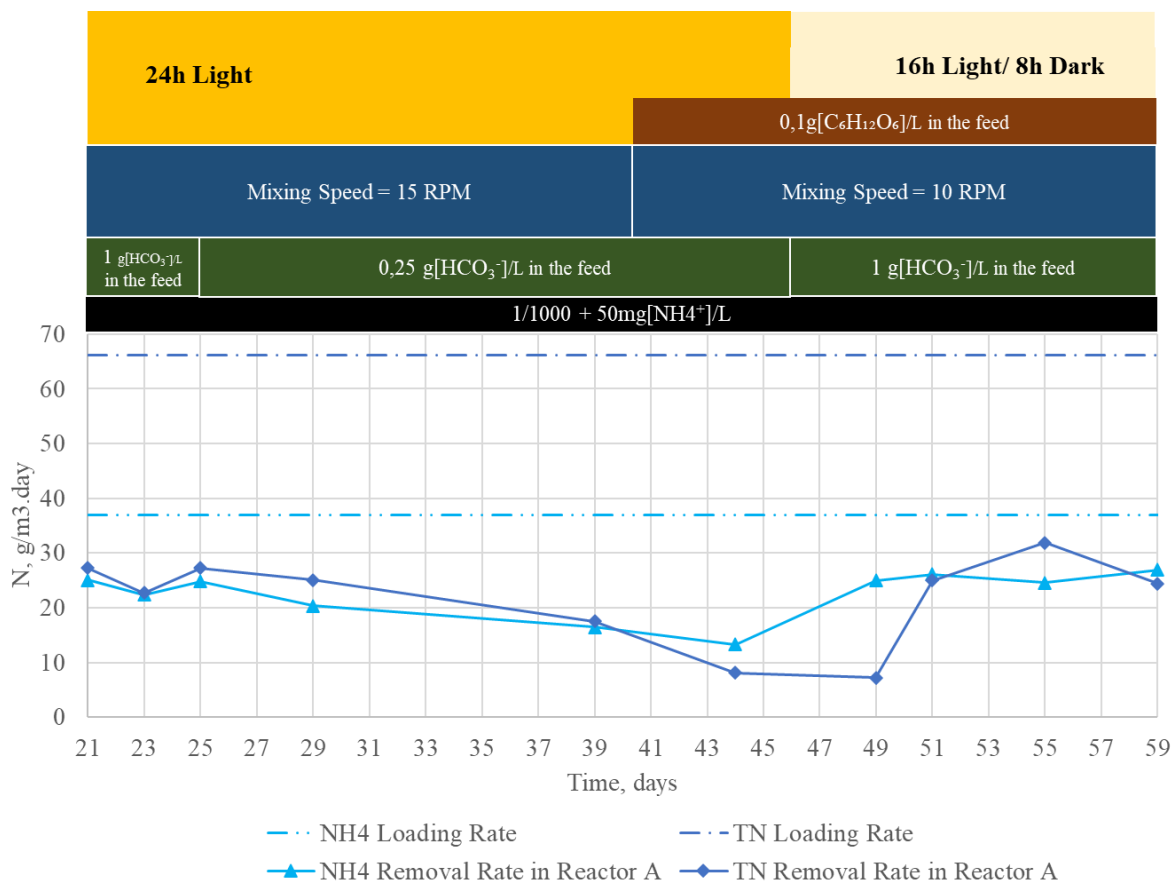


Figure 4.12–TN and N-NH₄⁺ Loading and Removal Rates in Reactor A during the 1st Stage

The progression indicates that under limiting concentration of carbon (between days 25 and 39), the assimilation of N-NH₄ by the culture suffers a decline.

The addition of glucose in the medium induced nitrification on day 44, and the increase verified from this day to day 49 appears to have been also attributed to the implementation of a period of darkness on day 46.

The increase in TN removal on day 51 indicates that nitrification has stopped and N-NH₄ assimilation is the dominant path on nitrogen removal in on this day.

The last 3 data points of this stage suggest that biomass assimilation was the main pathway for nitrogen removal in this system, with the exception of day 55, where the removal of nitrogen happening in the feed appears to have contributed to the overall nitrogen removal in this system.

With this analysis it is possible to conclude that carbon limitation negatively affects the assimilation of nitrogen by the culture which is consistent with the results from (LEE & Lee, 2002) that also observed that lower C/N negatively affects nitrogen uptake by *Chlorella kessleri*.

4.4.1.2 Total Nitrogen and Ammonium Nitrogen Removal in Reactor B

Figure 4.13 depicts the Total Nitrogen and Ammonium Nitrogen Loading and Removal Rates in Reactor B during the 1st Stage of the experiment.

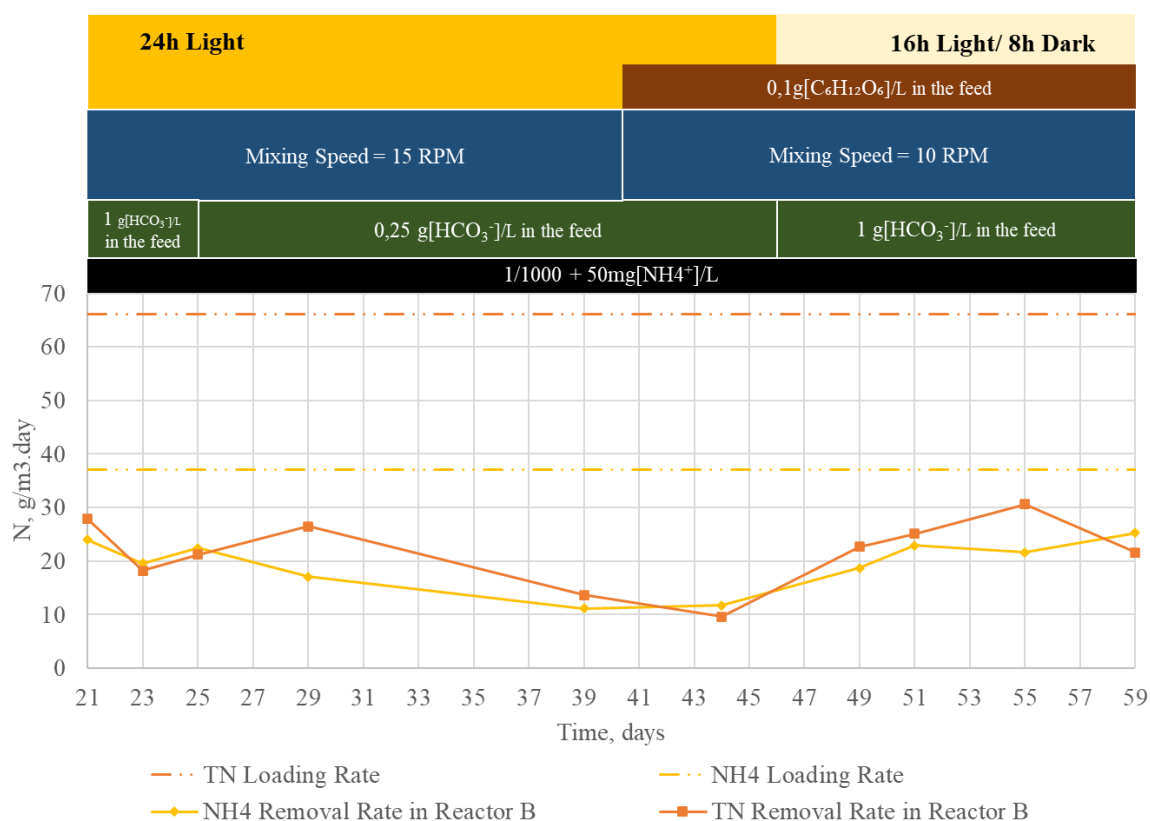


Figure 4.13- TN and N-NH₄⁺ Loading and Removal Rates in Reactor B during the 1st Stage

In the case of reactor B, it is possible to observe a higher TN removal on day 29, which did not occur in reactor A. This could be related to the experimental set up that requires longer tubes to connect the feed tank to reactor B. This, in turn allows for more bacteria to accumulate and consume the nutrients before it reaches the reactor.

Similarly to what happened in reactor A, the removal of N is compromised by the limiting carbon concentrations verified between days 25 and 39. In this case, the addition of glucose did not contribute to increased nitrogen removal and oppositely to what happened in A, nitrification was not observed in the system during this stage.

The addition of a period of darkness as well as the increase of bicarbonate concentration, led to a rise in nitrogen removal in this until day 55. On day 59, the nitrogen removal suffered a slight drop, resembling what occurred on reactor A on this same day.

The progression of this graph suggests that the configuration of the experiment allows for less nitrogen to be fed to this reactor. In addition, at this stage, no signs of nitrification were detected. Similarly to what happened in A, carbon limitation impacts the reactor's performance.

The increase in N-NH₄ removal after day 46 is believed to be due to the increase in bicarbonate concentration rather than the shortening of the light cycle as studies show that NH₄ assimilation is enhanced under longer periods of light exposure in microalgal- based cultures (Zhi et al., 2019; Gonçalves et al., 2014).

4.4.1.3 Ammonium Nitrogen Removal in Reactor A and B

Figure 4.14 depicts the Ammonium Nitrogen Loading and Removal Rates in Reactor A and B during the 1st Stage of the experiment.

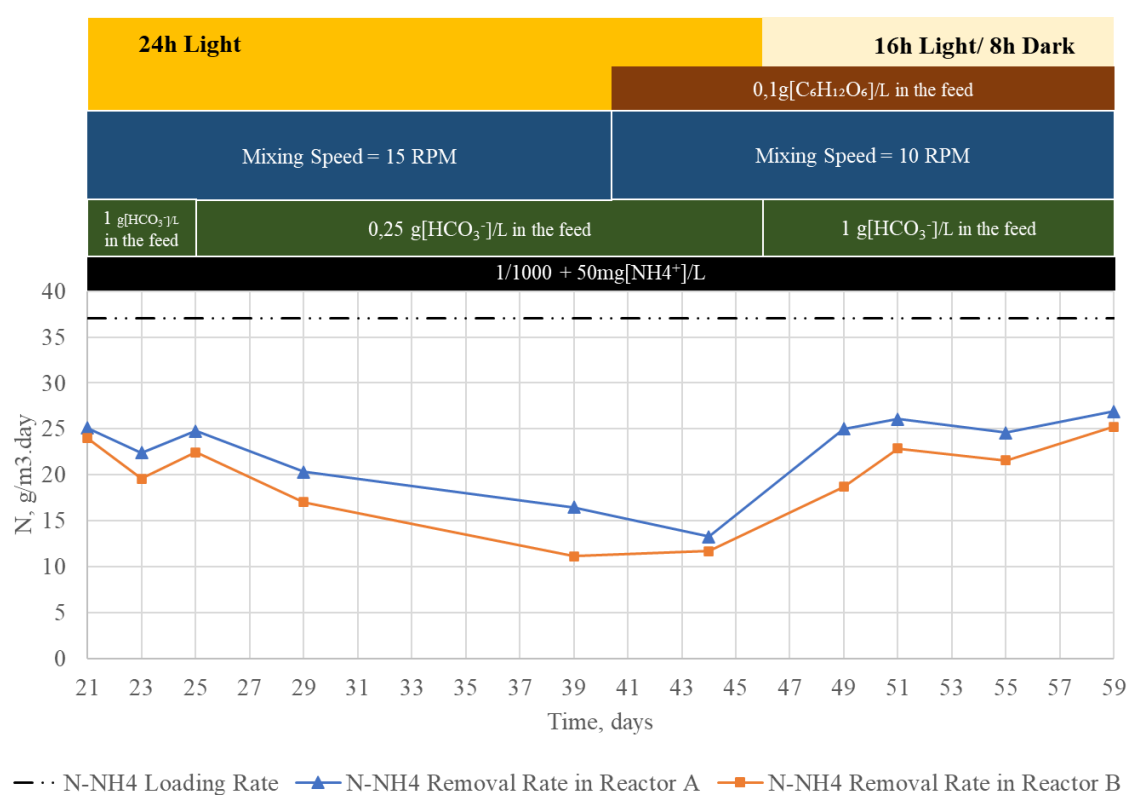


Figure 4.14- N-NH₄ Loading and Removal Rates in Reactor A and B during the 1st Stage

As stated before, ammonium removal is achieved by the culture mainly through biomass assimilation in both reactors during this stage. With this, the removal of ammonium should be proportional to the biomass accumulation in the system.

The progression displayed in Figure 4.14, shows a similar evolution of the ammonium removal in A and in B. In addition, reactor A displays higher rates of removal than B. This fact goes against what is expected for the performance of reactor B, as this system has consistently registered higher biomass accumulation than A during this time span.

Rada-Ariza et al., 2019 achieved ammonium removal efficiencies above 94%, with total nitrogen removal of over 70% across various SRTs in microalgae-bacterial consortia. Another study conducted by Zhang et al., 2022 was able to induce the growth of a 3-layer biofilm on a knotted carbon cloth through the manipulation of the photoperiod. The biofilm comprising of anammox bacteria, AOB and Algae observed in this study was able to remove up to 63% of the total nitrogen present in the medium.

Based on the literature, it is a well-established fact that the symbiotic relationship between algae and bacteria in biofilms enhances nutrient removal in the medium. This makes it intriguing why there appears to be an absence of a correlation between Sub-system B2 and ammonium removal in this present study.

4.4.1.4 Total Nitrogen Mass Balance in Reactor A and B

The mass balance made to the reactors (Figure 4.15) show that most of the nitrogen is trapped in the system, meaning that the systems are fed more nitrogen than their cultures can assimilate and/or remove. To close the balance, an alternative could be cell recycle, however, by the previous analysis, biomass is not the limiting factor for the removal of nitrogen during this stage. Another possibility is to implementation of longer darker periods which could induce an anoxic environment conducive of annamox and denitrification. The effects that the implementation of different photoperiods have on the culture will be explored next.

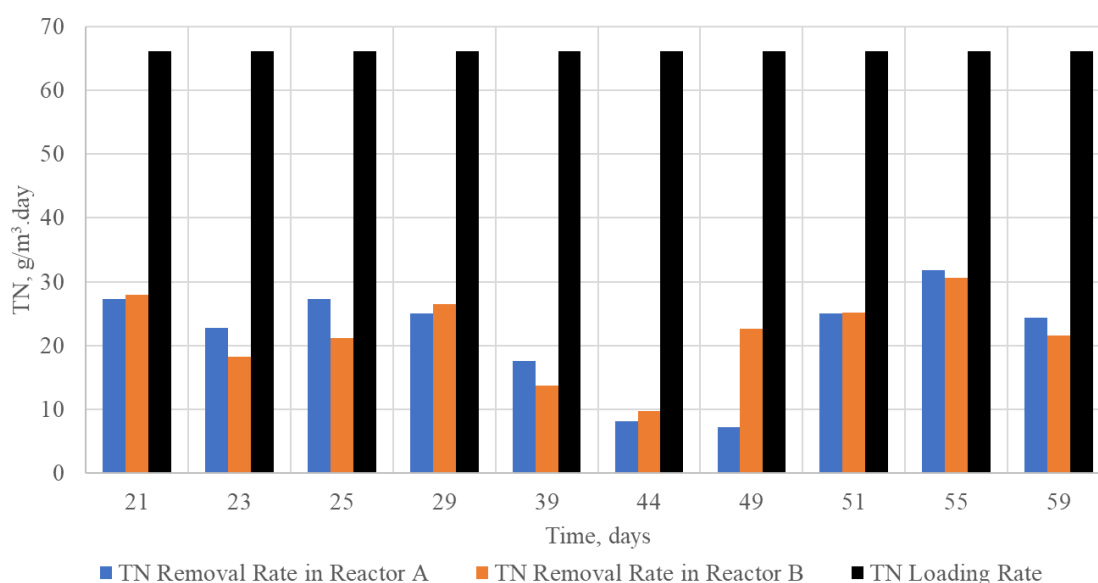


Figure 4.15– TN mass balance in Reactors A and B during the 1st Stage

4.4.1.5 Total Nitrogen Specific Removal Rate

The Specific Removal Rate of Nitrogen represents the portion of the biomass that is responsible for the removal of nitrogen from the system. By analysing the progression displayed in Figure 4.16, this parameter is consistently lower in B than it is in A. This fact indicates that the additional biomass that is

growing and accumulating in B is not up taking nitrogen as efficiently as it could which may be attributed to diffusion limitations in the biofilms. This is one of the drawbacks of the biofilm systems as has been reported by (N. C. Boelee, 2013). From day 49 onwards, the specific removal rate in B displayed an increase that linked to an increase in TN uptake by the culture after the additional bicarbonate supplied to the system.



