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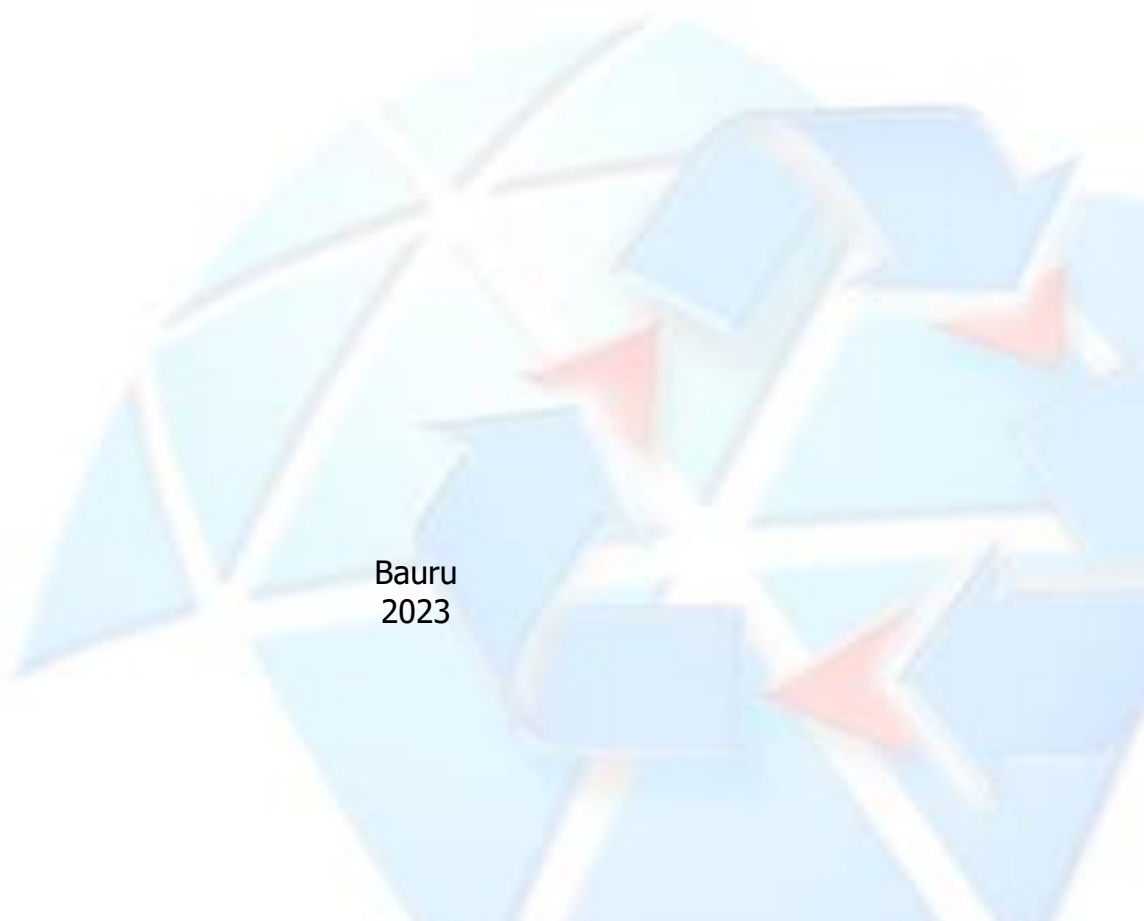
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**POST-TREATMENT OF MUNICIPAL WASTEWATER USING
MICROALGAE AND ACTIVATED SLUDGE SYNERGY**

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**POST-TREATMENT OF MUNICIPAL WASTEWATER USING
MICROALGAE AND ACTIVATED SLUDGE SYNERGY**

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ATA DA DEFESA PÚBLICA DA DISSERTAÇÃO DE MESTRADO DE ABRAHAM JAMES OLAMILEKAN, DISCENTE DO PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA CIVIL E AMBIENTAL, DA FACULDADE DE ENGENHARIA - CÂMPUS DE BAURU.

Aos 07 dias do mês de junho do ano de 2023, às 09:30 horas, por meio de Videoconferência, realizou-se a defesa de DISSERTAÇÃO DE MESTRADO de ABRAHAM JAMES OLAMILEKAN, intitulada **POST-TREATMENT OF MUNICIPAL WASTEWATER USING MICROALGAE AND ACTIVATED SLUDGE SYNERGY**. A Comissão Examinadora foi constituída pelos seguintes membros: Prof. Dr. GUSTAVO HENRIQUE RIBEIRO DA SILVA (Orientador(a) - Participação Virtual) do(a) Departamento de Engenharia Civil e Ambiental / Faculdade de Engenharia de Bauru UNESP, Profa. Dra. MARÍA DEL ROSARIO RODERO RAYA (Participação Virtual) do(a) Institute of Sustainable Processes / University of Valladolid, Prof^a. Dr^a. ANA TERESA LOMBARDI (Participação Virtual) do(a) Departamento de Botânica / Universidade Federal de São Carlos - UFSCar. Após a exposição pelo mestrando e arguição pelos membros da Comissão Examinadora que participaram do ato, de forma presencial e/ou virtual, o discente recebeu o conceito final: APROVADO. Nada mais havendo, foi lavrada a presente ata, que após lida e aprovada, foi assinada pelo(a) Presidente(a) da Comissão Examinadora.


Prof. Dr. GUSTAVO HENRIQUE RIBEIRO DA SILVA

DEDICATION

To God and my beloved mother of blessed memory, the pathfinder of this lofty height

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Summary

O problema das águas residuárias não tratadas ou tratadas inadequadamente ainda persiste globalmente. Isso não tem apenas contribuído para o aumento das cargas de poluição ambiental, mas também falhado em aproveitar os materiais bioeconômicos presentes nas águas residuárias. Embora a ciência da tecnologia de tratamento baseada em microalgas tenha se mostrado eficiente para o tratamento e recuperação de recursos potenciais, a maioria dos estudos foi realizada sob condições controladas. Portanto, o presente estudo avaliou o desempenho do crescimento de microalgas nativas-lodo ativado (MLA) para o tratamento terciário de águas residuárias digeridas anaerobiamente em um reator anaeróbio de fluxo ascendente com manta de lodo (UASB), a partir de fotobiorreatores externos em escala laboratorial (2 L). Sete diferentes condições com proporções distintas de inóculo (Condições: 1 a 3 é MLA; 4 a 6 são controles de microalgas; 7 é sem inóculo) foram operadas em bateladas em reator em triplicata, com tempo de retenção hidráulica (TRH) de 5 dias em 11,5:12,5 Foto-horas, para identificar a melhor proporção de inóculo a ser adotada no fotobiorreator piloto do tipo *flat panel*. A condição 1 (0,10 g.L⁻¹: microalga e 0,20 g.L⁻¹: lodo ativado), com menor proporção de MLA, apresentou o melhor resultado, com produtividade de densidade celular (2,03 x 10⁷ células.mL⁻¹) juntamente com biomassa de 0,13 g SST.L⁻¹.d⁻¹, remoção de fósforo total (85,07%) e nitrogênio total (66,14%) e remoção logarítmica (Log-Re) de bactérias patogênicas (indicadores de qualidade da água), com Log-Re 3,3 para coliformes totais (137E+02 UFC.100 mL⁻¹) e 4,7 para *Escherichia coli* (0,00E+00 UFC.100 mL⁻¹). O MLA de melhor desempenho (condição 1) foi testado em um fotobiorreator piloto do tipo *flat panel*, ao lado de um controle inoculado com a proporção de microalgas do MLA e operado sob TDH de 5 e 3 dias. O desempenho do sistema foi determinado pela eficiência de remoção e recuperação de nutrientes, sedimentação da biomassa e remoção de *Escherichia coli* e coliformes totais. Os resultados mostraram que o TRH mais alto (5 dias) favoreceu o desempenho do MLA em relação ao crescimento da biomassa, com eficiência de remoção significativamente maior ($p < 0,05$), 88,0; 79,0; 59,5% para fósforo dissolvido total (FDT), nitrogênio amoniacal (NH⁺₄-N) e demanda química de oxigênio (DQO_{filtrado}), respectivamente. A produtividade média de biomassa e taxa de crescimento para TRHs de 5 e 3 dias foi de 59,41±12,31 mg SST.L⁻¹.d⁻¹, 0,08±0,01 d⁻¹ e

925,56±11,09 mg SST.L⁻¹.d⁻¹, 0,03±0,01 d⁻¹, respectivamente. Os Log-Re > 4,0 UFCmL⁻¹ e > 3,5 UFCmL⁻¹ foram obtidos para *E. coli* e coliformes totais, respectivamente, em ambos os TRHs, e mostraram potencial para uso agrícola por atender ao padrão recomendado pela Organização Mundial da Saúde (OMS) de 10³ MPN.100mL⁻¹. No geral, os resultados mostraram uma viabilidade economicamente promissora do tratamento de águas residuárias à base de microalgas ao ar livre. No entanto, a eficiência de autofixação foi inversamente baixa de 33,0 e 14,0% para TRHs de 5 e 3 dias, respectivamente, e supostamente impactada pelo alto pH (> 10,0) na população bacteriana e pela dominância da não-mucilagem *Cyanobium* sp. (98%).

Abstract

The problem of untreated and inadequately treated wastewater still persists globally. This has not only contributed to the increasing environmental pollution loads, but has also failed to harness the bioeconomy materials present in wastewater. While the science of microalgae-based treatment technology has shown to be efficient for treatment and recovery of potential resource, the majority of the studies were conducted under controlled conditions. Therefore, this present study evaluated the performance of native microalgae-activated sludge (MAS) growth for tertiary treatment of anaerobically digested wastewater from an upflow anaerobic sludge blanket (UASB) in an outdoor lab-scale photobioreactors (2 L). Seven different conditions with distinct inoculum proportions (Conditions: 1 to 3 is MAS; 4 to 6 is microalgae controls; 7 is without inoculum) were operated in batch mode reactor in triplicate for 5-days of hydraulic retention time (HRT) at 11.5:12.5 Photo-hours, to identify the best inoculum proportion for adopted in pilot flat panel photobioreactor. Condition 1 (0.10gL⁻¹: microalgae and 0.20gL⁻¹: activated-sludge), with lowest MAS proportion showed the best outcome with cell density productivity (2.03 x 10⁷ cells.mL⁻¹) along with biomass of 0.13 g TSS.L⁻¹.d⁻¹ and the total phosphorus uptake (85.07%), total nitrogen uptake (66.14%), logarithmic removal (Log-Re) of bacterial pathogens (water quality indicators), which showed Log-Re (3.3 for total coliforms (137E+02 CFU.100 mL⁻¹) and 4.7 for *Escherichia coli* (0.00E+00 CFU.100 mL⁻¹)). The best performing MAS (condition 1) was tested in a pilot flat panel photobioreactor, alongside a control, inoculated with the microalgae proportion of the MAS, and operated under 5-days and 3-days HRT. The performance of the system was determined by the efficiency of nutrient removal and recovery, biomass sedimentation and removal of *Escherichia coli* and Total Coliforms. Results showed that high HRT favoured the performance of MAS with respect to biomass growth, with significantly higher ($p < 0.05$) removal efficiency, 88.0, 79.0, 59.5% for total phosphorus (TDP), ammonium nitrogen (NH⁺₄-N), chemical oxygen demand (COD_{filtered}), respectively at 5 days HRT. The average biomass productivity and growth rate for HRTs 5 days and 3 days are 59.41±12.31 mg TSS L⁻¹ d⁻¹, 0.08±0.01 d⁻¹ and 25.56±11.09 mg TSS L⁻¹ d⁻¹, 0.03±0.01 d⁻¹, respectively. The log-Re > 4.0 CFUmL⁻¹ and > 3.5 CFUmL⁻¹ were achieved for *E. coli* and total coliforms, respectively at both HRTs, and showed the potential for

agricultural use for complying with the WHO recommended standard of 10^3 MPN.100mL⁻¹. Overall, results showed a promising feasibility of outdoor microalgae-based treatment of wastewater cost effectively. However, self-settling efficiency was conversely low 33.0 and 14.0% for HRTs 5 days and 3 days, respectively, and suspiciously impacted by high pH (> 10.0) on bacterial population and the dominance of non-mucilage *Cyanobium* sp. (98%).