Figure 4.16- Total Nitrogen Specific Removal Rate over the course of Stage 1 for Reactor A and Reactor B

On the other hand, A displays a similar progression as the total removal rate of nitrogen that can be observed in figure 4.15. Although the specific removal rate in this present study are within the same magnitude as the ones observed in literature (Salgado et al., 2023), the values obtained are lower. This could be attributed to the fact that Salgado et al., 2023 used a monoculture of *C. Vulgaris*, therefore the ammonium available was not limited by AOB activity like it was observed in this study.

4.4.2 Total Nitrogen, Ammonium Nitrogen and Nitrogen as Nitrate Removal during Stage 2

4.4.2.1 Total Nitrogen and Ammonium Nitrogen Removal in Reactor A

Figure 4.17 depicts the Total Nitrogen and Ammonium Nitrogen Loading and Removal Rates in Reactor A during the 2nd Stage of the experiment.

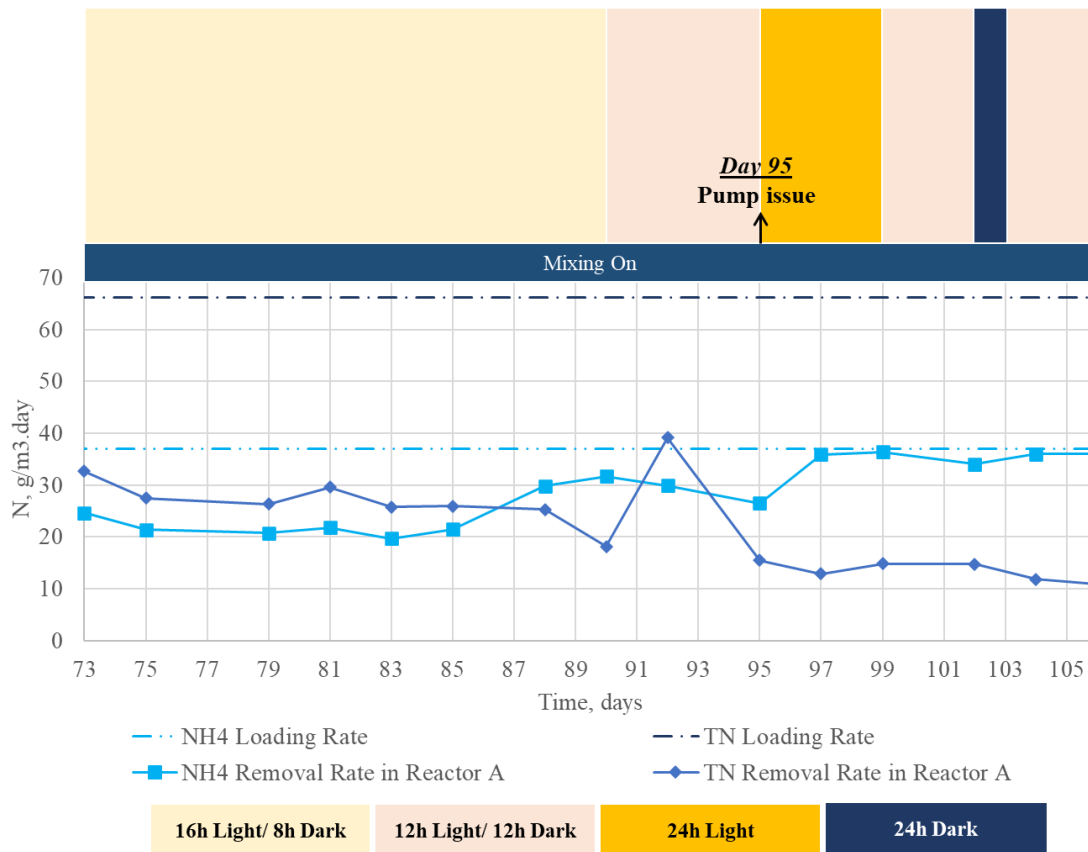


Figure 4.17 - TN and N-NH₄⁺ Loading and Removal Rates in Reactor A during the 2nd Stage

Up until day 85, the graph shows no signs of nitrification. The difference between TN and N-NH₄ removal highlights the occurrence of TN removal in the feed tank.

On days 88 and 90, nitrification takes place, surpassing assimilation rates, causing the TN removal to drop. Since no external adjustments have been made to the systems, the signs of nitrification could be a result of the introduction of AOB through external contamination.

On day 92, the TN removal reached a peak while the N-NH₄ removal suffers a slight drop. This indicates a prominent feed removal on this day, as well as a decrease in nitrification and the reestablishment of N assimilation. It is also possible to attribute this sudden peak to denitrification as on this day, the culture was put under an extended period of darkness that could result in anoxic conditions suitable for denitrifying bacteria. This fact will be investigated with the nitrate removal analysis conducted next.

Day 95 was marked by increasing flowrates in the reactor. From this day onwards, nitrification became prominent in the culture. This fact could be attributed to either increasing AOB entering the system due to pump malfunction and/or the adjustments made to the photoperiod, especially between day 95 and 99, when the phototrophic metabolism was taking place, increasing the DO levels in the culture and consequently, promoting the growth and development of heterotrophs. In addition, the attempt of driving the system to reach anoxic conditions with the shortening of the photoperiod and the 24-hour darkness exposure in the last days of the experiment was not successful, as the TN removal decreased, proving that the high rates of ammonium removal were indeed attributed to nitrification.

This data indicates that ammonium removal in this stage, for reactor A is also the main responsible for TN removal. In addition, bacterial activity is more prominent during this stage compared to Stage 1, as can be seen with the high rates of ammonium conversion in the last part of the experiment. It remains unclear if the nitrification was enhanced by the increased flowrate that resulted from the pump malfunction on day 95, or if it was from the adjustments made to the photoperiod, or even both.

4.4.2.2 Total Nitrogen and Nitrogen as Nitrate Removal in Reactor A

It is known that nitrogen removal is taking place in the feed solution, leading to an almost complete conversion of nitrates into nitrites. Once nitrites enter oxygen rich mediums, they are converted back to nitrates through partial nitrification. With this information the following scenarios can emerge from the nitrate removal curves:

- 1) Decreasing N-NO_3^- removal profile – Indicates that nitrates are accumulating in the system. In other words, the rate at which nitrites are being converted into nitrates is increasing over time.
- 2) Increasing N-NO_3^- removal profile – The conversion of nitrites to nitrates is complete, in addition, nitrates are undergoing denitrification and/ or are being assimilated as biomass. The latter only occurs if N-NH_4 is depleting in the system, which is not the case, therefore, this last removal pathway is highly unlikely. On the other hand, a more likely scenario would be lower rates of nitrite-nitrate conversion.
- 3) No N-NO_3^- Removal- When all of the nitrites as converted to nitrate and/or AOB converts ammonium into nitrate. In addition to this, no further removal pathway are taking place.
- 4) Negative N-NO_3^- Removal – In the presence of AOB and after complete conversion of nitrites into nitrates is conducted, the ammonium present in the system undergoes nitrification resulting in the increase of total N-NO_3^- concentration.

Figure 4.18 depicts the Total Nitrogen and Nitrogen as Nitrate Loading and Removal Rates in Reactor A during the 2nd Stage of the experiment.

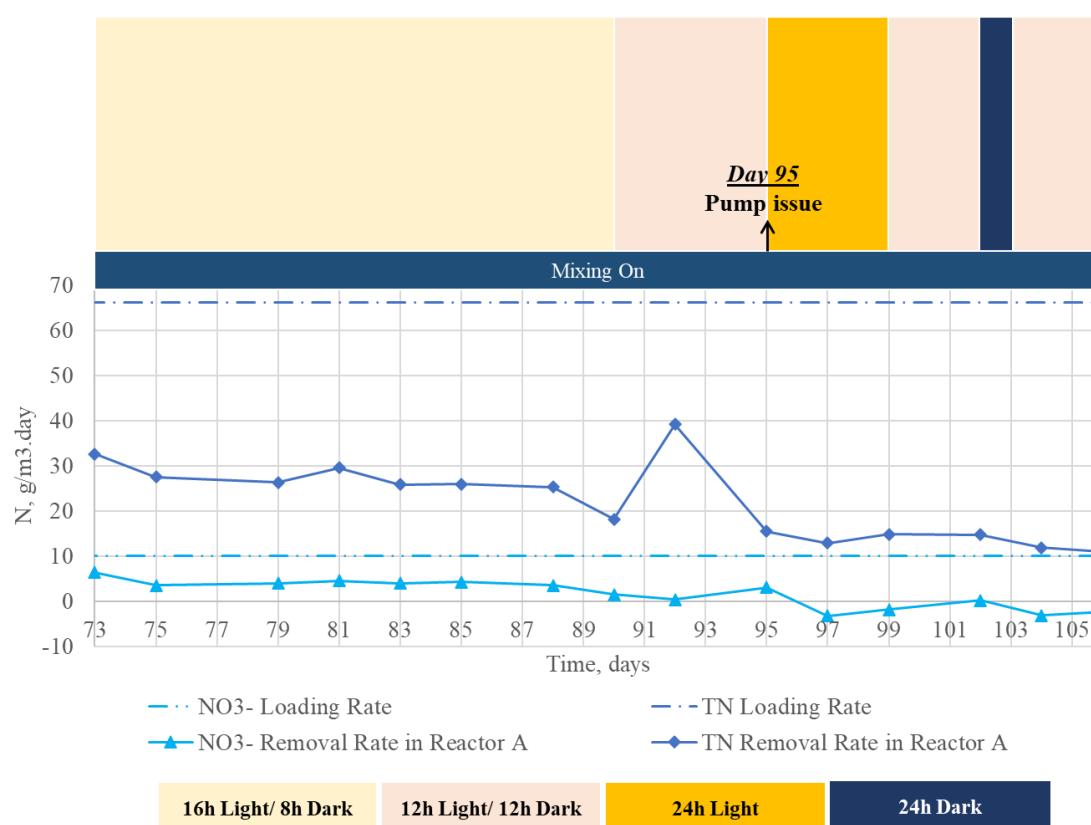


Figure 4.18- TN and N-NO₃⁻ Loading and Removal Rates in Reactor A during the 2nd Stage

Up until day 88, the progression of the nitrate removal remained relatively constant. From day 88 to day 92 a decrease in NO₃⁻ removal was observed, indicating higher rates of nitrite conversion. This conversion can be attributed to both AOB and NOB. This drop also confirms that denitrification did not take place, and the peak in TN removal observed on this day, can indeed be attributed to the removal of nitrogen that is happening in the feed tank.

On day 95 the removal of nitrate displayed a slight increase that could also be related to lower rates of nitrite conversion in the reactor due to lower bacterial activity. It is possible that the increase in the feed flowrate led to the washout of nitrifying bacteria from the medium.

From day 95 onwards, the removal of nitrates was equal or below zero, confirming the prominent presence of AOB in the medium. From this it is possible to assume that the exposure to 24 hours of constant light over a week promoted the development of a new population of these microorganisms.

With this analysis, it is possible to establish a close connection between the removal of ammonium and nitrates in reactor A during this stage. In addition, the lack of anoxic conditions in the system attributes the increases in nitrate removal rates to the partial denitrification that is taking place in the feed tank coupled with conversion of nitrites to nitrates once the solution is fed to the system.

4.4.2.3 Total Nitrogen and Ammonium Nitrogen Removal in Reactor B

Figure 4.19 depicts the Total Nitrogen and Ammonium Nitrogen Loading and Removal Rates in Reactor B during the 2nd Stage of the experiment.

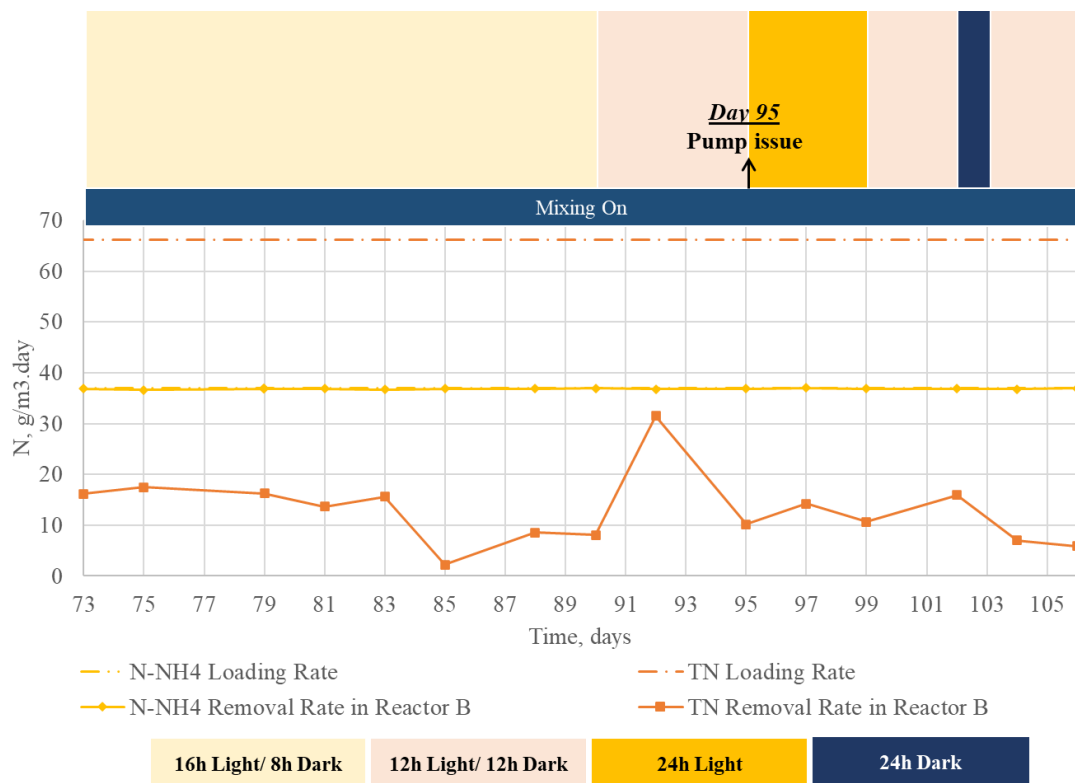


Figure 4.19- TN and N-NH₄⁺ Loading and Removal Rates in Reactor B during the 2nd Stage

In the graph presented in figure 4.19, the N-NH₄ Removal Rate curve overlaps with the N-NH₄ Loading Rate, indicating close to 100% removal throughout this entire stage. The fact that N-NH₄ removal > TN removal, also suggests that in this system, the conversion of ammonium is taking place and competing with nitrogen assimilation by culture.

4.4.2.4 Total Nitrogen and Nitrogen as Nitrate Removal in Reactor B

Figure 4.20 depicts the Total Nitrogen and Nitrogen as Nitrate Loading and Removal Rates in Reactor B during the 2nd Stage of the experiment.