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List of Abbreviations

CFU – Coliform Forming Unit

COD - Chemical Oxygen Demand

DAE - Department of Water and Sewage of Bauru

DO – Dissolved Oxygen

FAPESP - Fundação de Amparo à Pesquisa do Estado de São Paulo

HRT – Hydraulic Retention Time

IPMet - Instituto de Pesquisas Meteorológicas

MAS – Microalgae and Activated Sludge

n – Sample size

NTK - Total Nitrogen Kjeldahl

OD - Optical Density

PBRs – Photobioreactors

pH - Hydrogen Potential

R1 – Reactor 1

R2 – Reactor 2

SDG - Sustainable Development Goals

TDP - Total Dissolved Phosphorus

TDN - Total Nitrogen

TSS – Total Suspended Solids

UASB - Upflow Anaerobic Sludge Blanket

UNESP – State University “Júlio Mesquita Filho

UNICEF – United Nations International Children Emergency Fund

WHO – World Health Organization

WW – Wastewater

WWTP – Wastewater Treatment Plant

Organization of Dissertation

This thesis is organized in five chapters. Chapter 1 (Introduction) explained the background, research problem, the objectives and the hypothesis of this work. Chapter 2 (Literature review) provided the summary and highlights of relevant existing studies, and explained wastewater characteristics, importance of anaerobic sewage treatment systems, microalgae cultivations and microalgae-based system as a tertiary treatment technology, environmental factors affecting microalgae cultivation, bioeconomy, microalgae biomass recovery and industrial applications. Chapter 3 (Batch experiment study) detailed the laboratory scale experiment that evaluated the potential of microalgae-activated sludge (MAS) co-culture (seven conditions) in natural outdoor condition, and contained the introduction, methodology and results and discussion. Chapter 4 (Fed-batch experiment study) showed the performance of the best condition (identified at the laboratory scale) in pilot flat panel photobioreactor in natural outdoor condition with respect to hydraulic retention time, and contained the introduction, methodology, result and discussion and future perspective drawing from recorded findings. Chapter 5. The general conclusion of the experiments.

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1 INTRODUCTION

The management of wastewater is still a major challenge confronting the whole world. This could be attributed to volumetric increase of untreated wastewater from municipal, industrial, and agricultural activities, which constitutes a significant threat to the quality of water resources as well as public health (Aradhana and Kumar, 2015; Zhu et al., 2019; Pompei et al., 2022). The world population has doubled in the last 48 years, and survey has shown many of the African countries having the fastest growth rates, with the continent's population size doubling by 2050 (Routley, 2022). This raises more concern on the capacity of these countries to treat their wastewater and invariably necessitates the need for more explorative studies to be conducted on affordable and effective alternative treatment options to bridge existential treatment gap.

The growing human population has also continued to widen the supply gap of fertilizer. The survey separately conducted by the International Fertilizer Development Centre in 2010 and the United State Geological Survey in 2012 showed utilization of phosphorus would reach it peak between 2030 and 2040, with hard biting global scarcity effect and food insecurity (Desmidt et al., 2014; Neset and Cordell, 2011). Also, various authors have given different projections for outright depletion of the finite phosphate rock, ranging between 50 to 400 years (Kok et al., 2018), which is likely to have reduced due to increasing demand.

Alternatively, Moges et al. (2020) showed that repurposing wastewater to produce phosphorus is capable of closing the supply loop of phosphorus, which would in turn reduce mining of phosphate rock and accompanying environmental problems. Specifically, Kok et al. (2018) revealed that phosphorus recovery can sustainably satisfy the global demand, indicating 20% potential from all urban wastewater and 90% potential from animal manure

for total agricultural demands, making it a good substitute for phosphorus fertilizer. For the purpose of sustainability, the decentralized wastewater treatment plants are recommended for phosphorus recovery from human waste, owing to simple treatment processes and low-cost technologies and operated in close proximity to the flow of water, energy, and organic and inorganic material (Silva et al., 2019).

The war between Russia and Ukraine has further exacerbated the challenge of fertilizer scarcity and food insecurity. Recently, 20 percent hike in food prices was projected by the United Nations' Food and Agriculture Organization hinged on dwindled availability of fertilizer and food supplies from the supplying warring nations (Lu, 2022). This further underscores the significance of recovering valuable resources from municipal wastewater, especially nitrogen and phosphorus nutrients, the main ingredient of fertilizer, while ensuring adequate treatment, before being discharged into the environment. This has the potential to reduce dependence on external supplies to meet fertilizer needs (Ashley, 2009), as prevailing social or environmental related problems may cut short supply, just as it is currently (Shaddel et al., 2019). This has the potential to drive sustainable food supply and security, and would also boost the science of circular economy (Su, 2020).

Globally, approximately 80% of wastewater is released into the environment without adequate treatment (Oviedo et al., 2022). Specifically, in the Sub-Saharan and Latin America and the Caribbean between, over 50% of the wastewater generated from 2000 to 2017 was discharged into the environment without prior treatment (WHO and UNICEF, 2019). Furthermore, Brazil, being the most populous in Latin America and the Caribbean, scored fairly above half (56%) until 2017 in the treatment of domestic wastewater at secondary level, while, on the other hand, a damning 32% coverage was recorded for Nigeria for the same level of treatment of domestic wastewater. Moreover, the small percentage of treated wastewater fall short of the stipulated permissible level owing to the shortcoming of the conventional treatment technologies (Serejo et al. 2020).

This has led to the discharge of wide range of pollutants (nitrate, phosphate, microplastics, metals persistent organic pollutants) and pathogenic microorganisms (bacteria, protozoa, viruses) that are usually present in wastewater in concentrations above the critical safe limits, thus increasing the pollution loads of receiving water bodies. This poses serious challenges that border on environmental pollution, ecosystem obliteration resulting from algae bloom, oxygen depletion, death of aqua species, and public health

epidemics from ingestion of pathogenic organisms (Akor & Muchie, 2011; UNESCO, 2017; Ruas et al., 2017; Gonçalves et al., 2017).

The disproportionate discharge of untreated wastewater into the environment may be linked to high operational cost implication of conventional treatment process, particularly for municipal wastewater. This presents a major limitation in form of high cost of energy for aeration and frequent removal of accompanying increased sludge produced during treatment (Kehrein et al., 2020; Wang et al., 2020; Molinuevo-Salces et al., 2019). Additionally, the conventional biological treatment methods fall short in the nutrients removal efficiency required, thus contributing to nutrient pollution load of receiving water bodies (Quijano et al., 2017; Wang et al., 2020).

In specific terms, the technical and economic drawbacks of conventional biological treatment methods have been linked to (1) inadequate pollutant and pathogen removal (2) poor nutrient recovery (3) expensive and energy demanding, and (4) CO₂ production (Molinuevo-Salces et al., 2019). The activated sludge biological treatment process is adjudged the most used of the options (Maddela et al., 2021). On the other hand, the anaerobic counterpart, which excludes the need for aeration, effectively decomposes the organic matters in wastewater, but releases effluent that is rich in nutrients (de Oliveira et al., 2021). Therefore, the highlighted limitations have made the conventional treatment methods less desirable and effective to wastewater treatment. This has the potential to undermine the realisation of the United Nations Sustainable Development Goal (SDG) 6 due to associated polluting potential and consequent reduction in water quality meant for domestic and other purposes.

In response and considering that nutrients are unavoidable by-products of wastewater treatment plants and systems, biorefinery treatment technologies are being explored to ensure effective treatment. In addition to the offer of environmental protection attributable treatment efficacy, the potential resources in wastewater are concurrently recovered by biorefineries (Barros et al., 2022), thus projects an economic outlook inform of provision of materials and inputs for industrial symbiosis. The transformation of wastewater into high-value products for agriculture, aquaculture, and sundry other industrial processing inputs will incentivize maximum use of resources and ensure increased production value-chain. This will in-turn promote environmental sustainability by addressing the problems of

depleting natural resources and environmental pollution as well as it will improve agricultural productivity.

Microalgae biorefinery has received a lot of attention in recent decades owing to the empirical evidence as an efficient treatment alternative. More importantly, high value-added products are generated through nutrient cycling and valorisation (biofertilizer, biofuel and animal feeds) while wastewater is being treated, with the removal of ammonium, nitrates and phosphorus, lipids and pigments, protein (Liu and Hong, 2021; Dourou et al., 2020; Molinuevo-Salces et al., 2019; Matamoros et al., 2016; Wang et al., 2010). These microalgae utilize the nutrients in wastewater with the aid of solar energy or artificial light source to replicate exponentially, generate high biomass growth, and concomitantly treat the fed wastewater. The nutrients are stored in their cellular composition, forming a nutrient-rich biomass that can be used as feedstock for industrial purposes (Hwang, 2016; Andrade et al., 2021).

The biomass as a resource has a wide range of applicability, considering the potential of the high-value products encapsulated within the microalgae cells. The proteins, lipids, and polysaccharides contents can be used for the production of bio-based polymers; an environmentally friendly alternative packaging material due to their degradability (Sogut & Seydim, 2018). This offers the benefits of reduced environmental footprint of plastic proliferation and the corresponding municipal solid waste problem. Furthermore, it strengthens the fight against climate change, reducing the concentration of carbon emission by stalling the consumption of about 8% of the world's oil production, meant for the manufacture of synthetic plastics (Riera & Palma, 2021). Moreover, biomass is used as feedstock for bioenergy production, in cosmetics industry, and as biofertilizers (Rumin et al. 2020).

However, slow settling and consequent poor biomass harvesting of microalgae cells posits a serious challenge to biomass recovery. Microalgae are negatively charged, with small cell size (5 and 50 μm) and relatively low cell density, thus often dispersely distributed in suspension (Molinuevo-Salces et al., 2019; Quijano et al., 2017). To recover these microalgae, application of different techniques such as filtration, centrifugation, coagulation, are employed industrially (Fasaei et al., 2018), and amount to additional capital expenditure and energy consumption. Infact, the chemical harvesting method (chemical coagulants) have

shown the tendency of affecting the quality of harvested biomass (Quijano et al., 2017), which is liable to limit the scope of application of the biomass for industrial use.

To this effect, natural coagulants and auto-flocculation have been explored to recover nutrients rich biomass. Teixeira et al. (2021) achieved better floc formation with improved biomass recovery and over 90% turbidity and optical density removal, at a much-reduced sedimentation time, with Tannin-based coagulants. Likewise, high recovery percentage of microalgae biomass anaerobically cultivated in blackwater, alongside turbidity and optical density, was reported by Silva et al. (2020). Furthermore, auto-flocculation induced by altering the pH aqueous system using NaOH achieved about 94% recovery (Ummalyma et al., 2016). Biopolymers such as chitosan, cationic starches and polyacrylamide have equally displayed a promising recovery performance of algae biomass (Matter et al., 2019), but effectiveness is pH dependent and produces good outcome at a narrow range, with low performing efficiency for some microalgae species (Matter et al., 2019).

The highlighted techniques and technologies come with additional obligations in form of energy requirement, equipment and maintenance, and materials such as nature-based coagulants and NaOH in case of pH alteration, which exert cost implications. Meanwhile, the synergistic interaction of microalgae and bacteria (activated sludge) has shown to be economically viable with the aid of bio-flocculation, recovering high quality and nutrient rich biomass by sedimentation under gravity (Zhu et al., 2019). Interestingly, Quijano et al. (2017) provided a good account of how the synergy of microalgae and bacteria consortia produce stable and settleable aggregates. Microalgal-bacterial aggregates (MABAs) formed contained extracellular polymeric substances (EPS) separately produced by microalgae and bacteria, which are subsequently bonded together and mediate the formation of aggregates of efficient settling characteristics, settling out by gravity, thus yielding excellent outcome of biomass harvest.

Plethora of studies have explored the pollution mitigation potential of microalgae and bacteria consortia since the realization of treatment efficiency and effectiveness. Serejo et al. (2020) reported suitable surfactant removal, and optimum removal of contaminants of emerging concern was achieved in the studies conducted by Prosenc et al. (2021) and Matamoros et al. (2016). Also, high removal efficiency of pathogenic microorganism under the influence of CO₂ addition was reported by Ruas et al. (2017), the influence of temperature on treatment performance was explored by Ji et al. (2021), influence of hydraulic retention

time and nitrogen/carbon ration on treatment efficiency was conducted by Toledo-Cervantes et al. (2019), influence of solid retention time on the formation of microalgal-bacteria aggregate (Buitron and Coronado-Apodaca, 2022) and the defence mechanism of microalgal-bacterial granules to tetracycline (Wang et al., 2020). Notably, these studies were prominently undertaken in temperate regions and conducted largely under controlled environment in photobioreactors and High-Rate Algae Pond. Moreso, there is a dearth of information on the inoculation ratio of microalgae and activated sludge for wastewater treatment. The conducted by Su et al. (2012) in Germany with defined inoculum ratio was carried out in a controlled environment.

Although, temperature, pH and nutrients richness are also essential factors for microalgae growth, but the critical role light intensity plays in photosynthetic reaction; the process that generates organic compound required for biomass growth (Prasad et al., 2021; Dolganyuk et al., 2020; Coêlho et al., 2019), justifies the predetermined amount of light energy for cultivation. Because of the externality imposed by a controlled treatment environment, it becomes imperative to explore a more energy efficient method of treatment; evaluating the performance of Microalgae Activated Sludge (MAS) under a natural outdoor condition with solar radiation as the source of light.