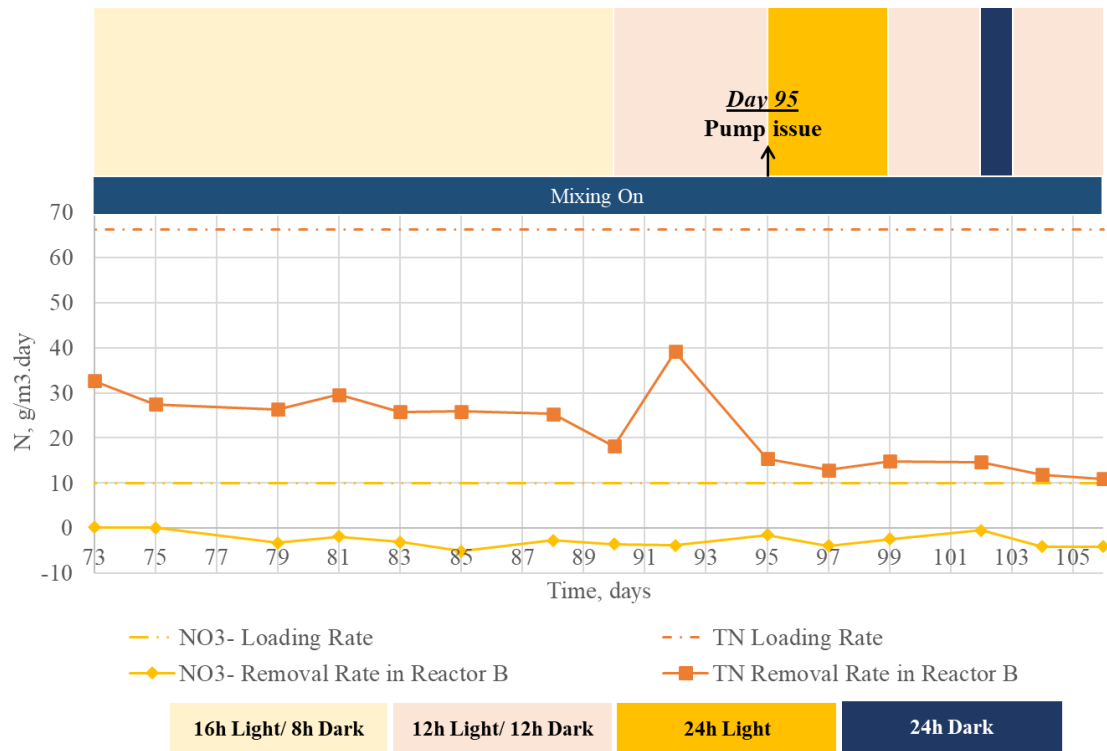


Figure 4.20- TN and N-NO₃- Loading and Removal Rates in Reactor B during the 2nd Stage

The removal rates of nitrate in this system vary between 0,2 g/m³.day and -5,05 g/m³.day which equates to approximately 14% of the total ammonium removed in this system during this time span. This 14% ammonium removal represents the additional nitrite that NOB is able to convert to nitrate, meaning that there is still more nitrite in the system that was not converted into nitrate.

4.4.2.5 Ammonium Nitrogen Removal in Reactor A and B

Figure 4.21 depicts the Ammonium Nitrogen Loading and Removal Rates in Reactor A and B during the 2nd Stage of the experiment.

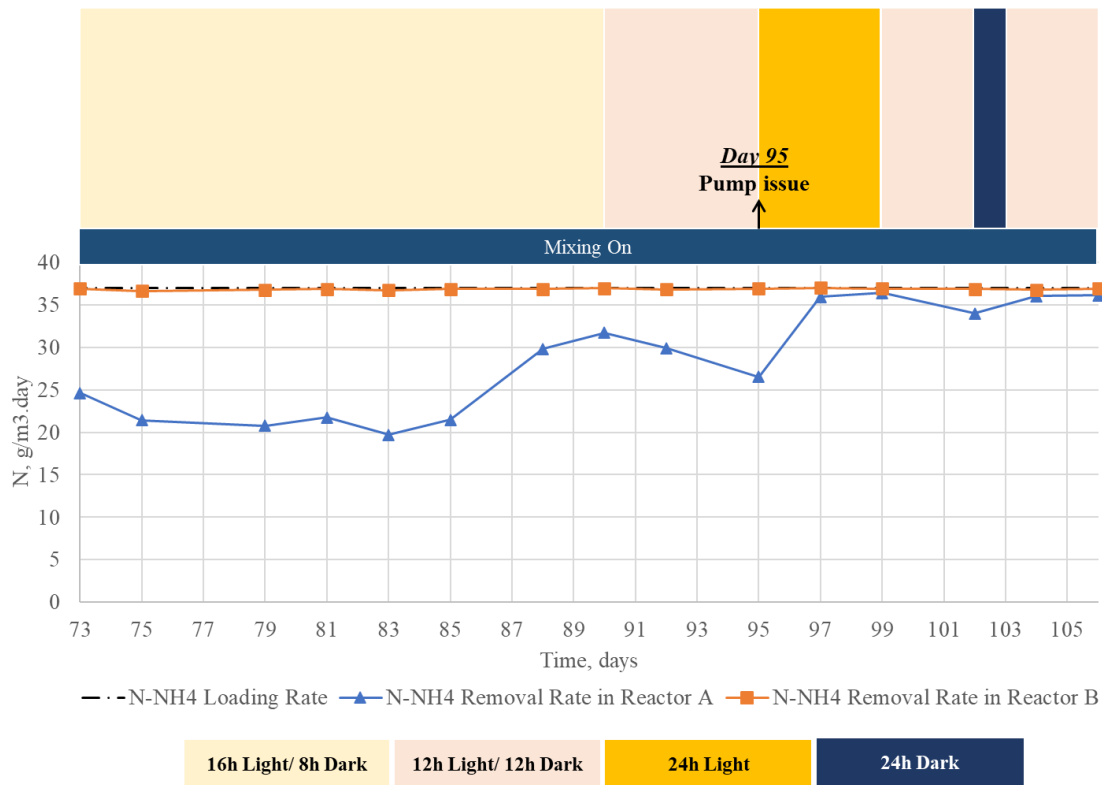


Figure 4.21- N-NH₄ Loading and Removal Rates in Reactor A and B during the 2nd Stage

By comparing the N-NH₄ removal in reactor A to reactor B, it becomes evident that the latter is more efficient at removing ammonium. This progression also reveals that the bacterial culture in this system is not responsive to the adjustments made to the photoperiod. The lower HRT that resulted from the pump incident on day 95 followed by the constant light supplementation did not seem to affect the removal of ammonium in this system as much as it did in reactor A, indicating that the population bacteria that evolved in this last part of the experiment, was already present in B from the beginning. In addition, this increase in AOB in A could be a result of the increase in oxygen concentrations due to higher rates of photosynthesis under constant light supplementation in the system, which is consistent with the observations made by French et al., 2012.

4.4.2.6 Nitrogen as Nitrate Removal in Reactor A and B

Figure 4.22 depicts the Nitrogen as Nitrate Loading and Removal Rates in Reactor A and B during the 2nd Stage of the experiment.

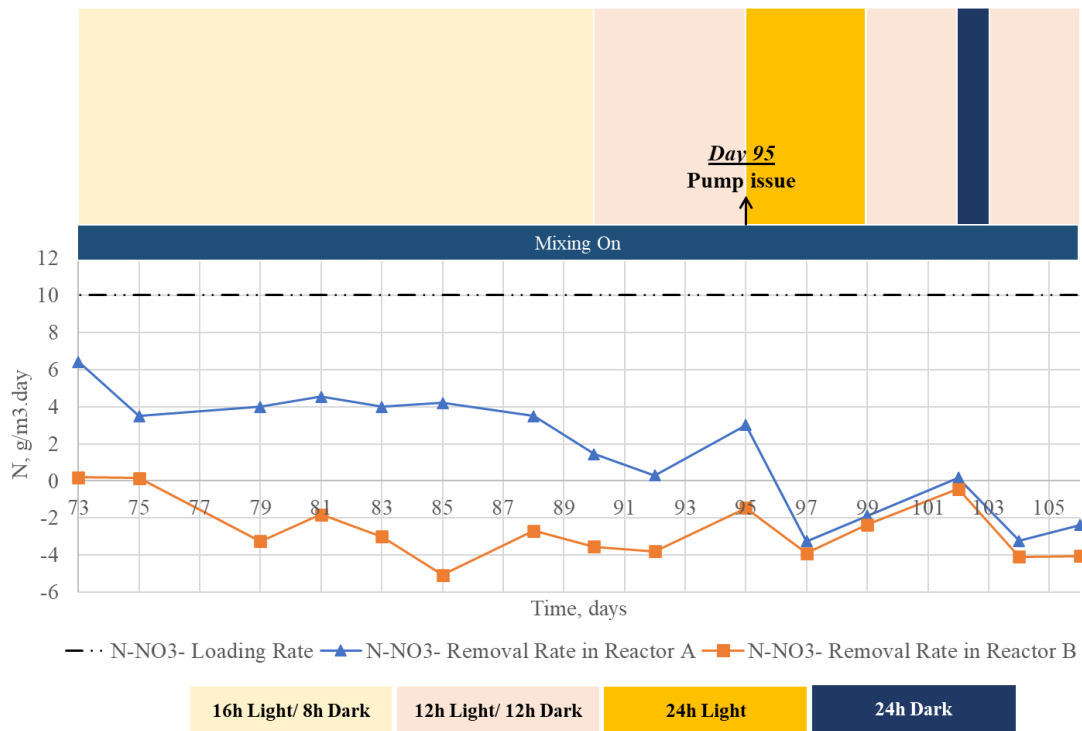


Figure 4.22- N-NO₃- Loading and Removal Rates in Reactor A and B during the 2nd Stage

The nitrate progression in A and B confirms that the microbial population that evolved in A after day 95 had a similar behaviour than the population that already existed in reactor B. Additionally, the negative removal of nitrates identified in reactor A and B suggest that more ammonium is undergoing complete nitrification in B than in A.

4.4.2.7 Total Nitrogen Mass Balance in Reactor A and B

The TN mass balance (Figure 4.23) indicates that TN removal in A is more efficient than B throughout the course of this stage. This indicates that more assimilation is taking place in reactor A than in B. Rada-Ariza et al., 2019 noticed that nitrification/denitrification was the primary pathway for nitrogen removal in algal bacteria consortia, followed by algal cell synthesis and bacterial nitrogen growth requirements.

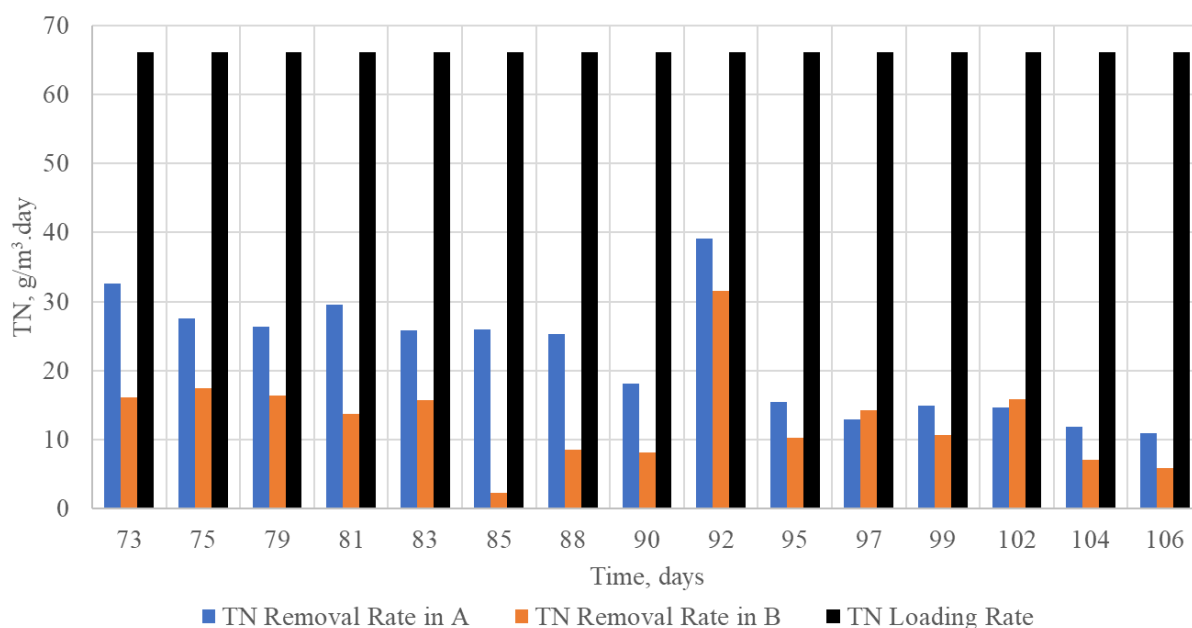


Figure 4.23- TN mass balance in Reactors A and B during the 2nd Stage

The previous analysis showed evidence of bacterial activity in B earlier than in A. The differences in microbial compositions in the reactors and the environmental conditions could have promoted system A's removal of nitrogen through assimilation in the first stage, and once the cultures displayed similar characteristics after day 95, it became evident that a more efficient nitrogen removal could only be achieved by adjusting the system's conditions in order to get rid of the excess nitrates that were being produced by the nitrifying bacteria. However, the attempts made to achieve anoxic conditions were not successful to close the mass balance.

This indicates the removal of nitrogen in the system is being limited not by the amount of microorganisms but rather the type of microorganisms present in the culture, as well as the environmental conditions to which they are being submitted.

4.4.2.8 Total Nitrogen Specific Removal Rate

Figure 4.24 depicts Total Nitrogen Specific Removal Rates in Reactor A and B during the 2nd Stage of the experiment.

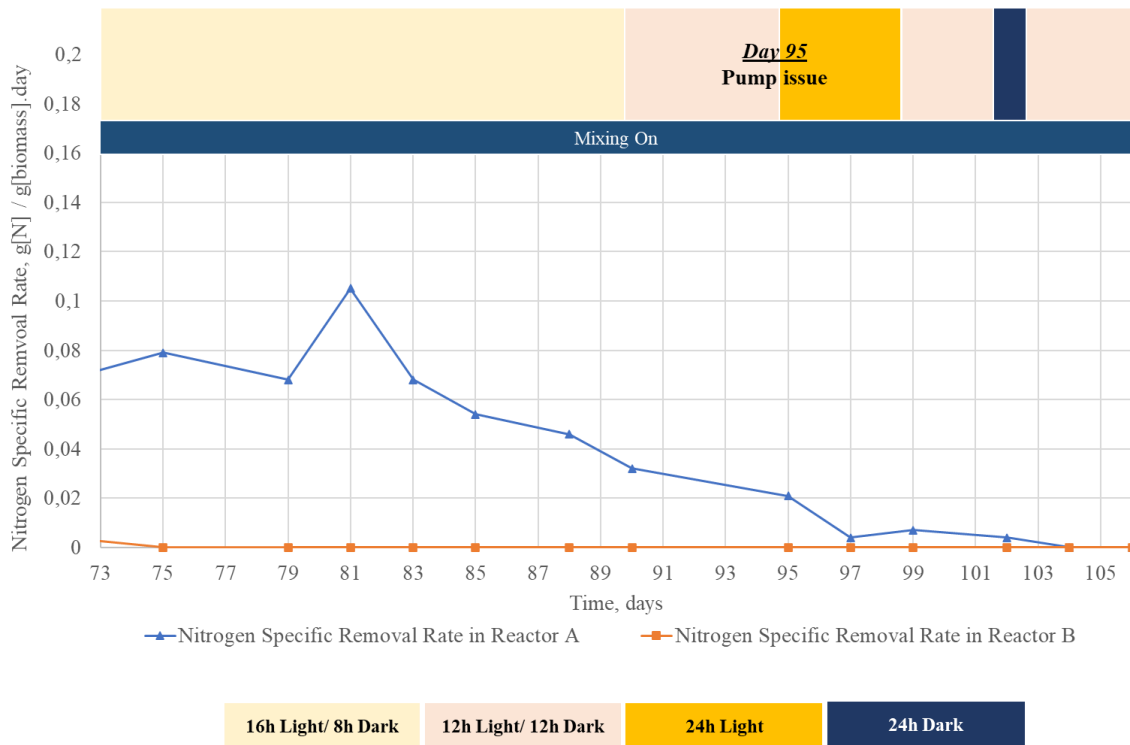


Figure 4.24- Total Nitrogen Specific Removal Rate over the course of Stage 2 for Reactor A and Reactor B

In the second stage, the specific removal rate in A still surpasses B. The decline observed in A is consistent with the increase in AOB activity which confirms that for both systems, assimilation is being limited by the nitrification. It is also possible that the increase in NO_2 in the culture is limiting the assimilation by the microorganisms due the NO_2/HNO_3 that is known to be harmful to microalgae (Blackburne et al., 2007; Yang et al., 2004).

4.5 Chemical Oxygen Demand

4.5.1 Chemical Oxygen Demand Removal during Stage 1

Figure 4.25 depicts the COD Loading and Removal Rates in Reactor A and B during the 1st Stage of the experiment.

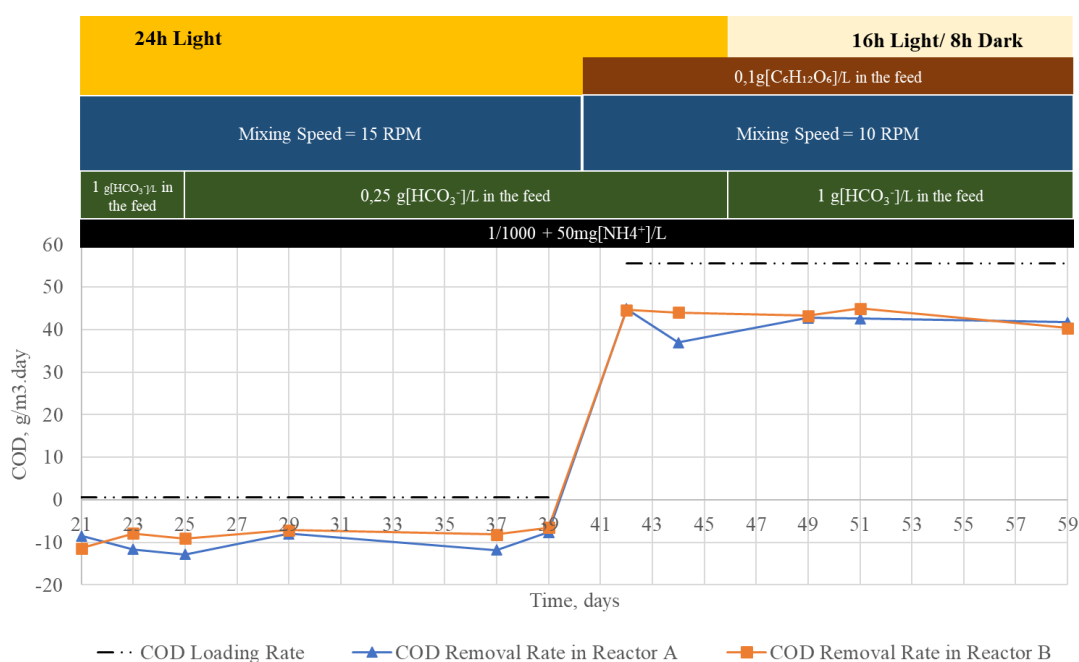


Figure 4.25– COD Loading and Removal Rates in Reactors A and B during the 1st Stage

During the initial 39 days of the experiment, negative removal rates were observed for COD, indicating that the concentration of organic matter in the effluents of both systems was significantly higher (12-18 times) than the influent concentration. This suggests that COD was being generated rather than consumed.

It was initially hypothesized that the discrepancy in COD values between the influent and effluent samples could be attributed to the presence of microbes in the tested filtrate. To investigate this further and prevent bacteria from getting to the filtrate, the samples underwent sterile filtration (Hahn, 2004) where a syringe filter with a pore size of 0,2 μm was used as an alternative filtration method on day 29. Surprisingly, even with the use of the 0,2 μm syringe filter, the COD value on this day remained similar to that of the previous days.

This observation indicates that the compounds responsible for the COD values in the filtrate were unlikely to be bacteria. Additionally, the size of these compounds was smaller than 0,2 μm , suggesting that these could be molecules resulting from the metabolic processes of the living organisms present in the culture, in other words, soluble COD was being produced by the microorganisms.

On day 42, the addition of glucose triggered the growth of bacteria, that in turn consumed the organic matter present in the medium, leading to higher rates of COD removal.

A study conducted by Lee et al., 2019, demonstrated that introducing sludge to an algal bacteria consortium at the “right time” can enhance the removal of COD. The study was based in the premise that the growth profile of microalgae is marked by two moments - 1) the COD consuming stage which occurs during the initial growth phase, and the 2) the COD release stage which occurs during the stationary growth phase. From this, it is possible to assume that the culture in the present study could have been undergoing the stationary growth phase and that the natural introduction of bacteria as able to counteract the COD release that was taking place.

4.5.2 Chemical Oxygen Demand Removal during Stage 2

Figure 4.26 depicts the COD Loading and Removal Rates in Reactor A and B during the 2nd Stage of the experiment.

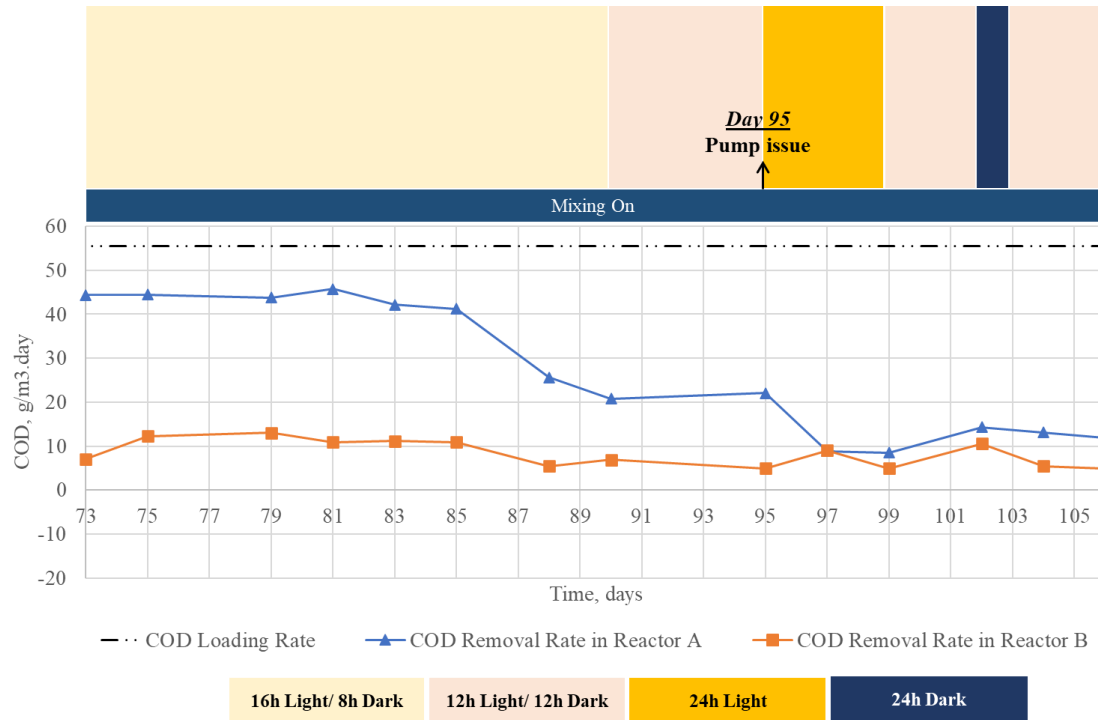


Figure 4.26- COD Loading and Removal Rates in Reactors A and B during the 2nd Stage

The progression of the graph indicates that up until day 97, A outperformed B in the removal of COD. It is also possible to see that the evolution in A is gradually declining over time.

As the previous analysis of nitrate removal suggests, the activity of nitrifying bacteria gradually increased during this stage in A, whereas in B, the bacterial activity remained relatively constant. In addition, the similar trend observed in A and B from day 97 onwards, was also observed in the nitrate removal curves. From this correlation, it is possible to assume that nitrification could be decreasing the COD removal. Similarly (Vossoghi & Daylami, 2003) found that COD removal decreases under high nitrification rates, and that enhanced COD removal is achieved during the denitrification stage when treating high loads of WW in a MBBR. (Dolinšek et al., 2013) found that chemolithoautotrophic nitrifying bacteria secrete soluble organic compounds that can be used by heterotrophic bacteria as a substrate. This could be the root cause as to why the removal of COD is being affected by the increasing population of nitrifiers in the system – the rate of production of these soluble organic compounds is higher than the rate of consumption, ultimately decreasing the COD removal capacity by the culture.

4.6 Total Phosphorus

4.6.1 Total Phosphorus Removal during Stage 1

Figure 4.27 depicts the Total Phosphorus Loading and Removal Rates in Reactor A and B during the 1st Stage of the experiment.

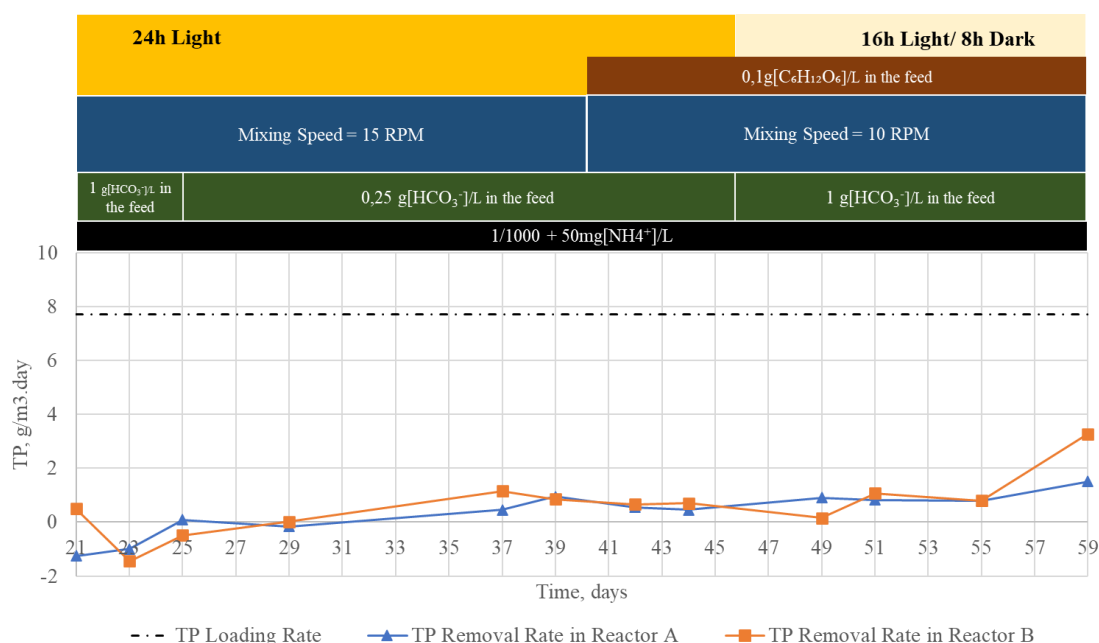


Figure 4.27- TP Loading and Removal Rates in Reactors A and B during the 1st Stage

During the first 8 days, the reactor registered a negative removal of phosphorus in both systems, meaning the phosphorus output was higher than the input.

It is suspected that there is bacterial activity in the feed tank. Furthermore, it is also known that under anoxic conditions, PAOS release phosphorus previously absorbed back into the medium (Saia et al., 2017). If there is indeed PAOS in the feed tank, and the environment is conducive of anaerobic bacterial activity, then it could be a possibility that every time the feed tank is emptied and refilled, the remaining bacteria that was not removed from the tank is releasing phosphorus back into the solution. In addition, this would be noticeable only if the phosphorus content is not being efficiently removed by the culture in the reactors. If that is the case, then negative removal rates would be observed in the systems. In order to prove this, a more detailed investigation needs to be conducted to understand the reactions that are taking place in the feed tank.

By taking a general look at the progression of the phosphorus removal curves in the reactors throughout the experiment, it is possible to see an overall increasing trend. In addition, both reactors display similar performances and do not appear to be significantly affected by the environmental adjustments made during this time span.

4.6.1.1 Total Phosphorus Mass Balance in Reactor A and B

Figure 4.28 depicts the Total Phosphorus mass balance during the 1st Stage of the experiment.

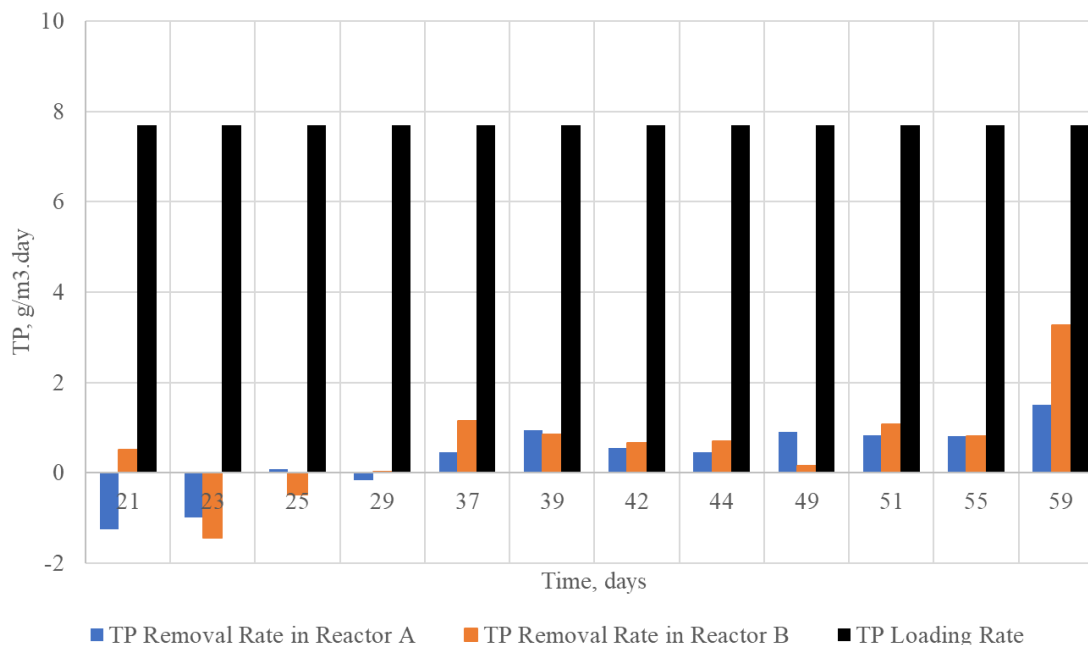


Figure 4.28- TP mass balance in Reactors A and B during the 1st Stage

The biological removal of phosphorus can be achieved directly through biomass assimilation or indirectly through the precipitation of phosphate salts in the presence of metals. Since the trace elements present in the fertilizer come in residual concentrations, it is expected that if any indirect removal takes place, it will not be significant. With this, assimilation would be the only possible pathway of TP removal in this study.

The biomass analysis recorded higher accumulation of phosphorus in B than in A, however this difference is not being translated into the rates of phosphorus removal during this time span as most of the TP is being retained in the system.

Similarly to what took place in the TN mass balance, it is possible that the culture is being supplied more phosphorus than it can uptake and that the biomass present in the systems are not these nutrients for their growth.