Solar radiation as the source of light intensity for photobioreactor treatment systems will be applicable in countries with tropical climatic conditions. This study therefore assessed the performance of the synergy of microalgae and activated sludge consortia under a natural outdoor working condition and light/dark cycle in a tropical environment in a small scale, to be used later in a pilot flat panel photobioreactor, and determine the experimental operations under outdoor conditions. Assessment will be based on rate of nutrients removal and recovery, settleability, removal potential of *Escherichia coli* and Total Coliforms, hydraulic retention time and the best inoculation ratio. This study is expected to contribute to the application of microalgae and activated sludge consortia in tropical regions for the treatment of municipal wastewater.

1.1 Justification of Study

The Sub-Saharan Africa and Latin America and the Caribbean still grapple with the menace of municipal wastewater treatment. According to the joint monitoring program report of the World Health Organization and UNICEF, 2019 over 50% of wastewater are

discharged into the environment without any form of treatment in many parts of the regions. This poses a potential risk of increased public health burden that may arise from the consumption of water contaminated with pathogens and environmental pollution.

Moreover, the widely practised conventional treatment technology lacks the capacity to stem the tide due to apparent disadvantages of high operation cost resulting from high energy consumption, resultant sludge production, inadequate removal and loss of nutrient. Although, the microalgal-based treatment technologies have been explored and proven to be an efficient alternative owing to comparatively low cost of operation, excellent nutrients removal and cycling, which conform with the principle of circular economy (Teixeira et al., 2021), but there exists limited information on the performance of photobioreactors under a natural tropical condition.

Many of the microalgae-based studies so far conducted were carried out in the temperate region and under a controlled environment. Also, these studies have been widely demonstrated at the laboratory scale (Min et al., 2022), which presents a question around the feasibility in real-life operability.

Based on the established robust potential benefits of microalgae-based treatment options, it is essential to optimise the treatment process and advance the recovery of accompanying high-value products, which are proven means of providing economically viable resources in an environmentally friendly manner, that supports the resilience of overburdening natural systems (World Bank, 2020; Chrispim et al., 2020).

Therefore, it becomes imperative to evaluate the performance of microalgae-activated sludge synergy for the treatment of municipal wastewater in pilot flat panel photobioreactors, under a natural tropical outdoor environment for treatment and recovery nutrient rich biomass which could be used as inputs for industrial processes. This would offer the benefits of providing information on the experimental design for adoption in tropical regions and as well bridge the wide treatment gap, especially in the Sub-Saharan Africa, which has posed serious environmental and public health threats. This also translates into recovery of bioeconomy potential products such as nitrogen and phosphorus nutrients, which can alleviate the burden of food scarcity and inflation linked to dwindled fertilizer provision from supplying countries due to prevailing social crisis.

1.2 References

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2 OBJECTIVES

The general objective of this study was to investigate the best inoculum proportion of microalgal-activated sludge consortia in a flat panel photobioreactor for the treatment of anaerobic municipal wastewater, in terms of nutrients removal, pathogens removal, biomass productivity and excellent settling characteristics.

2.1 Specific Objectives

The specific objectives are outlined as follows:

- To determine the best inoculum proportion for microalgae and activated sludge consortia in a lab-scale under outdoor condition;
- To investigate settleability of biofloc (microalgal-bacteria aggregate);
- To investigate the *Escherichia coli* and Total coliforms removal potential of microalgal-activated sludge consortia.
- To determine the best hydraulic retention time for removal and recovery of nutrients under outdoor condition.

2.2 Hypotheses

- Increased biomass settleability will be achieved in photobioreactor with Microalgae-bacteria consortia.
- Increased nutrients recovery rate will be in photobioreactor with Microalgae-bacteria consortia.
- Photobioreactors with Microalgae-bacteria consortia will produce excellent sedimentation.

3 LITERATURE REVIEW

3.1 Introduction

The increasing rise of human population and the accompanying behavioural change of civilization are translating to increase in wastewater generation, owing to increased anthropogenic activities bordering on agricultural practices, industrialization and urbanization, in pursuit of meeting insatiable needs (Gonçalves, et al., 2017). The resultant wastewater has been shown to contain high levels of personal care products, pesticides, industrial chemicals (Rizzo et al., 2018) and organic loads and nutrients, particularly ammonia, which are known deleterious potential when discharged into the environment without treatment (EPA, 2007 ; Rizzo et al., 2015).

According to Gonçalves et al. (2017), discharge of untreated wastewater into the environment will lead to the enrichment of nitrogen and phosphorus of the receiving water bodies, and cause a condition that is known as eutrophication. This manifests varying consequences ranging from depletion of dissolved oxygen, death of aqua lives to degradation of the affected water body, and by extension poses threat to public health.

Although, microalgae consortium and microalgal and activated sludge consortia have been adjudged efficient alternative to the conventional treatment methods, resulting from their metabolic flexibility and ensuing excellent treatment outcomes of wastewater from different sources (Wollmann et al., 2019), it is essential to examine the various secondary treatment technologies and varying microalgal treatment operating conditions, in relation to different environmental conditions.

3.2 Wastewater Characteristics

According to Sperling (2007), the characteristic nature of wastewater is defined by the presence of some pollutants (suspended solids, biodegradable organic matter, nitrogen

and phosphorus nutrients, pathogenic organisms, pesticides, detergents and metals), which varies across different locations, based on the degrees of activities of the contributing settlements. In addition to the aforementioned, pollutants of emerging concerns pharmaceutical and personal care products are also commonly found in wastewater (Chen et al., 2022; Shi et al., 2021).

Primarily, nutrients are unavoidable constituents of wastewater and are present in form of nitrogen and phosphorus. Nitrogen occurs in organic or inorganic forms, ammonium ($\text{NH}_4^+\text{-N}$), nitrate ($\text{NO}_3^-\text{-N}$), and nitrite ($\text{NO}_2^-\text{-N}$), and the ammonium and nitrate being the most assimilable forms for microorganisms, while phosphorus is present in inorganic form, orthophosphate ($\text{PO}_4^{3-}\text{-P}$) (Li et al., 2022; Wang et al., 2021; Chen et al., 2022).

The advent of circular economy initiative and the implementation in wastewater treatment process, using microalgae biomass recovery for example, has heralded quite a number of demonstrations to obtain the bioeconomic compounds present in wastewater such as nutrients, carbohydrate, protein etc., which also presents the prospect of environmental sustainability (Gao et al., 2021; Vargas-Estrada et al., 2021). Based on this, the looming exhaustion of phosphate rock, due to the rising demand for phosphorus, being the consequent effect of growing human population may be averted, exploring this environmentally friendly alternative of phosphorus recovery (Liu et al., 2021).

Moreso, the dissolved organic matter in wastewater also serves as a source of energy for microalgae (Lyu et al., 2021). Biochemical oxygen demand, chemical oxygen demand and total organic carbon make up the organic matter component of wastewater, which is a prominent part of wastewater properties (Kim et al., 2022; Gao et al., 2020). Also, the recovery of micronutrient metals such as Copper (Cu), Cadmium (Cd), Manganese (Mn), Chromium (Cr), Iron (Fe), Nickel (Ni) and Zinc (Zn) in wastewater by microalgae is essential as they are easily replenished into the soil by using microalgae biomass as biofertilizer (Huang et al., 2022; Liu et al., 2021). In particular, the significant role of Zn in agriculture has been established (Muhammad et al., 2022), however, the bioremediating and nutrients recovery potential of microalgae can be affected by the coexistence of high Zn concentration and nutrients in culture broth (Oliveira et al., 2023). Therefore, it is essential to study the compromising influence of Zn on microalgae growth and the overall treatment performance of wastewater.

3.3 Anaerobic Sewage Treatment Systems

According to de Oliveira et al. (2021), anaerobic treatment technology is one of the most common treatment process of wastewater. Particularly, this treatment method is used in tropical regions due to the favourable temperature. It is not suitable for the temperate regions owing to poor performance of anaerobic microorganisms at less than 20°C and the lack of feasibility to be heated (Chernicharo, 2007). Up-flow Anaerobic Sludge Blanket (UASB) is popular among the anaerobic treatment methods (Chernicharo, 2007) and this may be attributable to the conservation potential of the nutrients by converting to stable form that is recoverable through post-treatment technology.

Up-flow Anaerobic Sludge Blanket (UASB) reactor has a favourable temperature condition that makes it suitable for use in warm-climate condition (tropics), especially for the treatment of high organic loads wastewater from slaughter house, agricultural industry, food and beverage industry, urban settlements (Chernicharo, 2007). Hellal et al., (2021) reiterated that the operating temperature and condition of UASB makes it readily adoptable to the treatment of wastewater by tropical and sub-tropical countries and coupled with its cost effectiveness in terms of low operating cost and energy consumption, and biogas production which could serve as fuel.

Furthermore, while it was shown that UASB can effectively treat biodegrade organic and ensure significant removal of BOD and COD, the need for a post-treatment with an additional treatment technology that is capable of removing nutrients (Nitrogen and Phosphorus), heavy metals, microorganisms and emerging contaminants, in compliance with stipulated standard was established (Hellal et al., 2021). In a bid to upscale the operation of UASB, de Oliveira et al. (2021) integrated micro aeration into the operation mechanism of UASB and increased organic matter removal and methane production was recorded due to enhanced hydrolysis of the organic that was attributed to the micro-aeration. However, this did not overrule the need for post-treatment owing to the presence of high concentration of nutrients in the effluent, which can serve as alternative source of fertilizer when recovered.

On the other hand, aside the failings of aerated treatment technology highlighted above and the possibility of ecosystem chemical imbalance from inorganic chemical use, nutrients (phosphorus and nitrogen) are consumed during the treatment process, particularly phosphorus (Amenorfenyo et al., 2019). This presents a negative implication by reducing

the recovery rate of nutrients, loss of high-valued products and unsuitable for the circular economy model that prioritizes recovery of high-valued products and environmental sustainability. Removal of high amount of organic matter from wastewater by UASB than other anaerobic treatment technology is ascribed to high concentration of microorganisms, but it is restrained by the accumulation of Volatile Fatty Acids (VFA) (Hu et al., 2019).

3.4 Post-Treatment using Microalgae-Based Technologies

Up-flow Anaerobic Sludge Blanket (UASB) reactor has demonstrated capacity as an excellent alternative to conventional Septic tank wastewater treatment due to significant removal of organic matter and suspended solids (Vassalle et al., 2020). It was further shown that effluent from UASB reactor is not suitable for direct discharge into the environment due to high concentration of nutrients (nitrogen and phosphorus), microorganisms and emerging pollutants, which makes post-treatment compelling, aimed at improving the quality of effluent.

The poor removal of nutrients from anaerobic treatment (UASB) was linked to hydrolysis of organic nitrogen and phosphorus to ammonia and phosphate respectively, which are not removable from the system and as such become more concentrated (Khan et al., 2011). In this light, microalgae-based treatment technologies (open or closed system) were incorporated for the treatment of secondary effluent as they have shown to adequately remove the remaining contaminants. While this generates effluent that is fit for disposal into the environment without a damaging potential, it has paved room for circular economy through recovery of nutrients, carbohydrate, protein, lipids, cellulose carotenoids and reusability of generated water (Liu and Hong, 2021 ; Nagarajan et al., 2020; Speranza et al., 2022).

Microalgae based technologies operate as a complete system, breaking down organic matter and consuming nutrients by photodegradation, bioadsorption and biodegradation, with self-replenishing gaseous exchange between photosynthetic microalgae and heterotrophic bacteria, generating oxygen and carbon dioxide respectively (Vassalle et al., 2020; Wiczorek et al., 2015).

3.5 Microalgae Cultivation

A wide variety of culture methods is used to produce microalgae. Microalgae can be produced indoor where illumination, temperature, nutrient level, contamination are subject to control or under less predictable outdoor condition, and open culture systems like the High-Rate Algae Pond (HRAP) or tanks or closed culture systems like tubes, flasks, flat panels, or carboys (FAO, 1996). Juxtaposition of open and close photobioreactors showed that the former is prone to conditions that may undermine treatment efficiency when deployed under open roof and, which are (1) contamination with undesired microorganisms (2) dilution during heavy down pour (3) heat loss and evaporation and subsequent concentration of the effluent (Sepúlveda-Muñoz et al., 2020).

Therefore, closed photobioreactor, by virtue of the design, enjoys a relatively high temperature condition that influence biomass growth and removal of organic matter and nutrients in return (Sirohi et al. 2022). However, temperature for optimal growth and performance in photobioreactors is specie specific, but it is worth mentioning that the microalgae have the capability to adapt after a lag phase, which also varies, before commencing treatment operation (Sirohi et al., 2022; Sepúlveda-Muñoz et al., 2020).