4.6.2 Total Phosphorus Removal during Stage 2

Figure 4.29 depicts the Total Phosphorus Loading and Removal Rates in Reactor A and B during the 2nd Stage of the experiment.

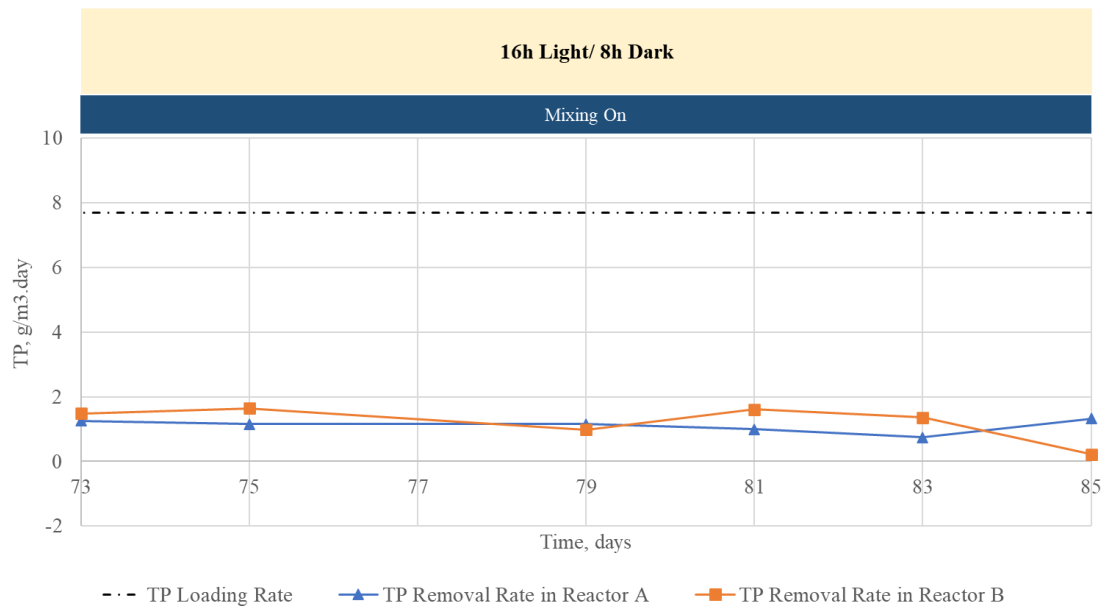


Figure 4.29- TP Loading and Removal Rates in Reactors A and B during the 2nd Stage

During the second stage of the experiment, the progression of TP removal displays minor fluctuations that could be a result of internal perturbations not related to the controlled parameters, as during this time frame, no adjustments were made to the surrounding environment.

4.6.2.1 Total Phosphorus Mass Balance in Reactor A and B

Figure 4.30 depicts the Total Phosphorus mass balance during the 2nd Stage of the experiment.

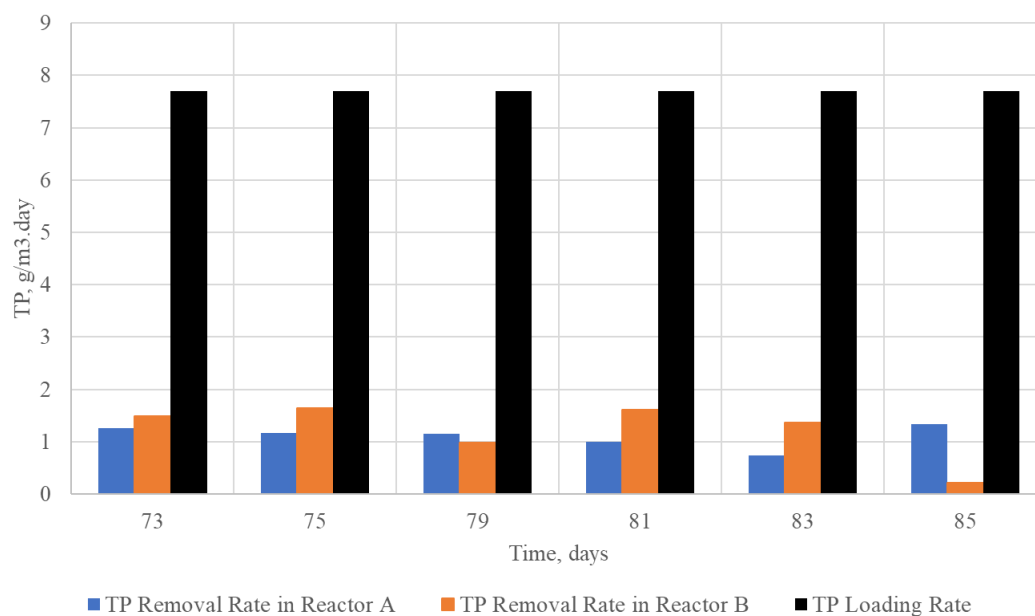


Figure 4.30- TP mass balance in Reactors A and B during the 2nd Stage

Similarly to what was observed in the previous stage, most of the phosphorus was retained in the system without being consumed, however it is possible to observe slightly higher rates of removal in B compared to A. On day 85, the accentuated drop on TP removal on B was also observed on TN removal. On this day, a drop in biomass was also observed in reactor B. This indicates that the removal of phosphorus is indeed related to assimilation, however it is unclear why the correlation between these two variables is not as prominent.

5 CONCLUSIONS

In this comparative analysis of a suspended and biofilm system, valuable insights were gained into the intricate dynamics governing nutrient removal, biomass growth, and environmental conditions.

The pH profiles allowed for an association to be drawn between the metabolic activities being performed by the cultures. System B was able to foster up to 23 times more biomass than reactor A due to biofilm growth. Moreover, the study revealed a nuanced interplay of factors influencing nutrient dynamics. Nitrogen species analysis unveiled a complex balance between assimilation, nitrification, and the presence of nitrifiers in the system. The TN removal in both systems was ultimately affected by the competition for ammonium by the AOB and the high DO levels that prevented the system from reaching anoxic conditions required for denitrification to be performed. Carbon limitation was identified as a key factor affecting nitrogen assimilation, corroborating earlier research findings.

The analysis of COD highlighted the significant role played by microbial communities in both the production and elimination of organic matter. It also accentuated the distinct phases involving COD utilization and subsequent release within microalgal cultures. Finally, the analysis of TP revealed the system's capacity to progressively eliminate phosphorus. However, it also unveiled instances of negative removal rates, indicating the possibility of phosphorus release under certain conditions. The mass balance analysis indicated a phenomenon of phosphorus retention, pointing towards a complex interplay of factors governing phosphorus assimilation.

The removal trends of TN, COD and TP were similar for A and B despite the higher quantity of biomass accumulated in system B. Furthermore, optimization of the higher biomass retained for intensification of nutrient removal is believed to be achieved through the manipulation of environmental conditions, opening the doors for more research to be conducted in the future in this field.

Overall, this study emphasizes the dynamic nature of microalgal-bacterial WWT systems and underscores the need for further research to optimize operational conditions and enhance system efficiency for practical WWT applications.

6 LIMITATIONS AND FUTURE RE- SEARCH

The most challenging aspect of this experience was finding suitable conditions that would lead to the selection of specific microorganisms for enhanced nutrient removal, especially TN. To achieve efficient TN bioremediation, both anoxic and aerobic conditions need to be met. In this study, attempts were made to reduce oxygen levels in the culture by adjusting the photoperiod, however, these adjustments were not sufficient to induce a low-oxygen environment. This occurrence is believed to be due to an imbalance between oxygen production and its consumption within the culture. Therefore, the suggestion for future research, outlined below, is based on extrapolating the conclusions drawn from this study and considering parameter adjustments and changes that would enhance TN removal in the system. The two proposed areas of future study are described as follows:

Proposal 1 - Optimizing Nitrogen Removal in WWT in Algal-bacterial MBBR through Carbon Supplementation and Photoperiod Adjustment

It is known that during the daytime, the culture assimilates nitrogen and performs nitrification. The preferred species to be assimilated is ammonia. However, ammonia is also used in nitrification. So, for efficient TN removal to be achieved during the day, assimilation rates must surpass nitrification rates. On the other hand, during the night, it is necessary for almost all oxygen to be consumed for anoxic conditions to be established.

One suggestion is to supplement the medium with an additional source of organic carbon to increase the respiration rate by the culture. Thus, there would be a need for two feeding tanks, one to feed the culture during the day, and another with a carbon rich solution to feed the culture during the night. Since the feeding tank allows for the growth of denitrifiers, this would facilitate the proliferation of these microorganisms in the culture during the nighttime.

Figure 6.1 illustrates a conceptual set-up of this experimental proposal.

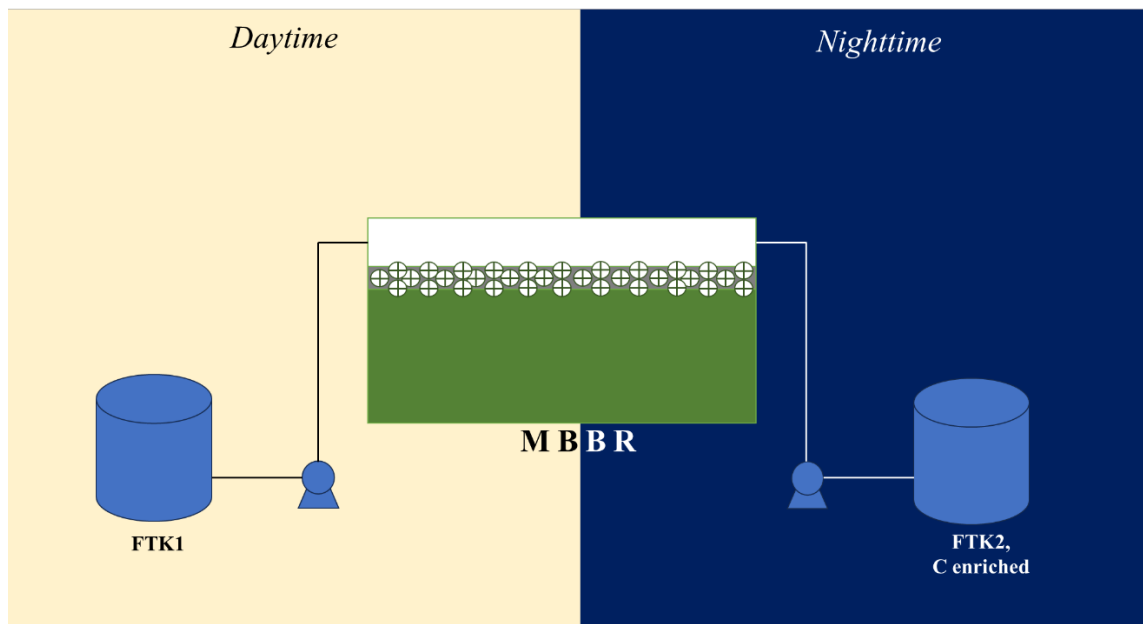


Figure 6.1 Proposal 1: Optimizing Nitrogen Removal in WWT in Algal-bacterial MBBR through Carbon Supplementation and Photoperiod Adjustment; FTK1- Feed Tank

To study the culture profile in detail, initially, both daytime and nighttime periods would need to be maximized (e.g., 24 hours day – 24 hours night). Important parameters to be tested would include nutrient concentration such as TN, N-NH_4^+ , N-NO_3^- , N-NO_2^- as well as COD during both daytime and nighttime, as well as the evolution of dissolved oxygen and pH levels. If a reduction in TN levels is indeed observed using this method, photoperiods can be reduced/adjusted to decrease electricity costs.

Proposal 2 - Enhancing Total Nitrogen Removal in WWT Using Multi-Functional Algal -Bacterial Biofilms and K1 Carrier-Based Inoculation

An experiment based on the formation of multi-functional biofilms capable of hosting both aerobic and anaerobic layers could intensify TN removal. An important step in this process would be cultivating carriers in a medium with the same composition as the feed solution, leveraging the biofilm formation capacity of the microorganisms to promote their growth on K1 carriers. After the formation of biofilms on these carriers is confirmed, they would be used to inoculate the mixed-culture moving bed biofilm reactor. This inoculation would occur during the nighttime, and as the daytime period re-establishes and oxygen levels gradually increase, the formation of an aerobic layer capable of not only protecting denitrifying microorganisms but also promoting a nitrification/denitrification chain similar to what was observed by Zhang et al., 2022 is expected to be observed.

Figure 6.2 illustrates a conceptual set-up of this experimental proposal.

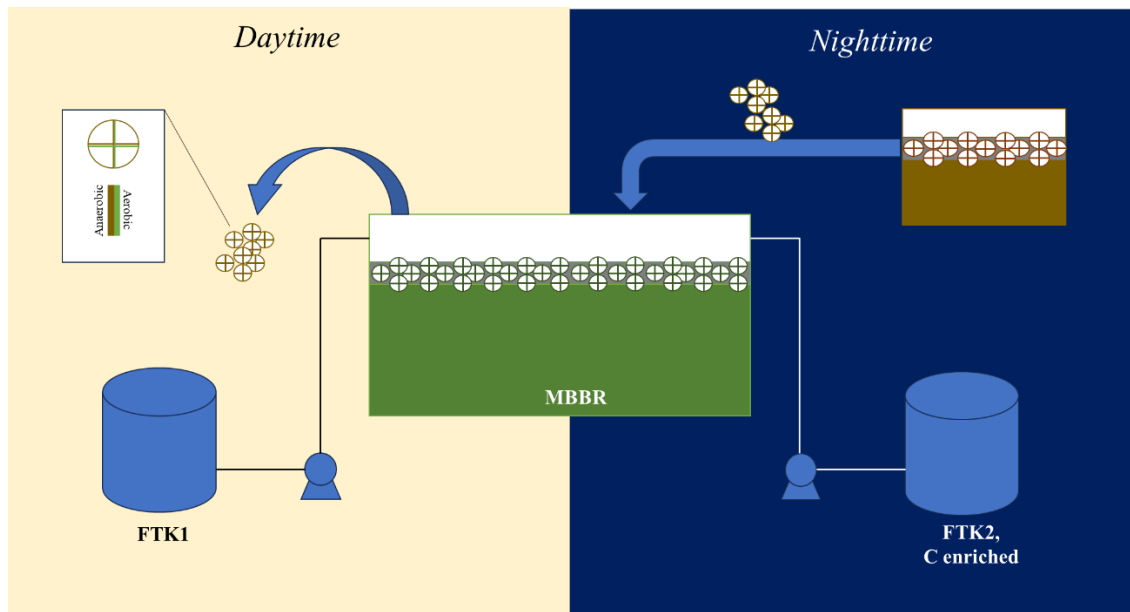


Figure 6.2- Proposal 2: Enhancing Total Nitrogen Removal in WWT Using Multi-Functional Algal -Bacterial Biofilms and K1 Carrier-Based Inoculation; FTK- Feed Tank

Similar to the first proposal, in addition to nutrient analysis and monitoring of DO and pH levels, the system would also benefit from metagenomic analysis of the biofilms present on the carriers, as done by Zhang et al. in 2022. This would allow for a comparison of carrier groups in the system to be done at a microbial composition level ultimately helping understand the feasibility of a multi-layered biofilm in the culture in question and its ability to enhance TN removal.

Both proposals in this study also foresee an improvement in COD removal since organic matter is one of the drivers of denitrification (Chen et al., 2023).

TP removal was also a challenge in nutrient removal performance by the system. It's suspected that the amount of phosphorus in feed solution into the system was excessive for the culture. Prolonged monitoring, with parameter adjustments such as HRT, and supplementation of phosphorus at different concentrations, will allow for a better understanding of phosphorus removal by the culture.