The pH condition of both open and closed photobioreactors tends towards alkaline due to the disintegration of volatile fatty acid and consumption of carbon dioxide, and it is relatively higher in the open system resulting from readily diffusion of carbon dioxide into the atmosphere (Sepúlveda-Muñoz et al., 2020). Hence, the limitation of carbon source and pH variability can interfere with photosynthesis and subsequently limit microalgae cell growth in both photobioreactors and with CO₂ supplementation (Fernández et al., 2012). However, a closed photobioreactor of microalgae culture could accumulated dissolved oxygen when may inhibit microalgae growth at > 250% air saturation (Solimeno et al., 2017).

Notably, penetration of light either from solar or artificial source, usually fluorescent lamps, may be minimized by microalgae fouling in closed photobioreactors by sticking to light receiving surfaces (Sirohi et al., 2022). For efficiency in terms of biomass production, gas exchange and energy transfer, wastewater treatment and nutrients removal, high light receiving surface to volume ratio is essential (Lam et al., 2019). The cubical shape and depth of flat-panel reduces the extent of light penetration, covering a short distance, but the translucent nature on both sides of the flat-panel compensate for it (Sirobi et al., 2022).

Considering the inhibitory impact of shadow by reducing the intensity of light, spacing range of 0.2 m to 0.4 m between flat-panels was recommended (Huang et al., 2019). Importantly, culturing microalgae in high-rate wastewater as substrate will lead to high yield of biomass and nutrients recovery, which will produce high value-added end product, usable for soil conditioning (Silver et al., 2019).

3.6 Environmental Factors Affecting Microalgae Cultivation

The environmental factors are critical to microalgae growth process and biomass productivity, hence the need for a review of the factors in order to identify means to optimise the influence of the factors on microalgae growth.

3.6.1 *Light Intensity*

Light, as the main source of energy input, plays a crucial role in microalgae cultivation, and has been shown to have a proportional impact on microalgae growth rate (Chowdury et al., 2020). In other words, increasing light intensity influences photosynthetic activities of microalgae which invariably increases microalgae productivity (Richmond, 2004; Chisti, 2007; Rai & Gupta, 2017). However, there are certain levels of intensity, although different across specie line, microalgae growth may be inhibited as due to exceedance of the maximum saturation level (Vejrazka, 2011; Vejrazka, 2012; Acien Fernandez et al., 2013). Also, light saturation requirement of a particular microalgae strain is location specific, considering the influence of environmental ambience on irradiance level, and as such location specific evaluation should be carried out for actual level of light requirement (Chowdury et al., 2020).

Besides the influencing impact of microalgae culturing ambience, exposure quality in terms of wavelength and source of light and quantity in terms of light intensity and duration of photoperiod are contributory to influencing microalgae growth rate (Schulze et al., 2014). The light source can either be natural or artificial and the photosynthetically active radiation usable for microalgae growth ranged from 400 to 700 nm (Scott et al., 2010). The artificial light from light emitting diode (LED) and fluorescent lamp sources have been shown to enhance microalgae biomass production (Chowdury et al., 2020).

From previous explorative studies of light intensity' influence of microalgae growth, the optimum intensity that fostered microalgae growth ranged from 26 to 400 $\mu\text{mol photon m}^2 \text{ s}^{-1}$, with only a few species such as *Dunaliella salina*, displaying the trait of

photoprotection mechanism for surviving under light intensity of $1000 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ (Maltsev et al., 2021). Additionally, lipid synthesis could be induced by varying the intensity of light in relation to the light requirement of the microalgae specie in use, with exposure to a range of 60 to $700 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ (Maltsev et al., 2021). It is essential to determine the optimum working light intensity for the microalgae specie to be used for treatment purpose, especially when dealing with a mix community of species, to achieve a desirable outcome.

3.6.2 Temperature

Alongside light intensity, temperature is considered an environmental factor critical to microalgae growth. Usually, light irradiation confers a level of temperature increment within a photobioreactor, which jointly influence biomass productivity (Deb et al., 2017). However, this can as well cause overheating of in the reactor which may pose a negative implication to microalgae cells, although more preponderant in large scale reactors, as the lab-scale is usually compensated by convection with ambient air (Chowdury et al., 2020). As a form of solution, dark-coloured netted shading, water sprinkling, partial water immersion of reactors and heat exchange have been recommended in places with low air humidity to whittle the possibility of reactor overheating (Becker, 1994; Torzillo, 1997; Watananbe et al., 2011). However, the tendency of net shading to over protect and reduce light irradiance was noted, which was implicated for reducing biomass productivity. On the other hand, water sprinkling guarantees good biomass productivity but imposes cost implications on microalgae cultivation (Chowdury et al., 2020).

Notably, microalgae grow significantly at the optimum temperature which ranged between 20 to 35°C , while the mesophilic species can thrive at 40°C (Chowdury et al., 2020). Therefore, this implies that microalgae culture may underperform below the optimum temperature range while the microalgae cells may be lysed at high temperature, leading to cell death (Sanchez et al., 2008; Bernard & Fernandez, 2012).

3.6.3 Nutrients

Nutrients are vital to microalgae growth and must be available is sufficient amount in culture medium for biomass productivity. Nutrients are presented largely in form of carbon, nitrogen and phosphorus, and metals such as iron, zinc etc., in wastewater culture medium with the proportion of carbon being the highest, as the microalgae biomass has

shown to contain about 50% of carbon (Richmond, 2004; Acien Fernandez et al., 2013). Culture medium can be enriched with inorganic compounds such as CO₂ and bicarbonates (HCO₃⁻) or organic compounds such as sugars, acids and alcohols as sources of carbon (Acien Fernandez et al., 2013) while nitrogen is presented in form of proteins, nucleic acids, vitamins as assimilable as NH₄⁺-N, NO₃⁻-N, NO₂⁻-N (Perez-Garcia et al., 2011), and phosphorus is presented in polyphosphate, pyrophosphate, orthophosphate, and metaphosphate (Markou et al., 2014) for microalgae uptake and growth. The proportionate ration of these nutrients, particularly nitrogen and phosphorus for optimum microalgae growth differs for different microalgae species (Silva et al., 2015), and must be evaluated to achieve the desire productivity outcome.

3.6.4 Value of pH

The influence of pH on microalgae culture is manifest in microalgae growth, dissolution of minerals and carbon dioxide (CO₂) (Qui et al., 2017), and ranged from 6.0 to 8.0 on the average for microalgae (Rai & Gupta, 2017; Zhu, 2015), although some species have been noted to thrive at high alkaline pH of 10.0 and some at acidic pH level of 3.0 (Lu et al., 2014). pH tolerance level differs for different microalgae species and respective pH level in cultures is determined by buffering capacity, CO₂ dissolution, temperature and metabolic activities (Singh & Dhar, 2011). Also, the pH of microalgae culture increases during the fixation of CO₂ by photosynthesis due to the release of hydroxide ion (OH⁻) and subsequent accumulation (Richmond, 2004).

3.7 Bioeconomy and Circular Economy

This is an emerging concept that is engineered towards the recovery of materials of economic value from products intended for disposal, and as such advancing the re-use and recycle prevention protocols, and by extension, reducing over reliance and exploitation on finite natural capital, which in turns promotes environmental sustainability. Puyol et al. (2017) provided a detailed account of the valued end products, among of which are, nutrients (phosphorus and nitrogen), biofuels (methane, diesel) and trace metals, that are derivable from waste products, with corresponding treatment technologies, and microalgae-based treatment technology was ranked among the most prominent and growing options.

The nitrogen and phosphorus content of municipal wastewater, excluding waste from animal production, can potentially cater for 20% of global nitrogen and phosphorus needs,

which really represents a sustainable alternative source of supplies. This was sustained in Kok et al. (2018) indicating the potential of 20% global agricultural needs from the nutrients load of urban wastewater and a humongous proportion, 90%, derivable from animal manure. Harnessing this resource from wastewater would prevent over stretching the finite natural phosphate rock which is projected to go out of supply in 50 to 400 years, and readily provide alternative means of fertilizer, particularly for the Sub-Saharan Africa, and other regions with known deficiency soil fertility (Kok et al., 2018).

A possibility of 100% recovery of nutrients (phosphorus and nitrogen) and significant proportions of other high-valued products (carbon, potassium, copper, cobalt, zinc, iron, polysaturated acids, pigments, carotenoids etc.) from wastewater was shown through microalgae assimilation (Fernandes et al., 2022). This further gives credence to microalgae-based treatment technology. Also in the same vein, Silva et al (2019) corroborated the high-rate removal potential of micronutrients and macronutrients by microalgae, making it suitable for agricultural soil conditioning and improved soil productivity, and by extension this will reduce the environmental impacts that accompany extraction of phosphate rock.

3.8 Microalgae Biomass Recovery

Microalgae treatment efficiency is detailed in extant literatures (Molinuevo-Salces et al., 2019; Quijano et al., 2017). The drawback of microalgae axenically for wastewater treatment is linked to poor biomass settling which is due to negatively charged of the micro cells and preventing coalescing into sizes large enough to settle under gravity (Yan et al., 2022; Molinuevo-Salces et al., 2019). To overcome this challenge, filtration, centrifugation and chemical coagulation are deployed industrially (Fasaei et al., 2018), while nature-based coagulants are being explored to reduce cytotoxicity (Teixeira et al., 2022; Silva et al., 2020), but these methods come with the implications of high energy consumption and high cost of implementation (equipment, maintenance, operation, personnel).

However, the symbiosis of microalgae and activated sludge bacteria presents energy efficient biomass recovery option, which is also accompanied with improved purification and biomass production (Yan et al., 2022). Chemical exchanges were shown to be parts of the symbiotic interaction, which have been very beneficial to both microalgae and bacteria, and ultimately producing good quality outcome (Yan et al., 2022). Indole-3-acetic acid

(IAA) and N-acyl-homoserine lactones (AHLs) chemicals produced by bacteria spike microalgae growth and gaseous exchange alternatively improve the activities of microalgae and bacteria, with increased biomass production (Chan et al., 2022; Barreiro-Vescovo et al., 2021).

Aside the increased biomass production induced by the symbiotic interaction of microalgae and bacteria, which is put at 10 to 70%, substantial amount of extracellular polymeric substances (EPS) is secreted by bacteria and cause the formation of aggregates (bioflocs) that are large enough to settle out of gravity, with efficient settling rate (95–99%) and high biomass yield (Zhang et al., 2021), providing and the economic value of the high-valued products (biodiesel, feed, fertilizer, and cosmetics). The EPS contains proteins and polysaccharides and are produced under reflex conditions of environmental stress and also without a precursor, which are produced in significant concentration to initiate the agglomeration of cells and subsequent settling (Wang et al., 2022).

3.9 Biomass and Agro-Industrial Applications

In general microalgae biomass has a number of potential uses in various industries such as biofuel production, bioplastics, cosmetics, animal feeds, etc. Wastewaters of various origins often contain abundant energy and nutrient sources that can be recovered and utilized within a circular bioeconomy standpoint, although, not usable in cosmetics and animal feeds due to probable presence of toxic substances. Microalgae cultivation can aid the recovery and reuse of recyclable nutrients from secondary sources, contributing significantly to global sustainable demands (Nagarajan et al., 2020). Microalgae serve as a rich source of lipids, proteins, carbohydrates, pigments, nutrients, cellulose, carotenoids which are synthetically processed and converted into the various agro-industrial uses (Fig. 1). These resources embedded in the cells of the microalgae biomass, and as a result, they are dewatered and dried after sedimentation, to obtain the bioproducts for their respective uses (Speranza et al., 2022).

3.9.1 Biofuel Production

Microalgae is a third-generation source of biofuels (Noraini et al., 2014) and it guarantees clean energy production and promotes environmental conservation, with significant reduction in greenhouse gas emission and reduced air pollution problems (Gavilanes et al., 2017; Peng et al., 2020). The first-generation (edible materials like corn,

palm, soybeans, beets, sugarcane, etc.) and second-generation (lignocellulosic material like jatropha and grass) sources were remarkably disadvantaged for low lipid yields, low growth rate, low CO₂ mitigation potential, low tolerance to wastewater, large expanse of land (Noraini et al., 2014; Bennion et al., 2015; Bharadwaj et al., 2020; Goh et al. 2019; Yin et al. 2020).

Based on the potential of microalgae biomass, they are used as feedstock for the production of various biofuels such as biodiesel, bioethanol, biohydrogen and biogas. Although, the best production route for optimum yield is yet to be defined, anaerobic digestion has shown to be the least complex, but microalgae strain is an essential factor that determines productivity (González-Fernández et al. 2012; Ayala-Parra et al. 2017). Moreover, the pH and temperature of substrate in anaerobic digestion process influence the production of biogas (methane) (Chu and Phang 2019). The alkaline range produces highly pure biogas due to the solubility of CO₂ from biogas as dissolved carbonate.

High microalgae biomass with high content of lipid within the cells increases the oil production strength of microalgae (Chisti, 2007). By dry weight, the lipid contents of microalgae biomass ranged between 2% and 41% (Gouveia et al. 2008), and they can be transformed into fatty acids methyl esters which serves as a feedstock for the production of biodiesel (Soares et al. 2019). However, if the proportion of free fatty acids > 5%, although often constitutes about 85% of lipid content depending on microalgae strain and condition of cultivation (Chen et al. 2012; Krohn et al. 2011), triggers saponification and renders it unsuitable for biodiesel (Huang et al. 2010). As a solution, two steps treatment processes are advised, first is to convert of free fatty acids to fatty acids methyl esters with methanol and followed with transesterification (Dong et al., 2013).

Another biofuel derivative is bioethanol. Bioethanol production from microalgae biomass is specie specific, and the species that produce high level of carbohydrates as reserve polymers instead of lipids are considered the ideal candidates (Andrade et al., 2020), as they can be extracted to produce fermentable sugars, to obtain bioethanol (Mussatto et al. 2010). Some of the highlighted species that stand out for this purpose are *Sargassum fulvellum*, *Hizikia fusiformis*, *Chlorella vulgaris*, *Chlorella sorokiniana*, etc. (Andrade et al., 2020).

3.9.2 **Biofertilizer**

Microalgae biomass as a source of fertilizer serves an environmentally friendly alternative for producing healthy foods in a sustainable manner by the release of nutrients for plant uptake from biomass, and potentially reducing the implication of sole reliance on chemical fertilizer (Singh et al., 2011). Additionally, Rachidi et al. (2020) showed that the metabolites such as phytohormones, polysaccharides, amino acids from green microalgae and cyanobacteria provides the benefits of high agricultural yields with nutritional values. Also, micronutrients such as zinc, copper manganese, iron from green microalgae and cyanobacteria enrich food crops, and are crucial for the nourishment of humans and animals (Renuka et al., 2018).

The potential of microalgae-based biofertilizer for high agricultural yields have been demonstrated for different food crops. High maize yields with high nutritional content were reported by Dineshkumar et al. (2019), celery plant (Rashad et al., 2019) and rice cultivation (Chittapun et al., 2018). Specifically, biomass *Acutodesmus dimorphus* microalgae specie have the potential of increased productivity in Roma tomato plant (Garcia-Gonzalez & Sommerfeld, 2016). In a comparative study, utilizing microalgae-based biofertilizer in crop production has yielded results that are similar to those of commercial treatment (Coppens et al., 2016).

According to Renuka et al. (2016), reliance on agrochemicals can be reduced by 75% with the use of microalgae-based biofertilizer. If the production of microalgae biomass is fully optimised for increased productivity, for application as biofertilizer, it will reduce excessive use of agrochemical, promotes sustainable crop production and minimise associated environmental degradation (Abideen et al., 2022).

3.9.3 **Bioplastics**

The popularity of bioplastics as an alternative to the synthetic plastic from fossil fuel is due to environmental pollution, high carbon footprint, non-degradability, toxicity ascribed to the later (Cinar et al., 2020). As at 2019, production of bioplastics stood at 1% of 2.11 MMt global production (European Bioplastics 2019; Payne et al. 2019). This is still at the infancy stage with a huge gap, and more work is needed to be done to match production demand from the fast-rising human population. It is important to decrease the production of

fossil fuel-based plastics as this will contribute towards ensuring environmental sustainability.

Among the bioplastic sources, microalgae appear to be the most preferred because of the advantages of less space for cultivation, which does not compete for land with food crop, growing on wastewater, high lipid yields (Rahman & Miller, 2017; Hempel et al., 2011). Moreso, microalgae-based bioplastics showed higher mechanical strength (stress and elongation) when compared with the conventional synthetic plastics (Abdo & Ali, 2019). However, the performance of different strains of microalgae for use as feed for bioplastics may differ in terms of biomass yields and accumulation of required high-valued products such as lipids, carbohydrate and polyhydroxybutrate (Abdo & Ali, 2019).

The feasibility of producing bioplastic from microalgae biomass, using the high-valued products derivatives such as lipids, cellulose, carbohydrate, protein polyhydroxybutrate was established in (Khoo et al., 2019). However, most of the studies so far conducted were at the lab scale, and therefore, the challenge of the economic viability bordering on large scale cultivation, harvesting, extraction and pre-treatment needs to be explored for the establishment of a promising pathway. In the light of this, microalgae biofilm technology, which is still at the infancy stage, is proposed owing to the advantages of low cost of production, low consumption of energy for harvesting and increased productivity over cultivation ponds (Chong et al., 2022).

3.9.4 *Cosmetics*

There are several bioactive compounds from microalgae and cyanobacteria biomass such as Polysaccharides, Methanolic extracts of exopolysaccharides, Chrysolaminarin, Sulfated polysaccharides, β -1,3-Glucan, β -carotenes, Asthaxanthin, Phycocyanobilin phycoerythrobilin, β -Cryptoxanthin, Chlorophyll, etc., that have potential uses for cosmetic products (Mourelle et al., 2017). This is due to their pharmacological activities capable of protecting and improving the appearance of human skin and hair (Mourelle et al., 2015; De Luca et al., 2021). In more specific terms, these bioactive compounds have both protective and healing potentials, correcting dermatitis (dandruff), preventing inflammation, external aging, and are moisturizing (Kim et al., 2008), but wastewater cultivated microalgae may not be applicable for this purpose.

Additionally, polysaccharides obtained from microalgae and cyanobacteria have potential for free-radical collection and immunostimulation, which make them a good fit for use in skincare cosmetics (Koller et al., 2014), and offers the benefits to stand in gap, protecting the skin against oxidative stress and damaging free-radicals due to compromising susceptibility of the skin's natural antioxidant system to external factors like UV from sunlight (Mourelle et al., 2017). Also, carotenoids, one of the bioactive substances from microalgae biomass have shown numerous skincare potentials such as photoprotection, antiallergen, antioxidant, etc. (Heydarizadeh et al., 2013; Rumin et al., 2020).

The interlinking beneficial effects of skin care products and formulations containing these bioactive compounds, which cut across pharmaceuticals and cosmetics, with medical and drug-like properties has led to the creation of cosmeceuticals in skincare parlance (De Luca et al., 2021). This reflects the appreciation of the benefits in the advancement of the cosmetics industry and as shown by Sathasivam et al. (2018), the naturally formulated cosmetic products enjoy more preference by consumers. This portends a fast-rising global demand and more research on how to optimize production and match demand should be conducted.

3.9.5 *Animal feeds*

Microalgae biomass have demonstrated the potential to sustainably provide livestock, poultry and aquaculture animals (Viegas et al., 2021). Microalgae grown in municipal wastewater can as well be used for this purpose considering the richness in protein, carbohydrates, lipids, carotenoids and microelements (Saadaoui et al., 2021). However, the biomass to be used for this purpose must be sure to be free from toxins and pathogens (Ahmad et al., 2022). Moreso, biomass harvested with the aid of chemical coagulants may not be suitable for this purpose, as the presence of chemical residue reduces the quality of biomass, rendering it toxic (Silver et al., 2020).

The nutritional benefits of microalgae biomass are evident in the sound physiological functioning, optimum growth and good health, and the overall product quality of animals fed with a blend of small portion of commercial feed with *Chlorella*, *Scenedesmus* and *Arthrospira* microalgae (Kotrbaček et al., 2015). This provides a basis for the increasing interest in microalgae feed-supplements for animals in countries like United State of America, India, Japan, China, Korea, Thailand, United Kingdom, Philippines, with the Asian countries taking the lead (Saadaoui et al., 2021). As a matter of fact, 30% of microalgae

biomass production is currently being used as animal feed-supplement globally (Becker, 2007; Dineshababu et al., 2019).

Notably, accruable nutritional benefits from microalgae biomass differ across specie line, due to differences in growth conditions such as illumination, temperature, and location, etc. (Saadaoui et al., 2021). The understanding of the cultivation condition will aid the optimization growth process, to achieve a sustainable animal feed alternative that is economically viable.

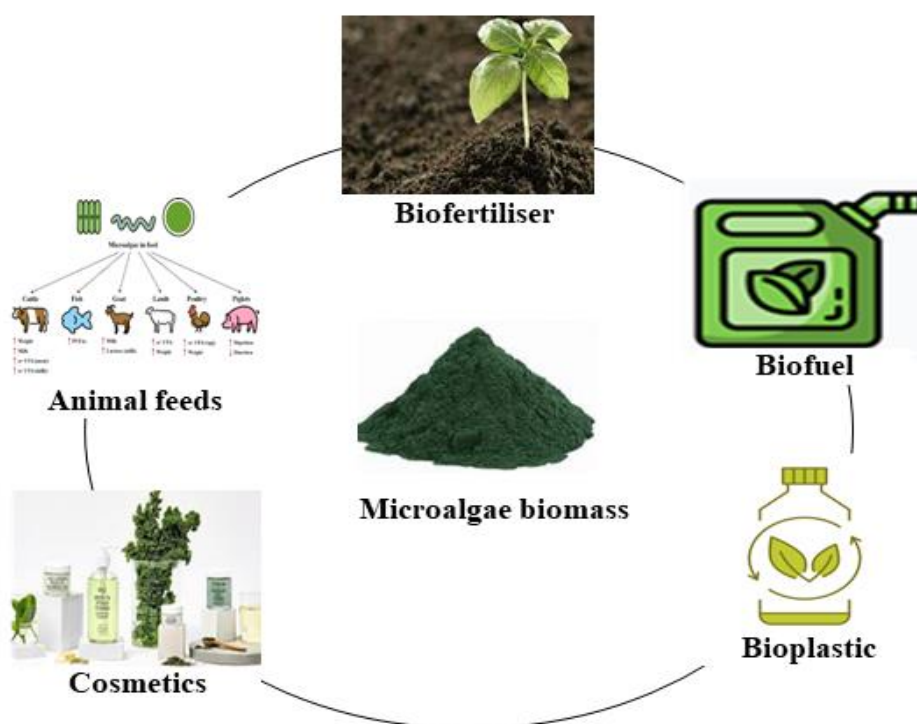


Figure 1: Agro-industrial application potential of microalgae biomass from wastewater

3.10 References

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4 LABORATORY SCALE BATCH EXPERIMENT

Exploration of microalgae-activated sludge (MAS) growth performance in lab-scale Photobioreactor at outdoor Environmental condition for wastewater biotreatment with respect to biocircular economy¹

Abstract

The problem of untreated and inadequately treated wastewater still persists globally. This has contributed to the growing environmental pollution and undermine the bioeconomy potential of the materials existing in wastewater, particularly in low-income region. Therefore, the present study was focused and evaluated the performance of native microalgae-activated sludge (MAS) growth for tertiary treatment of anaerobically digested wastewater from an upflow anaerobic sludge blanket (UASB) in an outdoor lab-scale photobioreactors (2 L). Seven different conditions with distinct inoculum proportions (Conditions: 1 to 3 is MAS; 4 to 6 is microalgae controls; 7 is without inoculum) were operated in batch mode reactor in triplicate for 5-days of hydraulic retention time (HRT) at 11.5:12.5 Photo-hours. Condition 1 (0.10gL⁻¹: microalgae and 0.20gL⁻¹: activated-sludge), with lowest MAS proportion showed the best outcome with cell density productivity (2.03 x 10⁷ cells.mL⁻¹) along with biomass of 0.13 g TSS.L⁻¹.d⁻¹ and the total dissolved phosphorus uptake (85.07%), total dissolved nitrogen uptake (66.14%), logarithmic removal (Log-Re) of bacterial pathogens (water quality indicators), which showed Log-Re (3.3 for total coliforms (137E+02 CFU.100 mL⁻¹) and 4.7 for *Escherichia coli* (0.00E+00 CFU.100 mL⁻¹)). The results revealed that the optimum remediation performance and recovery of potential nutrients may advancing the science of circular bioeconomy.

Keywords: Microalgae-Activated Sludge; Nutrients recovery; Pollutant Removal

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4.1 Introduction

The management of wastewater (WW) is still a major challenge confronting the whole world. This could be attributed to volumetric increase of untreated WW from municipal, industrial and agricultural activities which constitutes a significant threat to the quality of water resources and as well public health (Aradhana and Kumar, 2015; Zhu *et al.*, 2019; Pompei *et al.*, 2022).