Another limitation found in the experiment was the tracking of biofilm life cycles. More frequent and detailed monitoring of biofilm development phases can be useful for maximizing biomass recovery. This can be achieved through the adoption of indirect quantification methods in the sampling protocol. These methods assume that the substance to be quantified is related to the number of cells and is consistent from cell to cell (Trulear & Characklis, 1982). An example of indirect methods for biofilm quantification would be the quantification of Total Organic Carbon (TOC), DNA, RNA, polysaccharides, or metabolites (Wilson et al., 2017).

REFERENCES

- Abiusi, F., Trompetter, E., Pollio, A., Wijffels, R. H., & Janssen, M. (2022). Acid Tolerant and Acidophilic Microalgae: An Underexplored World of Biotechnological Opportunities. *Frontiers in Microbiology*, 13.
<https://www.frontiersin.org/articles/10.3389/fmicb.2022.820907>
- Abiusi, F., Wijffels, R. H., & Janssen, M. (2020). Oxygen Balanced Mixotrophy under Day–Night C. *ACS Sustainable Chemistry & Engineering*, 8(31), 11682–11691.
<https://doi.org/10.1021/acssuschemeng.0c03216>
- Agarwal, A., & Jain, A. (2013). Glucose & sodium chloride induced biofilm production & ica operon in clinical isolates of staphylococci. *Indian J Med Res*, 138(2), 262-266.
- Aqualia. (2016). All-gas Newsletter. In https://www.all-gas.eu/documents/15132649/15132891/New%20sletter_2016.12.pdf/8f2590da-0474-a9c2-93b4-c04bfc2b561d?T=1512465173000
(Accessed on June 13th 2023)
- Arrigo, K. R. (2005). Marine microorganisms and global nutrient cycles. *Nature*, 437(7057), 349-355.
<https://doi.org/10.1038/nature04159>
- Ashok, V., Gupta, S., & Shriwastav, A. (2019). Photobioreactors for WWT. In (pp. 383-409).
https://doi.org/10.1007/978-3-030-13913-1_18
- Babu, M. (2011). Effect of Algal Biofilm and Operational Conditions on Nitrogen Removal in Waste Stabilization Ponds: UNESCO-IHE phd Thesis. CRC Press.
- Bankston, E., Wang, Q., & Higgins, B. T. (2020). Algae support populations of heterotrophic, nitrifying, and phosphate-accumulating bacteria in the treatment of poultry litter anaerobic digestate. *Chemical Engineering Journal*, 398, 125550.
<https://doi.org/10.1016/j.cej.2020.125550>
- Berthod, L., Roberts, G., Sharpe, A., Whitley, D. C., Greenwood, R., & Mills, G. A. (2016). Effect of sewage sludge type on the partitioning behaviour of pharmaceuticals: a meta-analysis. *Environmental Science: Water Research & Technology*, 2(1), 154-163.
<https://doi.org/10.1039/c5ew00171d>
- Blackburne, R., Vadivelu, V. M., Yuan, Z., & Keller, J. (2007). Kinetic characterisation of an enriched Nitrospira culture with comparison to Nitrobacter. *Water Research*, 41(14), 3033-3042.
<https://doi.org/10.1016/j.watres.2007.01.043>
- Boelee, N., Temmink, H., Janssen, M., Buisman, C., & Wijffels, R. (2011). Nitrogen and phosphorus removal from municipal wastewater effluent using microalgal biofilms. *Water Research*, 45(18), 5925–5933.
<https://doi.org/10.1016/j.watres.2011.08.044>

- Chen, G., Lin, L., Wang, Y., Zhang, Z., Cao, W., & Zhang, Y. (2023). Unveiling the interaction mechanisms of key functional microorganisms in the partial denitrification-anammox process induced by COD. *Frontiers of Environmental Science & Engineering*, 17(8), 103.
<https://doi.org/10.1007/s11783-023-1703-3>
- Chen, R., Li, R., Deitz, L., Liu, Y., Stevenson, R. J., & Liao, W. (2012). Freshwater algal cultivation with animal waste for nutrient removal and biomass production. *Biomass and Bioenergy*, 39, 128–138.
<https://doi.org/10.1016/j.biombioe.2011.12.045>
- Chisti, Y. (2007). Biodiesel from microalgae. *Biotechnology Advances*, 25(3), 294-306.
<https://doi.org/https://doi.org/10.1016/j.biotechadv.2007.02.001>
- Chisti, Y. (2013). Constraints to commercialization of algal fuels. *Journal of Biotechnology*, 167(3), 201-214.
<https://doi.org/https://doi.org/10.1016/j.jbiotec.2013.07.020>
- Choi, O., Das, A., Yu, C.-P., & Hu, Z. (2010). Nitrifying bacterial growth inhibition in the presence of algae and cyanobacteria. *Biotechnology and Bioengineering*, 107(6), 1004-1011.
<https://doi.org/https://doi.org/10.1002/bit.22860>
- Chojnacka, K., & Marquez-Rocha, F. (2004). Kinetic and Stoichiometric Relationships of the Energy and Carbon Metabolism in the Culture of Microalgae. *Biotechnology(Faisalabad)*, 3, 21-34.
<https://doi.org/10.3923/biotech.2004.21.34>
- Chojnacka, K., & Zielińska, A. (2012). Evaluation of growth yield of *Spirulina* (*Arthrospira*) sp. In photoautotrophic, heterotrophic and mixotrophic cultures. *World Journal of Microbiology and Biotechnology*, 28(2), 437-445.
<https://doi.org/10.1007/s11274-011-0833-0>
- Claros, J., Jiménez, E., Aguado, D., Ferrer, J., Seco, A., & Serralta, J. (2013). Effect of pH and HNO₂ concentration on the activity of ammonia-oxidizing bacteria in a partial nitrification reactor. *Water Science and Technology*, 67(11), 2587-2594.
<https://doi.org/10.2166/wst.2013.132>
- Commision, C. E. (2011). Industrial scale demonstration of sustainable algae cultures for biofuel production
<https://cordis.europa.eu/project/id/268208>
(Consulted on 13/June/2023)
- Craggs, R., Park, J., Sutherland, D., & Heubeck, S. (2015). Economic construction and operation of hectare-scale WWT enhanced pond systems. *Journal of Applied Phycology*, 27(5), 1913-1922.
<https://doi.org/10.1007/s10811-015-0658-6>
- Davies-Colley, R. J., Craggs, R. J., & Nagels, J. W. (2003). Disinfection in a pilot-scale “advanced” pond system (APS) for domestic sewage treatment in New Zealand. *Water Science and Technology*, 48(2), 81-87.
<https://doi.org/10.2166/wst.2003.0091>
- Davis, R., Aden, A., & Pienkos, P. T. (2011). Techno-economic analysis of autotrophic microalgae for fuel production. *Applied Energy*, 88(10), 3524–3531.
<https://doi.org/10.1016/j.apenergy.2011.04.018>

- De Godos, I., Blanco, S., García-Encina, P. A., Becares, E., & Muñoz, R. (2010). Influence of flue gas sparging on the performance of high rate algae ponds treating agro-industrial wastewaters. *Journal of Hazardous Materials*, 179(1), 1049-1054.
<https://doi.org/https://doi.org/10.1016/j.jhazmat.2010.03.112>
- Dhaouefi, Z., Toledo-Cervantes, A., García, D., Bedoui, A., Ghedira, K., Chekir-Ghedira, L., & Muñoz, R. (2018). Assessing textile WWT in an anoxic-aerobic photobioreactor and the potential of the treated water for irrigation. *Algal Research*, 29, 170-178.
<https://doi.org/10.1007/s13762-018-2130-z>
- Dolinšek, J., Lagkouravdos, I., Wanek, W., Wagner, M., & Daims, H. (2013). Interactions of nitrifying bacteria and heterotrophs: identification of a Micavibrio-like putative predator of Nitrospira spp. *Appl Environ Microbiol*, 79(6), 2027-2037.
<https://doi.org/10.1128/aem.03408-12>
- Ennaceri, H., Ishika, T., Mkpuma, V. O., & Moheimani, N. R. (2023). Microalgal biofilms: Towards a sustainable biomass production. *Algal Research*, 72, 103124.
<https://doi.org/10.1016/j.algal.2023.103124>
- Eze, V. C., Velasquez-Orta, S. B., Hernández-García, A., Monje-Ramírez, I., & Orta-Ledesma, M. T. (2018). Kinetic modelling of microalgae cultivation for WWT and carbon dioxide sequestration. *Algal Research*, 32, 131-141.
<https://doi.org/10.1016/j.algal.2018.03.015>
- Fallahi, A., Rezvani, F., Asgharnejad, H., Khorshidi Nazloo, E., Hajinajaf, N., & Higgins, B. (2021). Interactions of microalgae-bacteria consortia for nutrient removal from wastewater: A review. *Chemosphere*, 272, 129878.
<https://doi.org/10.1016/j.chemosphere.2021.129878>
- Fleischer, W. E. (1935). The relation between chlorophyll content and rate of photosynthesis. *The Journal of General Physiology*, 18(4), 573-597.
<https://doi.org/10.1085/jgp.18.4.573>
- French, E., Kozłowski, J. A., Mukherjee, M., Bullerjahn, G., & Bollmann, A. (2012). Ecophysiological characterization of ammonia-oxidizing archaea and bacteria from freshwater. *Appl Environ Microbiol*, 78(16), 5773-5780.
<https://doi.org/10.1128/aem.00432-12>
- Galès, A., Bonnafous, A., Carré, C., Jauzein, V., Lanouguère, E., Le Floch, E., Pinoit, J., Poullain, C., Roques, C., Sialve, B., Simier, M., Steyer, J.-P., & Fouilland, E. (2019). Importance of ecological interactions during WWT using High Rate Algal Ponds under different temperate climates. *Algal Research*, 40, 101508.
<https://doi.org/https://doi.org/10.1016/j.algal.2019.101508>
- Gillett, A., Waugh, D. G., & Lawrence, J. (2016). 7 - Laser surface modification of polymeric surfaces for microbiological applications. In R. Vilar (Ed.), *Laser Surface Modification of Biomaterials* (pp. 197-220). Woodhead Publishing.
<https://doi.org/https://doi.org/10.1016/B978-0-08-100883-6.00007-1>

- Gim, G. H., Kim, J. K., Kim, H. S., Kathiravan, M. N., Yang, H., Jeong, S.-H., & Kim, S. W. (2014). Comparison of biomass production and total lipid content of freshwater green microalgae cultivated under various culture conditions. *Bioprocess and Biosystems Engineering*, 37(2), 99-106.
<https://doi.org/10.1007/s00449-013-0920-8>
- Godos, I. D., Blanco, S., García-Encina, P. A., Becares, E., & Muñoz, R. (2009). Long-term operation of high rate algal ponds for the bioremediation of piggery wastewaters at high loading rates. *Biore-source Technology*, 100(19), 4332-4339
<https://doi.org/https://doi.org/10.1016/j.biortech.2009.04.016>
- Gonçalves, A. L., Simões, M., & Pires, J. C. M. (2014). The effect of light supply on microalgal growth, CO₂ uptake and nutrient removal from wastewater. *Energy Conversion and Management*, 85, 530–536.
<https://doi.org/10.1016/j.enconman.2014.05.085>
- González-Camejo, J., Aparicio, S., Pachés, M., Borrás, L., & Seco, A. (2022). Comprehensive assessment of the microalgae-nitrifying bacteria competition in microalgae-based WWT systems: Relevant factors, evaluation methods and control strategies. *Algal Research*, 61, 102563.
<https://doi.org/https://doi.org/10.1016/j.algal.2021.102563>
- González-Camejo, J., Aparicio, S., Ruano, M. V., Borrás, L., Barat, R., & Ferrer, J. (2019). Effect of ambient temperature variations on an indigenous microalgae-nitrifying bacteria culture dominated by *Chlorella*. *Bioresource Technology*, 290, 121788.
<https://doi.org/https://doi.org/10.1016/j.biortech.2019.121788>
- Gonzalez-Camejo, J., Montero, P., Aparicio, S., Ruano, M. V., Borrás, L., Seco, A., & Barat, R. (2020). Nitrite inhibition of microalgae induced by the competition between microalgae and nitrifying bacteria. *Water Research*, 172, 115499.
<https://doi.org/10.1016/j.watres.2020.115499>
- Grima, E. M., Belarbi, E.-H., Fernández, F. A., Medina, A. R., & Chisti, Y. (2003). Recovery of microalgal biomass and metabolites: Process options and economics. *Biotechnology Advances*, 20(7–8), 491–515.
[https://doi.org/10.1016/S0734-9750\(02\)00050-2](https://doi.org/10.1016/S0734-9750(02)00050-2)
- Gross, M., Mascarenhas, V., & Wen, Z. (2015). Evaluating algal growth performance and water use efficiency of pilot-scale revolving algal biofilm (RAB) culture systems. *Biotechnology and Bioengineering*, 112(10), 2040-2050.
<https://doi.org/https://doi.org/10.1002/bit.25618>
- Guerrero, M. A., & Jones, R. D. (1996). Photoinhibition of marine nitrifying bacteria. II. Dark recovery after monochromatic or polychromatic irradiation. *Marine Ecology Progress Series*, 141(1/3), 193-198.
https://elischolar.library.yale.edu/journal_of_marine_research/1541
- Hahn, M. W. (2004). Broad diversity of viable bacteria in ‘sterile’ (0.2 µm) filtered water. *Research in Microbiology*, 155(8), 688-691.
<https://doi.org/https://doi.org/10.1016/j.resmic.2004.05.003>
- Haider, M. N., Iqbal, M. M., Nishimura, M., Ikemoto, E., Ijichi, M., & Kogure, K. (2023). Bacterial response to glucose addition: growth and community structure in seawater microcosms from North Pacific

- Ocean. Scientific Reports, 13(1).
<https://doi.org/10.1038/s41598-022-27251-2>
- Hasport, N., Krahe, D., Kuchendorf, C. M., Beier, S., & Theilen, U. (2022). The potential impact of an implementation of microalgae-based WWT on the energy balance of a municipal WWT plant in Central Europe. *Bioresource Technology*, 347, 126695.
<https://doi.org/https://doi.org/10.1016/j.biortech.2022.126695>
- Heyman, B., Tulke, H., Putri, S. P., Fukusaki, E., & Büchs, J. (2020). Online monitoring of the respiratory quotient reveals metabolic phases during microaerobic 2,3-butanediol production with *Bacillus licheniformis*. *Eng Life Sci*, 20(3-4), 133-144.
<https://doi.org/10.1002/elsc.201900121>
- Hoang, M. D., Riessner, S., Oropeza Vargas, J. E., von den Eichen, N., & Heins, A.-L. (2023). Influence of Varying Pre-Culture Conditions on the Level of Population Heterogeneity in Batch Cultures with an *Escherichia coli* Triple Reporter Strain. *Microorganisms*, 11(7).
<https://doi.org/10.3390/microorganisms11071763>
- Hultberg, M., Asp, H., Marttila, S., Bergstrand, K. J., & Gustafsson, S. (2014). Biofilm formation by *Chlorella vulgaris* is affected by light quality. *Curr Microbiol*, 69(5), 699-702.
<https://doi.org/10.1007/s00284-014-0645-1>
- International, B. B. (2017). All-Gas enters next phase of biogas from algae project. Retrieved 19 September from <https://bdi-bioenergy.com/en/newsroom/detail/all-gas-enters-next-phase-of-biogas-from-algae-project>
 (Accessed on June 13th 2023)
- Jeon, C. O. K., Lee, D. S., Lee, M. W., & Park, J. M. (2001). Enhanced Biological Phosphorus Removal in an Anaerobic–Aerobic Sequencing Batch Reactor: Effect of pH. *Water Environment Research*, 73(3), 301-306.
<https://doi.org/https://doi.org/10.2175/106143001X139407>
- Ji, B., Zhang, M., Wang, L., Wang, S., & Liu, Y. (2020). Removal mechanisms of phosphorus in non-aerated microalgal-bacterial granular sludge process. *Bioresource Technology*, 312, 123531.
<https://doi.org/https://doi.org/10.1016/j.biortech.2020.123531>
- Kesari, K. K., Soni, R., Jamal, Q. M. S., Tripathi, P., Lal, J. A., Jha, N. K., Siddiqui, M. H., Kumar, P., Tripathi, V., & Ruokolainen, J. (2021). WWT and Reuse: a Review of its Applications and Health Implications. *Water, Air, & Soil Pollution*, 232(5).
<https://doi.org/10.1007/s11270-021-05154-8>
- Krausfeldt, L. E., Farmer, A. T., Castro Gonzalez, H. F., Zepernick, B. N., Campagna, S. R., & Wilhelm, S. W. (2019). Urea Is Both a Carbon and Nitrogen Source for *Microcystis aeruginosa*: Tracking ¹³C Incorporation at Bloom pH Conditions. *Frontiers in Microbiology*, 10, 1064.
<https://doi.org/10.3389/fmicb.2019.01064>
- Kuhl, M., Glud, R. N., Ploug, H., & Ramsing, N. B. (1996). Microenvironmental control of photosynthesis and photosynthesis-coupled respiration in an epilithic cyanobacterial biofilm¹. *Journal of Phycology*, 32(5), 799-812.
<https://doi.org/10.1111/j.0022-3646.1996.00799.x>

- Kwon, G., Kim, H., Song, C., & Jahng, D. (2019). Co-culture of microalgae and enriched nitrifying bacteria for energy-efficient nitrification. *Biochemical Engineering Journal*, 152, 107385.
<https://doi.org/10.1016/j.bej.2019.107385>
- Lee, K., & Lee, C. (2002). Nitrogen Removal from Wastewaters by Microalgae Without Consuming Organic Carbon Sources. *Journal of Microbiology and Biotechnology*, 12.
- Lee, S. A., Lee, N., Oh, H. M., & Ahn, C. Y. (2019). Enhanced and balanced microalgal WWT (COD, N, and P) by interval inoculation of activated sludge [Article]. *Journal of Microbiology and Biotechnology*, 29(9), 1434-1443.
<https://doi.org/10.4014/jmb.1905.05034>
- Li, J., Li, C., Lan, C. Q., & Liao, D. (2018). Effects of sodium bicarbonate on cell growth, lipid accumulation, and morphology of *Chlorella vulgaris*. *Microbial Cell Factories*, 17(1), 111.
<https://doi.org/10.1186/s12934-018-0953-4>
- Li, M., Wang, Y., Zhang, J., Liu, B., Xue, H., Wu, L., & Li, Z. (2023). Knowledge Mapping of High-Rate Algal Ponds Research. *Water*, 15(10).
<https://doi.org/10.1016/j.ecolind.2023.11096>
- Li, X., Li, W., Zhai, J., Wei, H., & Wang, Q. (2019). Effect of ammonium nitrogen on microalgal growth, biochemical composition and photosynthetic performance in mixotrophic cultivation. *Bioresource Technology*, 273, 368-376.
<https://doi.org/https://doi.org/10.1016/j.biortech.2018.11.042>
- Li, Y., Chen, Y.-F., Chen, P., Min, M., Zhou, W., Martinez, B., Zhu, J., & Ruan, R. (2011). Characterization of a microalga *Chlorella* sp. Well adapted to highly concentrated municipal wastewater for nutrient removal and biodiesel production. *Bioresource Technology*, 102(8), 5138–5144.
<https://doi.org/10.1016/j.biortech.2011.01.091>
- Lu, S., Liu, X., Liu, C., Cheng, G., & Shen, H. (2020). Influence of photoinhibition on nitrification by ammonia-oxidizing microorganisms in aquatic ecosystems. *Reviews in Environmental Science and Bio/Technology*, 19(3), 531-542.
<https://doi.org/10.1007/s11157-020-09540-2>
- Luo, Y., Le-Clech, P., & Henderson, R. K. (2020). Characterisation of microalgae-based monocultures and mixed cultures for biomass production and WWT [Article]. *Algal Research*, 49, Article 101963.
<https://doi.org/10.1016/j.algal.2020.101963>
- Ma, X., Mi, Y., Zhao, C., & Wei, Q. (2022). A comprehensive review on carbon source effect of microalgae lipid accumulation for biofuel production. *Science of The Total Environment*, 806, 151387.
<https://doi.org/10.1016/j.scitotenv.2021.151387>
- Madan, S., Madan, R., & Hussain, A. (2022). Advancement in biological WWT using hybrid moving bed biofilm reactor (MBBR): a review. *Applied Water Science*, 12(6), 141.
<https://doi.org/10.1007/s13201-022-01662-y>
- Markou, G., Vandamme, D., & Muylaert, K. (2014). Microalgal and cyanobacterial cultivation: The supply of nutrients. *Water Research*, 65, 186-202.
<https://doi.org/https://doi.org/10.1016/j.watres.2014.07.025>

- Matter, I. A., Bui, V. K., Jung, M., Seo, J. Y., Kim, Y.-E., Lee, Y.-C., & Oh, Y.-K. (2019). Flocculation Harvesting Techniques for Microalgae: A Review. *Applied Sciences*, 9(15).
<https://doi.org/10.3390/app9153069>
- Meier, L., Vilchez, C., Cuaresma, M., Torres-Aravena, Á., & Jeison, D. (2022). Effect of pH Change on the Microalgae-Based Biogas Upgrading Process. *Applied Sciences*, 12(23), 12194.
<https://doi.org/10.3390/app122312194>
- Meng, F., Xi, L., Liu, D., Huang, W., Lei, Z., Zhang, Z., & Huang, W. (2019). Effects of light intensity on oxygen distribution, lipid production and biological community of algal-bacterial granules in photo-sequencing batch reactors. *Bioresource Technology*, 272, 473-481.
<https://doi.org/https://doi.org/10.1016/j.biortech.2018.10.059>
- Miyatake, T., Moerdijk-Poortvliet, T. C. W., Stal, L. J., & Boschker, H. T. S. (2014). Tracing carbon flow from microphytobenthos to major bacterial groups in an intertidal marine sediment by using an in situ C pulse-chase method. *Limnology and Oceanography*, 59(4), 1275-1287.
<https://doi.org/10.4319/lo.2014.59.4.1275>
- Mohsenpour, S. F., Hennige, S., Willoughby, N., Adeloye, A., & Gutierrez, T. (2021). Integrating microalgae into WWT: A review. *Science of the Total Environment*, 752, 142168.
<https://doi.org/10.1016/j.scitotenv.2020.142168>
- Mokashi, K., Shetty, V., George, S. A., & Sibi, G. (2016). Sodium Bicarbonate as Inorganic Carbon Source for Higher Biomass and Lipid Production Integrated Carbon Capture in *Chlorella vulgaris*. *Achievements in the Life Sciences*, 10(1), 111-117.
<https://doi.org/10.1016/j.als.2016.05.011>
- Mokashi, K., Shetty, V., George, S. A., & Sibi, G. (2016). Sodium Bicarbonate as Inorganic Carbon Source for Higher Biomass and Lipid Production Integrated Carbon Capture in *Chlorella vulgaris*. *Achievements in the Life Sciences*, 10(1), 111-117.
<https://doi.org/https://doi.org/10.1016/j.als.2016.05.011>
- Mondal, M., Ghosh, A., Sharma, A. S., Tiwari, O. N., Gayen, K., Mandal, M. K., & Halder, G. N. (2016). Mixotrophic cultivation of *Chlorella* sp. BTA 9031 and *Chlamydomonas* sp. BTA 9032 isolated from coal field using various carbon sources for biodiesel production. *Energy Conversion and Management*, 124, 297-304.
<https://doi.org/https://doi.org/10.1016/j.enconman.2016.07.033>
- Moreira, J. M. R., Gomes, L. C., Araújo, J. D. P., Miranda, J. M., Simões, M., Melo, L. F., & Mergulhão, F. J. (2013). The effect of glucose concentration and shaking conditions on *Escherichia coli* biofilm formation in microtiter plates. *Chemical Engineering Science*, 94, 192–199.
<https://doi.org/10.1016/j.ces.2013.02.045>
- Mousavian, Z., Safavi, M., Salehirad, A., Azizmohseni, F., Hadizadeh, M., & Mirdamadi, S. (2023). Improving biomass and carbohydrate production of microalgae in the rotating cultivation system on natural carriers. *AMB Express*, 13(1), 39.
<https://doi.org/10.1186/s13568-023-01548-5>
- Namdarimonfared, M., Zilouei, H., & Tondro, H. (2023). Biological hydrogen production from paper mill effluent via dark fermentation in a packed bed biofilm reactor. *Fuel*, 338, 127231.
<https://doi.org/10.1016/j.fuel.2022.127231>

- Novoveská, L., Zapata, A. K., Zabolotney, J. B., Atwood, M. C., & Sundstrom, E. R. (2016). Optimizing microalgae cultivation and WWT in large-scale offshore photobioreactors. *Algal Research*, 18, 86–94.
<https://doi.org/10.3390/md21080445>
- Olsson, J., Schwede, S., Nehrenheim, E., & Thorin, E. (2018). Microalgae as biological treatment for municipal WW– effects on the sludge handling in a treatment plant. *Water Science and Technology*, 78(3), 644-654.
<https://doi.org/10.2166/wst.2018.334>
- Osachoff, H. L., Mohammadali, M., Skirrow, R. C., Hall, E. R., Brown, L. L., van Aggelen, G. C., Kennedy, C. J., & Helbing, C. C. (2014). Evaluating the treatment of a synthetic wwcontaining a pharmaceutical and personal care product chemical cocktail: compound removal efficiency and effects on juvenile rainbow trout. *Water Res*, 62, 271-280.
<https://doi.org/10.1016/j.watres.2014.05.057>
- Oswald, W. J. (1991). Introduction to Advanced Integrated ww ponding Systems. *Water Science and Technology*, 24(5), 1-7
- Oswald, W. J., Gotaas, H. B., Golueke, C. G., Kellen, W. R., Gloyna, E. F., & Hermann, E. R. (1957). Algae in waste treatment. *Sewage and industrial wastes*, 29(4), 437-457.
- Pachés, M., Martínez-Guijarro, R., Gonzalez-Camejo, J., Seco, A., & Barat, R. (2018). Selecting the most suitable microalgae species to treat the effluent from an anaerobic membrane bioreactor. *Environmental Technology*.
<https://doi.org/10.1080/09593330.2018.1496148>
- Paula, A. J., Hwang, G., & Koo, H. (2020). Dynamics of bacterial population growth in biofilms resemble spatial and structural aspects of urbanization. *Nature Communications*, 11(1).
<https://doi.org/10.1038/s41467-020-15165-4>
- Pereira, S. F. L., Gonçalves, A. L., Moreira, F. C., Silva, T. F. C. V., Vilar, V. J. P., & Pires, J. C. M. (2016). Nitrogen Removal from Landfill Leachate by Microalgae. *International Journal of Molecular Sciences*, 17(11).
<https://doi.org/10.3390/ijms17111926>
- Perez-Garcia, O., Escalante, F. M. E., de-Bashan, L. E., & Bashan, Y. (2011). Heterotrophic cultures of microalgae: Metabolism and potential products. *Water Research*, 45(1), 11-36.
<https://doi.org/https://doi.org/10.1016/j.watres.2010.08.037>
- Petrova, O. E., & Sauer, K. (2016). Escaping the biofilm in more than one way: desorption, detachment or dispersion. *Curr Opin Microbiol*, 30, 67-78.
<https://doi.org/10.1016/j.mib.2016.01.004>
- Posadas, E., García-Encina, P.-A., Soltau, A., Domínguez, A., Díaz, I., & Muñoz, R. (2013). Carbon and nutrient removal from centrates and domestic wwusing algal–bacterial biofilm bioreactors. *Biore-source Technology*, 139, 50–58.
<https://doi.org/10.1016/j.biortech.2013.04.008>

- Powell, N., Shilton, A., Pratt, S., & Chisti, Y. (2011). Phosphate release from waste stabilisation pond sludge: significance and fate of polyphosphate. *Water Science and Technology*, 63(8), 1689-1694.
<https://doi.org/10.2166/wst.2011.336>
- Qi, M., Yao, C., Sun, B., Cao, X., Fei, Q., Liang, B., Ran, W., Xiang, Q., Zhang, Y., & Lan, X. (2019). Application of an in situ CO₂-bicarbonate system under nitrogen depletion to improve photosynthetic biomass and starch production and regulate amylose accumulation in a marine green microalga *Tetraselmis subcordiformis*. *Biotechnology for Biofuels*, 12(1), 184.
<https://doi.org/10.1186/s13068-019-1523-7>
- Qian, J., Wan, T., Ye, Y., Li, J., Toda, T., Li, H., Sekine, M., Takayama, Y., Koga, S., Shao, S., Fan, L., Xu, P., & Zhou, W. (2023). Insight into the formation mechanism of algal biofilm in soy sauce wastewater. *Journal of Cleaner Production*, 394, 136179.
<https://doi.org/10.1016/j.jclepro.2023.136179>
- Rada-Ariza, A. M., Fredy, D., Lopez-Vazquez, C. M., Van der Steen, N. P., & Lens, P. N. L. (2019). Ammonium removal mechanisms in a microalgal-bacterial sequencing-batch photobioreactor at different solids retention times. *Algal Research*, 39, 101468.
<https://doi.org/https://doi.org/10.1016/j.algal.2019.101468>
- Ratzke, C., & Gore, J. (2018). Modifying and reacting to the environmental ph can drive bacterial interactions. *Plos Biol*, 16(3), e2004248.
<https://doi.org/10.1371/journal.pbio.2004248>
- Rawat, I., Ranjith Kumar, R., Mutanda, T., & Bux, F. (2011). Dual role of microalgae: Phycoremediation of domestic wastewater and biomass production for sustainable biofuels production. *Applied Energy*, 88(10), 3411-3424.
<https://doi.org/https://doi.org/10.1016/j.apenergy.2010.11.025>
- Ren, Q., Gao, J., Imtiaz, S. A., Li, C., & Huang, H. (2021). Increasing importance of anammox process: the present status and its development trend in municipal WWT system. *H2Open Journal*, 4(1), 302-320.
<https://doi.org/10.2166/h2oj.2021.093>
- Rosa, R. M., Machado, M., Vaz, M. G. M. V., Lopes-Santos, R., Nascimento, A. G. Do, Araújo, W. L., & Nunes-Nesi, A. (2023). Urea as a source of nitrogen and carbon leads to increased photosynthesis rates in *Chlamydomonas reinhardtii* under mixotrophy. *Journal of Biotechnology*, 367, 20–30.
<https://doi.org/10.1016/j.jbiotec.2023.03.009>
- Rossi, S., Díez-Montero, R., Rueda, E., Cascino, F. C., 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, 123046.
<https://doi.org/10.1016/j.biortech.2020.123046>
- Rosso, D., Stenstrom, M., & Larson, L. (2008). Aeration of large-scale municipal wwtps: State of the art. *Water science and technology : a journal of the International Association on Water Pollution Research*, 57, 973-978.
<https://doi.org/10.2166/wst.2008.218>