Globally, approximately 80% of WW is released into the environment without adequate treatment (Oviedo *et al.*, 2022). Moreso, in the Sub-Saharan and Latin America and the Caribbean, over 50% of the wastewater generated between the years 2000 and 2017 was discharged into the environment without prior treatment (WHO and UNICEF, 2019). Recently, survey showed the world population has doubled in the last 48 years with African countries having the fastest growth, and the continent's population size was projected double by 2050 (Routley, 2022). This raises more concern on the capacity of these countries to treat their WW and invariably necessitates the need for more explorative studies to be conducted on affordable and effective alternative treatment options, to bridge existential treatment gap.

This has led to the discharge of wide range of pollutants (nitrate, phosphate, microplastics, metals, persistent organic pollutants) and pathogenic microorganisms (bacteria, protozoa, viruses) that are usually present in WW in concentrations above the critical safe limits, and are capable of causing environmental pollution, ecosystem obliteration and public health epidemics (Akpore & Muchie, 2011; UNESCO, 2017).

The disproportionate discharge of untreated WW into the environment, particularly municipal WW, may be attributed to the high operational cost implication of the conventional activated sludge treatment process, which is presented in form of energy reliance for aeration and sludge production (Kehrein *et al.*, 2020; Wang *et al.*, 2020; Molinuevo-Salces *et al.*, 2019). This poses serious threat to the achievement of the United Nations Sustainable Development Goal (SDG) 6 due to associated polluting potential and consequent reduction in water quality meant for domestic and other purposes.

However, the advent of microalgae-based treatment systems has heralded a promising pathway to overcoming the shortcomings of conventional treatment method. Aside the relatively low cost of operation and the attenuation of offending nutrients loads (causing alga bloom), the resultant effluent and biomass may be good feedstock for

symbiotic industrial production processes, with accompanying benefits of environmental sustainability (Boom-Cárcamo & Peñabaena-Niebles, 2022).

Microalgae entrap ammonium, nitrates and phosphorus (P), lipids and pigments, protein in their cellular compositions which are repurposed into high value products (biofertilizer, biofuel and animal feeds) through nutrient cycling and valorisation (Liu & Hong, 2021; Serejo *et al.* 2020; Dourou *et al.*, 2020; Molinuevo-Salces *et al.*, 2019; Quijano *et al.*, 2017; Matamoros *et al.*, 2016; Wang *et al.*, 2010).

Moreover, bio-flocculation potential of the synergistic interaction of microalgae and activated sludge (MAS) bacteria have been explored to optimize microalgae biomass recovery and improve the science of circular economy in a sustainable manner (Zhu *et al.*, 2019; Yan *et al.*, 2022; Zhang *et al.*, 2021; Wang *et al.*, 2022).

Over the years, the cultivation of MAS for growing and recovering biomass, and treating WW and is usually undertaken in a controlled condition, with the bulk of the studies in temperate regions, particularly for light intensity, in photobioreactors. Although, temperature, pH and nutrients richness are also essential factors for light intensity, the intensity plays a critical role in photosynthetic reaction; the process that generates organic compound required for biomass growth (Prasad *et al.*, 2021; Dolganyuk *et al.*, 2020; Coêlho *et al.*, 2019). This may have influenced the cultivation pattern to be carried out under a pre-determined condition for optimum performance. However, replication of such experimental design in Low Income Countries (LICs) may be challenging due to the problem around power supply and additional cost externality imposed by a controlled treatment environment.

Therefore, it becomes imperative to explore a more energy efficient method of treatment; evaluating the performance of MAS under a natural outdoor condition with solar radiation as the source of light. The solar radiation as the source of light intensity for photobioreactor treatment systems for the treatment of WW will be applicable in countries with tropical climatic conditions, especially in the Sub-Sahara Africa with high proportion of untreated sanitary wastewater discharged into the environment (WHO & UNICEF, 2019; Bankole *et al.*, 2023).

Therefore, the aim and novelty of this study was to evaluate the performance of different MAS proportions in laboratory scale photobioreactors (n=21) under natural outdoor conditions and using a real anaerobically digested effluent with respect to (a) biomass

growth, (b) nutrient removal, (c) removal of total coliforms and *Escherichia coli*, and (d) suitability of the treated effluent for discharge to the environment.

4.2 Materials and Methods

4.2.1 *Anaerobically Digested Wastewater*

Anaerobically digested municipal wastewater obtained from an upflow anaerobic sludge blanket (UASB) reactor was used as substrate for culturing microalgae-activated sludge MAS. The wastewater was collected from a Wastewater Treatment Plant (WWTP), located in Bauru City, São Paulo, Brazil. The activated sludge was obtained from a WWTP located at Botucatu city, São Paulo, Brazil. The UASB reactor sits on 6,683.14 square meters space (22°16'01.0" S, 49°05'12.05" W), and serve a population of between 30,000 and 50,000 inhabitants, at a flow rate of 78L s⁻¹. The operating temperature ranged between 17 - 25 °C at 8 hours hydraulic retention time (HRT). The wastewater from the UASB was collected in the morning at 9:00 AM and taken to the laboratory at the Universidade Estadual Paulista (UNESP). The wastewater was kept stationary for 1 hour under room condition to allow sedimentation of particles before it was transferred to the photobioreactors (PBRs). The characteristics of the anaerobically digested municipal wastewater are presented in Table 1.

Table 1: Mean concentration and standard deviation of substrate wastewater characteristics, being that: TDN = total dissolved nitrogen; TDP = total dissolved phosphorus; DO = dissolved oxygen; TSS = total suspended solids.

Parameter	Unit	Average value
pH	-	6.99±0.04
TDN	(mg N L ⁻¹)	58.24±0.58
TDP	(mg PO ₄ ³⁻ L ⁻¹)	6.00±0.16
DO	(mg O ₂ L ⁻¹)	0.59±0.25
TSS	(g TSS L ⁻¹)	0.07±0.05
Total Alkalinity	(mg CaCO ₃ L ⁻¹)	339.30±19.50
Volatile Fatty Acid	(mg L ⁻¹)	61.26±8.45

n=3

4.2.2 *Experimental Set-up*

Seven conditions were evaluated in triplicate in a laboratory scale, Duran® bottles photobioreactors (n = 21). Each Duran® bottle has 2.2 L capacity (27 cm - length and 13 cm -diameter), and was operated with 2 L.

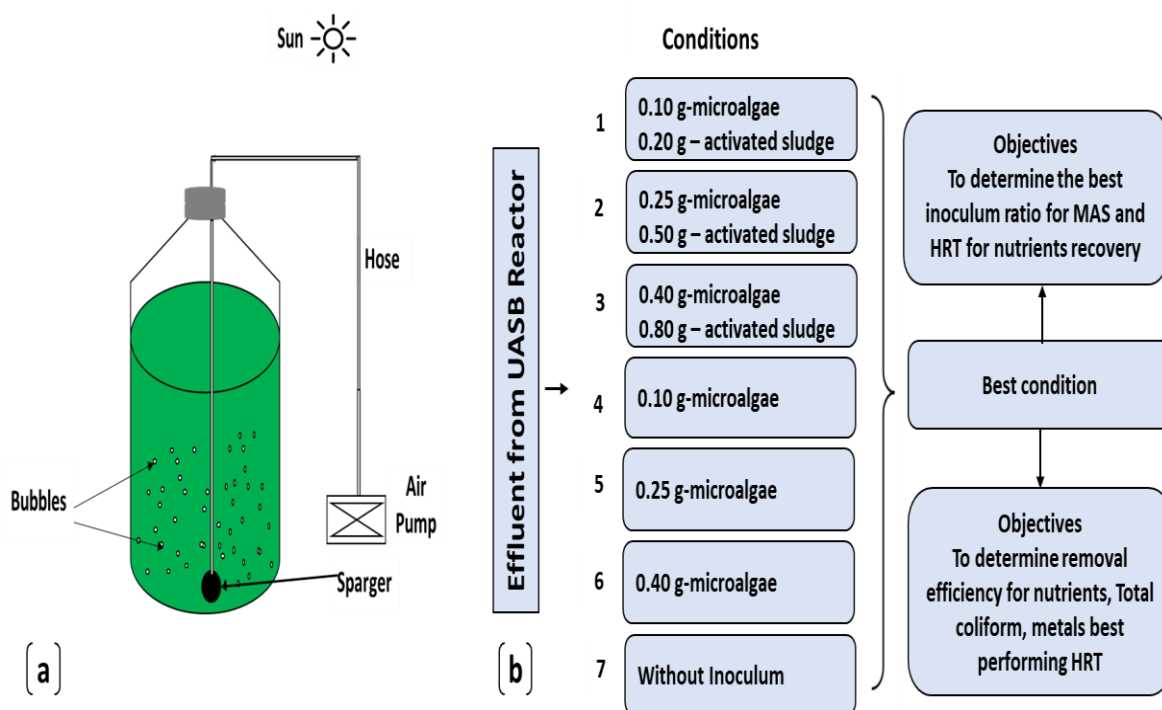


Figure 2: (a) Schematic diagram of Duran® bottle photobioreactor set-up (b) Composition of inoculum ratio for each condition evaluated.

Table 2 : Composition of operational volume per condition

	Conditions	Microalgae	Activated sludge	Wastewater
1	Microalgae - 0.10 g L ⁻¹ + Activated sludge - 0.20 g L ⁻¹	0.118 L	0.016 L	1.866 L
2	Microalgae - 0.25 g L ⁻¹ + Activated sludge - 0.50 g L ⁻¹	0.296 L	0.039 L	1.665 L
3	Microalgae - 0.40 g L ⁻¹ + Activated sludge - 0.80 g L ⁻¹	0.473 L	0.062 L	1.465 L
4	Microalgae - 0.10 g L ⁻¹	0.118 L	---	1.882 L
5	Microalgae - 0.25 g L ⁻¹	0.296 L	---	1.704 L
6	Microalgae - 0.40 g L ⁻¹	0.473 L	---	1.527 L
7	No inoculum	---	---	2.000 L

Concentrations of microalgae (1.69 g L⁻¹) and activated sludge (25.76 g L⁻¹) inoculum

4.2.3 *Microorganisms and Culture Conditions*

Phycoprospecting of the native microalgae used as inoculum was carried out by cultivating in the anaerobically digested municipal wastewater used for the treatment. Maturity level of microalgae cells (>1) was determined with the ratio of optical density (OD) at 680 nm and 540 nm (OD_{680nm}/OD_{540nm}). Gravimetric analysis using 30 mL of culture sample was used to determine the concentration of microalgae and activated sludge. Prior to this, microalgae culture and activated sludge were allowed to wait for one hour to sediment (Su *et al.*, 2012). Therefore, the obtained concentrations of microalgae and activated sludge were 1.69 g L⁻¹ and 25.76 g L⁻¹, respectively. Then, the proportion of each condition was determined according to Table 2.

The seven conditions (n = 21; twenty-one lab-scale PBRs) were inoculated with their respective proportions based on the calculation of equation 1, and operated in batch mode under a natural outdoor condition. Light intensity from the average photosynthetically active radiation (PAR) provided by the sun and the ambient temperature were $719.05 \pm 64.22 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 20.4 ± 2.0 °C, respectively. The ambient temperature and light intensity readings

were obtained from the automatic weather forecast located at latitude 22°21'27.6" and longitude 49°01'40.8, through the Meteorology center at UNESP in Bauru (IPMet). Obtained data were recorded at 5 minutes intervals, and the solar radiation data were converted from R_s ($W\ m^{-2}$) to PAR ($\mu mol\ m^{-2}\ s^{-1}$) using the conversion factor of 2.02, according to Reis & Ribeiro (2020).

4.2.4 Analytical Methods

The growth pattern in cultures were evaluated daily with optical density 680 nm wavelength (OD_{680nm}), (NANOCOLOR UV/VIS II Spectrophotometer), and TSS (2540 D, APHA, 2017) and cell count ($cell\ mL^{-1}$) (Optical microscope (Leica microsystems, D. 35578, Wetzlar, Germany. Monitoring of nutrients removal (4500-P, 8507 HACH, 8039 HACH, 8039 HACH- Spectrophotometer NANOCOLOR UV/VIS II, APHA, 2017) was carried out daily ($n=105$). Total Alkalinity (Titration potentiometric with acid sulfuric) was evaluated at the beginning, middle and the end of the experiment ($n = 42$), while the removal of Total coliforms and *E. coli* were determined at the beginning and end of the experiment using Chromocult Coliform Agar (Pour Plate 9215B, Merck KGaA, Germany, APHA, 2017) in triplicate ($n=42$). The pH (4500-HB, APHA, 2017) was monitored daily ($n=105$).