- Rusten, B., Eikebrokk, B., Ulgenes, Y., & Lygren, E. (2006). Design and operations of the Kaldnes moving bed biofilm reactors. *Aquacultural Engineering*, 34(3), 322-331.
<https://doi.org/https://doi.org/10.1016/j.aquaeng.2005.04.002>
- Saia, S. M., Sullivan, P. J., Regan, J. M., Carrick, H. J., Buda, A. R., Locke, N. A., & Walter, M. T. (2017). Evidence for polyphosphate accumulating organism (PAO)-mediated phosphorus cycling in stream biofilms under alternating aerobic/anaerobic conditions. *Freshwater Science*, 36(2), 284-296.
<https://doi.org/10.1086/691439>
- Salgado, E. M., Esteves, A. F., Gonçalves, A. L., & Pires, J. C. M. (2023). Microalgal cultures for the remediation of wastewaters with different nitrogen to phosphorus ratios: Process modelling using artificial neural networks. *Environmental Research*, 231, 116076.
<https://doi.org/10.1016/j.envres.2023.116076>
- Salgar-Chaparro, S. J., Lepkova, K., Pojtanabuntoeng, T., Darwin, A., & Machuca, L. L. (2020). Nutrient Level Determines Biofilm Characteristics and Subsequent Impact on Microbial Corrosion and Biocide Effectiveness. *Applied and Environmental Microbiology*, 86(7), e02885-02819.
<https://doi.org/doi:10.1128/AEM.02885-19>
- Saúco, C., Cano, R., Marín, D., Lara, E., Rogalla, F., & Arbib, Z. (2021). Hybrid WWT system based in a combination of high rate algae pond and vertical constructed wetland system at large scale. *Journal of Water Process Engineering*, 43, 102311.
<https://doi.org/https://doi.org/10.1016/j.jwpe.2021.102311>
- Sauer, K., Stoodley, P., Goeres, D. M., Hall-Stoodley, L., Burmølle, M., Stewart, P. S., & Bjarnsholt, T. (2022). The biofilm life cycle: expanding the conceptual model of biofilm formation. *Nature Reviews Microbiology*, 20(10), 608-620.
<https://doi.org/10.1038/s41579-022-00767-0>
- Sauer, K., Stoodley, P., Goeres, D. M., Hall-Stoodley, L., Burmølle, M., Stewart, P. S., & Bjarnsholt, T. (2022). The biofilm life cycle: expanding the conceptual model of biofilm formation. *Nature Reviews Microbiology*, 20(10), 608-620.
<https://doi.org/10.1038/s41579-022-00767-0>
- Schultze, L. B., Maldonado, A., Lussi, A., Sculean, A., & Eick, S. (2021). The Impact of the pH Value on Biofilm Formation. *Monogr Oral Sci*, 29, 19-29.
<https://doi.org/10.1159/000510196>
- Sepehri, A., Sarrafzadeh, M.-H., & Avateffazeli, M. (2020). Interaction between *Chlorella vulgaris* and nitrifying-enriched activated sludge in the treatment of wastewater with low C/N ratio. *Journal of Cleaner Production*, 247, 119164.
<https://doi.org/https://doi.org/10.1016/j.jclepro.2019.119164>
- Spaargaren, D. H. (1996). The design of culture media based on the elemental composition of biological material. *Journal of Biotechnology*, 45(2), 97-102.
[https://doi.org/https://doi.org/10.1016/0168-1656\(95\)00152-2](https://doi.org/https://doi.org/10.1016/0168-1656(95)00152-2)
- Su, Y., Mennerich, A., & Urban, B. (2011). Municipal WWT and biomass accumulation with a wastewater-born and settleable algal-bacterial culture. *Water Research*, 45(11), 3351-3358.
<https://doi.org/https://doi.org/10.1016/j.watres.2011.03.046>

- Swale, H. B. A. E. (1982). Culture Collection of Algae and Protozoa. Institute of terrestrial ecology
https://nora.nerc.ac.uk/id/eprint/5236/1/Culturing_algae.pdf
- Tang, Y., Song, L., Ji, X., Huang, S., Yu, Y., Ye, J., Xu, W., & Hou, M. (2022). Algal-bacterial consortium mediated system offers effective removal of nitrogen nutrients and antibiotic resistance genes. *Bio-resour Technol*, 362, 127874.
<https://doi.org/10.1016/j.biortech.2022.127874>
- Taziki, M., Ahmadzadeh, H., Murry-Ewers, M., & Lyon, S. (2015). Nitrate and Nitrite Removal from wastewater using Algae. *Current Biotechnology*, 04, 1-1.
<https://doi.org/10.2174/2211550104666150828193607>
- Torpey, W. N., Heukelekian, H., Kaplovsky, A. J., & Epstein, R. (1971). Rotating Disks with Biological Growths Prepare ww for Disposal or Reuse. *Journal (Water Pollution Control Federation)*, 43(11), 2181–2188. JSTOR.
- Trulear, M. G., & Characklis, W. G. (1982). Dynamics of Biofilm Processes. *Journal (Water Pollution Control Federation)*, 54(9), 1288-1301.
<http://www.jstor.org/stable/25041684>
- Trulear, M. G., & Characklis, W. G. (1982). Dynamics of Biofilm Processes. *Journal (Water Pollution Control Federation)*, 54(9), 1288-1301.
<http://www.jstor.org/stable/25041684>
- Tsagkari, E., Connelly, S., Liu, Z., mcbride, A., & Sloan, W. T. (2022). The role of shear dynamics in biofilm formation. *Npj Biofilms and Microbiomes*, 8(1), Article 1.
<https://doi.org/10.1038/s41522-022-00300-4>
- Tyrrell, T. (2001). Redfield Ratio. In J. H. Steele (Ed.), *Encyclopedia of Ocean Sciences* (pp. 2377-2387). Academic Press.
<https://doi.org/https://doi.org/10.1006/rwos.2001.0271>
- Umetani, I., Janka, E., Sposób, M., Hulatt, C. J., Kleiven, S., & Bakke, R. (2021). Bicarbonate for microalgae cultivation: a case study in a chlorophyte, *Tetrademus wisconsinensis* isolated from a Norwegian lake. *Journal of Applied Phycology*, 33(3), 1341-1352.
<https://doi.org/10.1007/s10811-021-02420-4>
- Van Der Lee, G. H., Kraak, M. H. S., Verdonchot, R. C. M., Vonk, J. A., & Verdonchot, P. F. M. (2017). Oxygen drives benthic-pelagic decomposition pathways in shallow wetlands. *Scientific Reports*, 7(1).
<https://doi.org/10.1038/s41598-017-15432-3>
- Vargas, G., Donoso-Bravo, A., Vergara, C., & Ruiz-Filippi, G. (2016). Assessment of microalgae and nitrifiers activity in a consortium in a continuous operation and the effect of oxygen depletion. *Electronic Journal of Biotechnology*, 23, 63-68.
<https://doi.org/https://doi.org/10.1016/j.ejbt.2016.08.002>
- Vossoghi, M., & Daylami, N. (2003). Study on Nitrification and Denitrification of High Nitrogen and COD Load Wastewater in Moving Bed Biofilm Reactor. *Iranian Journal of Biotechnology*, 1(2), 115-120.

- Wang, L., Wang, Y., Chen, P., & Ruan, R. (2010). Semi-continuous cultivation of *Chlorella vulgaris* for treating undigested and digested dairy manures. *Applied Biochemistry and Biotechnology*, 162, 2324–2332.
<https://doi.org/10.1007/s12010-010-9005-1>
- Wang, S., Parajuli, S., Sivalingam, V., & Bakke, R. (2020). Biofilm in Moving Bed Biofilm Process for WWT. In. Intechopen.
<https://doi.org/10.5772/intechopen.88520>
- Wang, W., Shen, H., Shi, P., Chen, J., Ni, L., & Xie, P. (2016). Experimental evidence for the role of heterotrophic bacteria in the formation of *Microcystis* colonies. *Journal of Applied Phycology*, 28(2), 1111–1123.
<https://doi.org/10.1007/s10811-015-0659-5>
- Weber, S. D., Ludwig, W., Schleifer, K. H., & Fried, J. (2007). Microbial composition and structure of aerobic granular sewage biofilms. *Appl Environ Microbiology*, 73(19), 6233–6240.
<https://doi.org/10.1128/aem.01002-07>
- Wehr, J. D. (2007). *Algae: Anatomy, Biochemistry, and Biotechnology* by Barsanti, L. & Gualtieri, P. *Journal of Phycology*, 43(2), 412–414.
<https://doi.org/10.1111/j.1529-8817.2007.00335.x>
- Weon, S. Y., Lee, S. I., & Koopman, B. (2004). Effect of temperature and dissolved oxygen on biological nitrification at high ammonia concentrations. *Environmental Technology*, 25(11), 1211–1219.
- Wilczak, A. J., JG; Marcinko, JP; Odell, LH; Kirmeyer, GJ; Wolfe, RL (1996). Occurrence of nitrification in chloraminated distribution systems *Journal of the American Water Works Association*, 88 (7), 74–85
<http://://WOS:A1996UX95800013>
- Wilson, C., Lukowicz, R., Merchant, S., Valquier-Flynn, H., Caballero, J., Sandoval, J., Okuom, M., Huber, C., Brooks, T. D., Wilson, E., Clement, B., Wentworth, C. D., & Holmes, A. E. (2017). Quantitative and Qualitative Assessment Methods for Biofilm Growth: A Mini-review. *Res Rev J Eng Technol*, 6(4).
- Winkler, M.-K. H., Boets, P., Hahne, B., Goethals, P., & Volcke, E. I. P. (2017). Effect of the dilution rate on microbial competition: r-strategist can win over k-strategist at low substrate concentration. *Plos ONE*, 12(3), e0172785.
<https://doi.org/10.1371/journal.pone.0172785>
- Wu, D., Cheng, M., Zhao, S., Peng, N., Hu, R., Hu, J., & Liang, Y. (2020). Algal Growth Enhances Light-Mediated Limitation of Bacterial Nitrification in an Aquaculture System. *Water, Air, & Soil Pollution*, 231(2), 73.
<https://doi.org/10.1007/s11270-020-4436-y>
- Wu, M., Wu, G., Lu, F., Wang, H., Lei, A., & Wang, J. (2022). Microalgal photoautotrophic growth induces pH decrease in the aquatic environment by acidic metabolites secretion. *Biotechnology for Biofuels and Bioproducts*, 15(1).
<https://doi.org/10.1186/s13068-022-02212-z>

- Xu, Y., Wu, Y., Esquivel-Elizondo, S., Dolfing, J., & Rittmann, B. E. (2020). Using Microbial Aggregates to Entrap Aqueous Phosphorus. *Trends in Biotechnology*, 38(11), 1292–1303.
<https://doi.org/10.1016/j.tibtech.2020.03.012>
- Yang, S., Wang, J., Cong, W., Cai, Z., & Ouyang, F. (2004). Utilization of nitrite as a nitrogen source by *Botryococcus braunii*. *Biotechnology Letters*, 26, 239–243.
<https://doi.org/10.1023/b:bile.0000013722.45527.18>
- Yeh, K.-L., Chang, J.-S., & chen, W.-m. (2010). Effect of light supply and carbon source on cell growth and cellular composition of a newly isolated microalga *Chlorella vulgaris* ESP-31. *Engineering in Life Sciences*, 10(3), 201–208.
<https://doi.org/https://doi.org/10.1002/elsc.200900116>
- Ylla, I., Borrego, C., Romanã, A. M., & Sabater, S. (2009). Availability of glucose and light modulates the structure and function of a microbial biofilm. *FEMS Microbiology Ecology*, 69(1), 27–42.
<https://doi.org/10.1111/j.1574-6941.2009.00689.x>
- Young, P., Taylor, M., & Fallowfield, H. J. (2017). Mini-review: high rate algal ponds, flexible systems for sustainable WWT. *World Journal of Microbiology and Biotechnology*, 33(6), 117.
<https://doi.org/10.1007/s11274-017-2282-x>
- Zerveas, S., Mente, M. S., Tsakiri, D., & Kotzabasis, K. (2021). Microalgal photosynthesis induces alkalization of aquatic environment as a result of H⁺ uptake independently from CO₂ concentration – New perspectives for environmental applications. *Journal of Environmental Management*, 289, 112546.
<https://doi.org/https://doi.org/10.1016/j.jenvman.2021.112546>
- Zhang, Y., Wang, J., Peng, S., Zhao, D., & Miao, L. (2022). Autotrophic biological nitrogen removal in a bacterial-algal symbiosis system: Formation of integrated algae/partial-nitrification/anammox biofilm and metagenomic analysis. *Chemical Engineering Journal*, 439, 135689.
<https://doi.org/https://doi.org/10.1016/j.cej.2022.135689>
- Zhang, Y., Wang, J., Peng, S., Zhao, D., & Miao, L. (2022). Autotrophic biological nitrogen removal in a bacterial-algal symbiosis system: Formation of integrated algae/partial-nitrification/anammox biofilm and metagenomic analysis. *Chemical Engineering Journal*, 439, 135689.
<https://doi.org/https://doi.org/10.1016/j.cej.2022.135689>
- Zhao, X., Kumar, K., Gross, M. A., Kunetz, T. E., & Wen, Z. (2018). Evaluation of revolving algae biofilm reactors for nutrients and metals removal from sludge thickening supernatant in a municipal WWT facility. *Water Research*, 143, 467–478.
<https://doi.org/10.1016/j.watres.2018.07.001>
- Zhao, Y., Liu, D., Huang, W., Yang, Y., Ji, M., Nghiem, L. D., Trinh, Q. T., & Tran, N. H. (2019). Insights into biofilm carriers for biological WWT processes: Current state-of-the-art, challenges, and opportunities. *Bioresource Technology*, 288, 121619.
<https://doi.org/10.1016/j.biortech.2019.121619>
- Zhi, R., Yang, A., Zhang, G., Zhu, Y., Meng, F., & Li, X. (2019). Effects of light-dark cycles on photosynthetic bacteria WWT and valuable substances production. *Bioresource Technology*, 274, 496–501.
<https://doi.org/10.1016/j.biortech.2018.12.021>

APPENDIX A – SYNTHETIC WASTEWATER RECIPES

This appendix provides supplementary information regarding the synthetic wastewater recipes employed in the cultivation of the inoculum for the research study. The three distinct tables presented herein detail the composition of each synthetic wastewater, encompassing essential parameters that influenced the development and characteristics of the microbial inoculum.

A.1 Synthetic WW Medium Recipe

The recipe present in table A.1 was used in Protocol 1 for the preparation of the inoculum.

Table A.1 – Synthetic WW medium recipe

Compound	Chemical formula	Mass
-	-	mg
Yeast Extract	-	28,0
Starch	C ₆ H ₁₀ O ₅	96,0
Sodium acetate anhydrous	NaCH ₃ COO	230,0
Monopotassium phosphate	KH ₂ PO ₄	22,0
Sodium bicarbonate	NaHCO ₃	155,0
Ammonium chloride	NH ₄ Cl	620,0
Ferric chloride	FeCl ₃	12,0
Magnesium chloride	MgCl ₂ .6H ₂ O	25,0
Calcium chloride	CaCl ₂ .2H ₂ O	37,0
Zinc sulphate	ZnSO ₄ .7H ₂ O	0,2
Add 1000 mL of Deionised Water		

A.2 Knop's Media Recipe

The recipe present in table A.2 was used in Protocol 2 for the preparation of the inoculum.

Table A.2- Knop's Media Recipe

Compound	Chemical formula	Mass
-	-	<i>mg</i>
Sodium Bicarbonate	NaHCO ₃	1380
Calcium Nitrate	Ca(NO ₃) ₂	250
Magnesium Sulfate	MgSO ₄	60
Dipotassium phosphate	K ₂ HPO ₄	60
Potassium nitrate	KNO ₃	30
Iron(III) chloride	FeCl ₃	1
Add 1000 mL of Deionised Water		

A.3 Strawberry Fertiliser Recipe

The recipe present in table A.3 was used in Protocol 3 for the preparation of the inoculum.

Table A.3 Strawberry Fertiliser Media Recipe

Compound	Chemical formula	Mass FEED
-	-	<i>mg</i>
Sodium Bicarbonate	NaHCO ₃	1380
Add 200 mL of Strawberry Fertiliser		
Add 1000 mL of Deionised Water		



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ASSESSING THE POTENTIAL OF ALGAL BIOFILMS FOR NUTRIENT REMOVAL IN WASTEWATERS