The nutrients analysed were the dissolved nutrients (nitrate, nitrite, TKN and phosphate), which were determined according to APHA 2017. The samples were first filtered through 1.2 μm glass fiber pore size membrane filter and then through 0.45 μm pore size cellulose acetate membrane, to determine the dissolved nutrients. Additionally, total suspended solids gravimetric analysis for productivity (dry weight) represented the biomass in culture medium.

4.2.5 Statistical Analysis

The results of laboratory analysis were entered into Excel Spread sheet, and SPSS Version 22.0 was employed for the statistical analysis. Data were analysed for mean and analysis of variance (ANOVA). The t-test was used to determine variations in the concentrations of parameters analysed, with 5% level of significance. The relationship between microalgal-activated sludge (MAS) bacteria inoculum and nutrients recovery were performed using Pearson's correlation analysis.

Geometric mean was used to calculate the average total coliforms and *E. coli* bacteria population to avoid probable distortion from the varying triplicate values (Sperling, 2005), using equation 2.

$$\text{Geometric mean} = (x_1 \cdot x_2 \cdot x_3 \cdot x_4 \dots x_n)^{1/n} \text{----- Equation 1}$$

Where n = number of sample replicate.

4.3 Results and Discussion

4.3.1 Influence of Temperature and Light Intensity Measurement

The experiment was conducted during the winter weather season in Brasil for a period of five days (27th June to 2nd July, 2022). The daily average temperature values ranged from 18.0±4.0 to 20±3.0 °C, and the average light intensity ranged from 694.29 to 841.31 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 3).

Microalgae culture was grown in a moderate temperature range as evidenced in the temperature range. While it is worth noting that different microalgae species require different temperature values for photosynthetic efficiency and optimal growth (Masojídek *et al.*, 2022; Dolganyuk *et al.*, 2020), the recorded temperature values are considered suitable for most microalgae species (Dolganyuk *et al.*, 2020; Carneiro *et al.*, 2020). This is significantly essential as temperature and light intensity values play crucial roles in the growth and productivity of microalgae culture for an outdoor photobioreactor treatment, in addition to nutrient adequacy and appropriate mixing (Coronado-reyes *et al.*, 2022; Chowdury *et al.*, 2020).

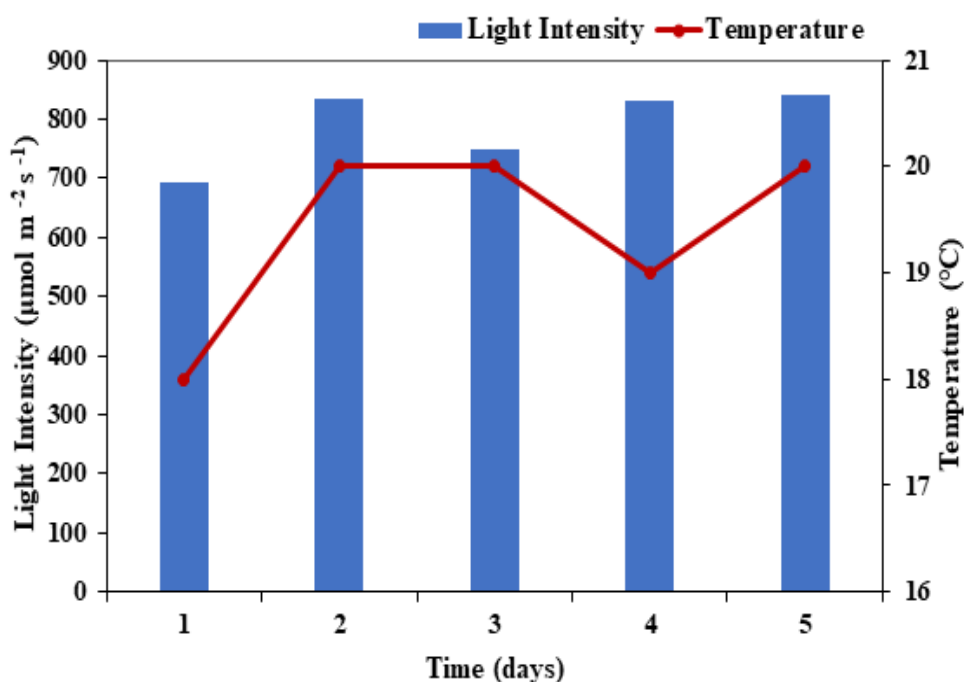


Figure 3: Daily average temperature and light intensity during the experiment

The average value of light intensity monitored ($719.05 \pm 65.93 \mu\text{mol m}^{-2} \text{s}^{-1}$) is considered adequate for microalgae growth and photosynthesis (Kliphuis *et al.*, 2010; Carneiro *et al.*, 2020). Moreover, the potential for increased growth with higher light intensity was demonstrated after a stepwise increase from 600 to $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ significantly yielded a percentage growth difference (~92%) in Kliphuis *et al.* (2010). The positive growth response was possibly due to the penetrative strength of the new light intensity, which restrained biomass shadowiness that may have limited the photosynthetic response of microalgae at $600 \mu\text{mol m}^{-2} \text{s}^{-1}$. This suggests the possibility of increased biomass productivity in summer weather season in an outdoor treatment situation.

While increasing light intensity presents the potential of high microalgae biomass growth from the resulting photosynthetic efficiency, the microalgae culture is also liable to photo-inhibition beyond the maximum coping level, with consequent decreased growth and productivity. Although, net-shading as a form of solution provides a cost-effective mitigation, the type and appropriateness of the net has been identified critical to its attenuation potential, as it can adversely affect microalgae growth by over protection and light inhibition (Casagli *et al.*, 2021).

Examining the average weather condition, the Sub-Saharan Africa with average temperature range 25 - 40°C (varies across the region, equatorial to semi-arid), presents a year-round warm temperature requirement for cost-effective outdoor microalgae-based treatment of wastewater. This suggests the provisioning of the minimum receivable amount of light intensity from solar radiation necessary for efficient photosynthesis of microalgae cultures and biomass growth without light-imposed cost externality, while ensuring adequate wastewater treatment and subsequent resource recovery.

Likewise, although having a degree of moderate temperature because of tropical and temperate climatic conditions, as in Latin America, with temperature range 10 to 30 °C (Valenti *et al.*, 2021), presents a similar potential for growing microalgae culture outdoor. Overall, the temperature range is largely within the near-optimal for high growth rate of microalgae which is capable of producing and accumulating useful biomaterials of commercial interests (Masojídek *et al.*, 2022; Elisabeth *et al.*, 2021; Nogueira *et al.*, 2015).

By implication, the extant wastewater treatment gap and associated health and environmental problems within these regions can be reduced to the barest minimum by

leveraging on microalgae-based treatment. This also presents the opportunity for the uptake or advancement of wastewater management with admiration to bioeconomy, and the economic potential it entails for industrial utilization of the ensuing nutrient rich microalgae biomass.

4.3.2 Operational and Environmental Conditions

Values of pH for conditions 1 to 7 varied significantly ($p = 0.046$) from 8.49 ± 0.91 to 9.55 ± 1.25 . The lowest and highest pH values observed (8.49 and 9.55) were in Condition 7 (without inoculum) and 6 (highest proportion of microalgae inoculum), respectively (Table 3). The recorded values are similar to the pH values reported for uncontrolled pH conditions in the study that evaluated the effect of different pH levels on microalgae cultivation and biomass recovery operated in batch mode for 30 days (Yu *et al.*, 2022).

An increase of pH was observed on the second day of the experiment, and remained in the alkaline zone till the end of the batch experiment. Moreover, pH values for MAS cultures differed in this order $1 > 2 > 3$ (Table 3), although not significantly ($p > 0.5$). The activated sludge proportions of the inoculum could be responsible for the varying degree of microalgae activities in the cultures, as they were markedly darkest in appearance.

Previous studies have attributed pH increase in cultures to reduction of bicarbonates in form of CO_2 fixation in the cultures, enabled by photosynthesis (Li *et al.*, 2015; Ronda *et al.*, 2012). In the light of this, the concentration of alkalinity is invariably reduced, as total alkalinity is consumed in this form by Microalgae (Li *et al.*, 2015; Ronda *et al.*, 2012) or directly as HCO_3^- (Paquette *et al.*, 2022; Rodero *et al.*, 2018) as a source of inorganic carbon for growth.

From Table 3, deviation reflects alkalinity reduction across the seven conditions (not statistically significant, $p = 0.064$), which is an indication of consumption of inorganic carbon in form of carbonate and bicarbonate present in the effluent (Slompo *et al.*, 2020).

Table 3: Mean and standard deviation of pH, total alkalinity, biomass productivity and cell density at optical density 680nm, and nutrients removal efficiencies found in the seven conditions

Condition	pH	Total Alkalinity (mg CaCO₃ L⁻¹)	Biomass productivity (g TSS L⁻¹ d⁻¹)	Cell density (OD_{680nm})	TDP removal (%)	TDN removal (%)
1	9.41±1.25	214.83±100.32	0.130±0.01	1.069±0.12	85.07±1.04	66.14±6.43
2	9.38±1.16	240.30±76.31	0.060±0.02	0.349±0.04	40.65±10.33	16.42±5.81
3	8.85±0.91	235.27±91.56	0.040±0.03	0.167±0.12	-43.65±15.73	-62.92±10.04
4	9.43±1.28	250.30±66.05	0.120±0.01	0.959±0.33	83.94±10.39	43.22±13.59
5	9.48±1.20	246.20±69.61	0.130±0.04	1.125±0.02	85.48±8.80	58.30±7.00
6	9.55±1.18	213.93±80.86	0.132±0.02	1.103±0.02	92.28±1.19	60.57±5.09
7	8.49±0.91	316.37±19.86	0.048±0.01	0.474±0.23	76.20±3.35	32.34±5.64

n = 105; Last day of experiment

TDP and TDN indicate the dissolved proportion Phosphorus-P and Nitrogen-N, respectively, in the solution as samples were filtered through 0.45 µm membrane pore size prior to determination

4.3.3 **Effect of Inoculum on Biomass Productivity**

The biomass productivity followed the order recorded $6 > 5 = 1 > 4 > 2 > 7 > 3$ in the different conditions tested (Table 3), which corresponded with the proportion of inoculation for the controls (native microalgae inoculated). Conversely, condition 1 with the lowest inoculum proportion had the highest productivity for the MAS.

A significant variation in biomass productivity ($p = 0.000$) was noted across the seven conditions. Biomass productivity correspondingly increased with increasing proportion of microalgae inoculum for controls, and conversely for the MAS conditions (Table 3). The markedly dark appearance of conditions 2 and 3 may have interfered with productivity rate in the reactors. This could be attributed to the proportions of activated sludge inoculated.

Based on this, the proportion of MAS inoculum for wastewater treatment is critical to the translucence of cultures in reactors and light energy receivable from solar radiation, which is required for replication and growth (Khan *et al.*, 2018; Lage *et al.*, 2021). While the adequacy of light intensity is integral to microalage biomass productivity (Metsoviti *et al.*, 2019; Nzayisenga *et al.*, 2020), the appearance of cultures in photobioreactors seems to play an essential role in the amount of irradiance (light received) and energy available.

This imposes the implication of limited growth rate, microalgae cells die-off from shadowiness and turbidity, and consequently reduces the remediation activities of microalgae in WW, due to limited consumption of materials such as nutrients in WW (Chowdury *et al.*, 2020). Notably, howbeit not high, condition 7 (without inoculum) showed potential for remediation in 5 days HRT as evidenced by the production of microalgae cells. An increase in the HRT would translate to steady rise in microalgae cell growth under the intensity of light and subsequent remediation.

The results of productivity in conditions 1, 4, 5 and 6 were similar to the values in the existing literatures for microalgae cultivations conducted outdoor (Fuentes *et al.*, 2020; Mazzelli *et al.*, 2020). Fuentes *et al.* (2020) reported $0.14 \text{ g TSS L}^{-1} \text{ d}^{-1}$ productivity in a large-scale cultivation of *Coccomyxa onubensis* with a synthetic commercial NPK fertilizer solution that was operated for 30 days outdoor.

Also, Mazzelli *et al.* (2020) reported a productivity range of 0.09 to $0.19 \text{ g TSS L}^{-1} \text{ d}^{-1}$ in the pilot study that cultivated *Tetradismus obliquus* and *Graesiella emersonii* in local

tap water for 9 months. In our study, native microalgae and activate sludge (MAS) in different proportions (seven condition) were cultivated in municipal wastewater in laboratory scale photobioreactors, and evaluated the best MAS condition, and biomass productivity $0.130 \text{ g TSS L}^{-1} \text{ d}^{-1}$, $0.120 \text{ g TSS L}^{-1} \text{ d}^{-1}$, $0.130 \text{ g TSS L}^{-1} \text{ d}^{-1}$, and $0.132 \text{ g TSS L}^{-1} \text{ d}^{-1}$ were obtained for conditions 1, 4, 5 and 6.

The pattern of biomass growth in terms of optical density 680 nm wavelength ($\text{OD}_{680\text{nm}}$) followed the same trend noted for productivity (Table 3). Also, the level of growth varied significantly ($p = 0.000$) across the seven conditions. The potential of $\text{OD}_{680\text{nm}}$ for microalgae cell growth estimation of mixed cultures (microalgae and bacteria) was demonstrated on the premise of high correlation between a model predicted and estimated growth trends at $\text{OD}_{680\text{nm}}$ (Casagli *et al.*, 2021).

As previously noted above, conditions 2 and 3 (high MAS inoculum proportions) had limited microalgae cell growth, as evidenced by low cell growth estimates at $\text{OD}_{680\text{nm}}$. A potential explanation for this is the die-off of microalgae and subsequent cell ruptures due to photoinhibition from shadowiness (Lage *et al.*, 2021). Also, the fragments of intracellular pigments released into the cultures from ruptured cells were considered minute and undetected at 440 nm and 680 nm wavelengths, as established in the sonication monitoring study of three microalgae species (*M. aeruginosa*, *C. pyrenoidosa*, and *C. reinhardtii*) using optical density estimate of microalgal suspension, intracellular pigments and protein, and cell counting (Tan *et al.*, 2018). Moreover, Slompo *et al.* (2020) has shown that biomass measurement at $\text{OD}_{682\text{nm}}$ is commonly indicative of biomass growth and not cell rupture.

The photoautotrophic growth of microalgae cells in the cultures was shown in Fig. 4. No lag phase was observed except for condition 3 (culture with highest MAS inoculum proportion), with slight decline on the second day of the experiment, suggesting die-off of some microalgae cells. Microalgae cell density varied significantly across the conditions ($p = 0.000$) and ranged between 2.53×10^6 and $2.03 \times 10^7 \text{ cells mL}^{-1}$, 6.02×10^6 x $1.47 \times 10^7 \text{ cells mL}^{-1}$, 1.07×10^7 and $1.83 \times 10^7 \text{ cells mL}^{-1}$, 2.25×10^6 and $1.50 \times 10^7 \text{ cells mL}^{-1}$, 6.30×10^6 and $2.13 \times 10^7 \text{ cells mL}^{-1}$, 1.06×10^7 and $2.32 \times 10^7 \text{ cells mL}^{-1}$, 5.11×10^5 and $4.66 \times 10^6 \text{ cells mL}^{-1}$, respectively for conditions 1 to 7, respectively. Among the cultures, conditions 2 and 3 decreased in cell density on day 4 of the experiment as evidenced on the growth trend (Fig. 4), suggesting growth of microalgae cells are limited by irradiance. This could be

attributed to shadowiness and poor translucence caused by turbidity, and linked to high MAS proportions of inoculums (Lage *et al.*, 2021).

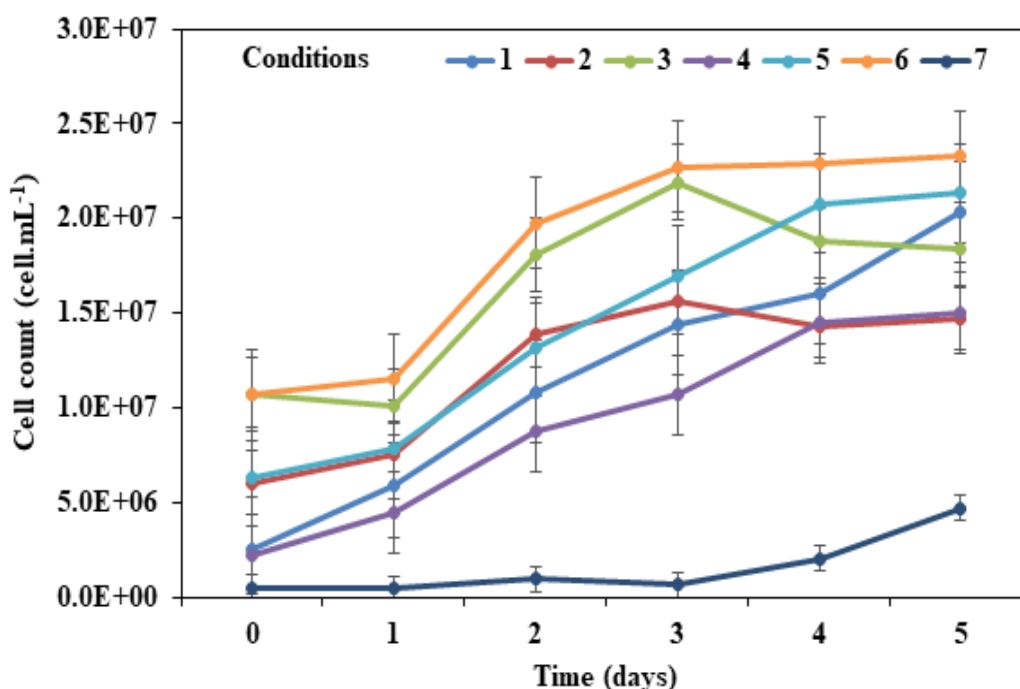


Figure 4: Average cell density expressed in cell mL⁻¹ for the seven experimental conditions

For conditions 1, 4, 5 and 6 as indicated above, microalgae cell growths were similar to the maximum mixotrophic (1.887×10^7 cells mL⁻¹) and heterotrophic (1.473×10^7 cells mL⁻¹) cell production of native microalgae (predominantly *Scenedesmus*) that was cultivated in municipal wastewater in pilot-scale photobioreactor outdoor (Pacheco *et al.*, 2019). Also, condition 7 (without inoculum) showed the same growth potential for microalgae. Similarly, in the study conducted by Vella *et al.* (2019), where *T. weissflogii* was cultivated outdoor all year round in batches in seawater enriched medium (f/2 medium) in transparent poly-methyl methacrylate photobioreactor, the obtained cell growth results in (June - August) aligned with the results obtained for conditions 1, 4, 5 and 6 of this present study. However, a definitive comparison is challenging because of limited information and varying cultivation techniques employed. Therefore, it becomes imperative to explore the commercial feasibility of this experiment in a pilot scale for improved outcome and competitiveness.

4.3.4 Effect of Inoculum on Nutrients Removal

The end of the experiment was premised on the attainment of $\geq 90\%$ TDP removal (mg P-PO₄ L⁻¹). It is a known fact that microalgae cells consume the P and N in culture

medium for growth and production of biomass. The total dissolved phosphorus (TDP) and total dissolved nitrogen (TDN) (nitrate, nitrite and Total Kjeldahl nitrogen) in the nutrient rich medium (anaerobically digested municipal wastewater from UASB) were used up by MAS cells.

The TDN removal varied significantly across the conditions ($p = 0.000$) while TDP removal was not statistically significant ($p = 0.194$). The highest TDP removal was achieved in condition 6 (92.28%), while condition 1 had the highest TDN removal (66.14%) (Table 3). Comparatively, condition 1 performed fairly within the same range (85.07%) with condition 6 (92.30%), which had the highest total phosphorus uptake. It is noteworthy to mention that condition 1 had the lowest proportion of MAS inoculum.

Notably, condition 3, which contained the highest proportion of MAS inoculum, had the TDP and TDN concentrations increased to 43.51% and 62.92%, respectively. This could be as a result of rupture cells that received little or no sun energy due to the viscosity and opaqueness of the reactors, thus injecting nutrients within the cells into the solution. This is a reflection of the decrease of cell density on the second day of the experiment (Fig. 4). The TDP concentration of microalgae inoculum used was ($11.61 \text{ mg P-PO}_4 \text{ L}^{-1}$), explained by weeks of cultivation and concentrations. Based on this, increased nutrient load of culture would result from cell ruptures, which was the case of condition 3. Meanwhile, the greenery appearance of other reactors (conditions 1, 4, 5, 6 and 7) at the end of the experiment showed the potential for increased nutrients removal beyond day 5.

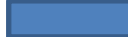
Furthermore, it has been shown that the cultures with highest biomass productivity and better growth condition demonstrate best nutrients removal performance during cultivation (Dębowski *et al.*, 2018; Yu *et al.*, 2019; Murray *et al.*, 2010; Yadav *et al.*, 2019). Based on the results obtained in nutrients removal, observable increases in nutrients uptake were recorded for conditions 1, 4, 5, 6, and 7, while the cultures with highest MAS proportions (conditions 2 and 3), had a decline in nutrients uptake. This explains the strong negative relationship between optical density ($\text{OD}_{680\text{nm}}$) and P uptake, with obvious increased biomass production leading to reduced concentration of P ($r = -0.7$ for condition 1, $r = -0.8$ each for conditions 4, 5 and 6), which was the reverse for conditions 2 and 3, ($r = 0.5$, 0.7 , respectively) (Table 4).

Thus, increasing the proportions of MAS inoculum for wastewater treatment seems to interfere with the process of photosynthesis, which consequently limits microalgae cell growth and replication. These results suggest that the volume of MAS proportion to be used as inoculum for microalgae cultivation and bioremediation of wastewater constitutes an essential condition that should be factored into experimental set-up. These is in agreement with previously reported findings by Soroosh *et al.* (2022).

Table 4: Correlation between TDP removal and OD₆₈₀ of the seven conditions.

	TDP1	TDP2	TDP3	TDP4	TDP5	TDP6	TDP7
TDP1	1						
TDP2	0.6	1					
TDP3	-0.3	0.5	1				
TDP4	0.9	0.3	-0.7	1			
TDP5	0.9	0.3	-0.7	1.0	1		
TDP6	0.9	0.3	-0.6	1.0	1.0	1	
TDP7	0.9	0.4	-0.6	1.0	1.0	1.0	1
OD1	-0.7	0.0	0.7	-0.7	-0.7	-0.8	-0.6
OD2	-0.2	0.5	0.8	-0.4	-0.4	-0.4	-0.3
OD3	0.0	0.6	0.7	-0.2	-0.1	-0.2	0.0
OD4	-0.8	-0.1	0.7	-0.8	-0.8	-0.8	-0.7
OD5	-0.8	-0.2	0.6	-0.8	-0.8	-0.8	-0.7
OD6	-0.8	-0.2	0.6	-0.8	-0.8	-0.8	-0.7
OD7	-0.5	-0.4	0.1	-0.4	-0.4	-0.4	-0.4

TDP = Total dissolved phosphorus, OD = Optical density

Negative  Neutral  Positive 

Moreover, high pH of culture medium was shown to contribute to P removal, as phosphate precipitates at high pH level (Soroosh *et al.*, 2022). This may have been contributory to the removal of TDP in this study as the pH values of the best conditions were relatively > 10.0.

The obtained results clearly showed the feasibility of nutrients assimilation of microalgae-based system in an outdoor environment, when the appropriate inoculum proportion is used. This is without prejudice to other growth influencing factors such as temperature, light intensity, pH. By implication of these factors, this presents potential benefits for nutrients cycling in an efficient manner, usable as bio-fertilizers for the production of food and cash crops (Cakirsoy *et al.*, 2022; Dineshkumar *et al.*, 2017; Dineshkumar *et al.*, 2018).

4.3.5 *Assessment of Total Coliforms and Escherichia coli Removal*

The potential of MAS to remove total coliforms and *E. coli* from municipal wastewater was evaluated D0 and D5 (Table 5) and log removal (Log-Re) performance is shown in Fig. 5. Complete Log-Re was achieved for *E. coli* in all the conditions tested (1 to 7). Also, a complete Log-Re 5.2, 5.0, 5.2, was achieved for total coliforms in conditions 5, 6, and 7. Whereas in condition 4, total coliforms were reduced by 4.9 log unit. Moreover, total coliforms in condition 1 system were reduced by 3.3 log unit, while conditions 2 and 3 had 2.3 and 2.2 Log-Re for total coliforms, respectively.

Table 5: Geometric mean of total coliform and *E. coli* at D0 and D5 in the seven experimental conditions

Day 0 (D0)		Day 5 (D5)	
Total coliform (CFU 100 mL ⁻¹)	<i>Escherichia coli</i> (CFU 100 mL ⁻¹)	Total coliform (CFU 100 mL ⁻¹)	<i>Escherichia coli</i> (CFU 100 mL ⁻¹)
3.55E+05	4.58E+04	1.37E+02	0.00E+00
1.68E+05	4.03E+04	5.94E+02	0.00E+00
1.83E+05	4.33E+04	8.81E+02	0.00E+00
2.11E+05	4.87E+04	0.00E+00	0.00E+00
2.06E+05	4.03E+04	0.00E+00	0.00E+00
1.20E+05	3.04E+04	0.00E+00	0.00E+00
2.17E+05	5.32E+04	0.00E+00	0.00E+00

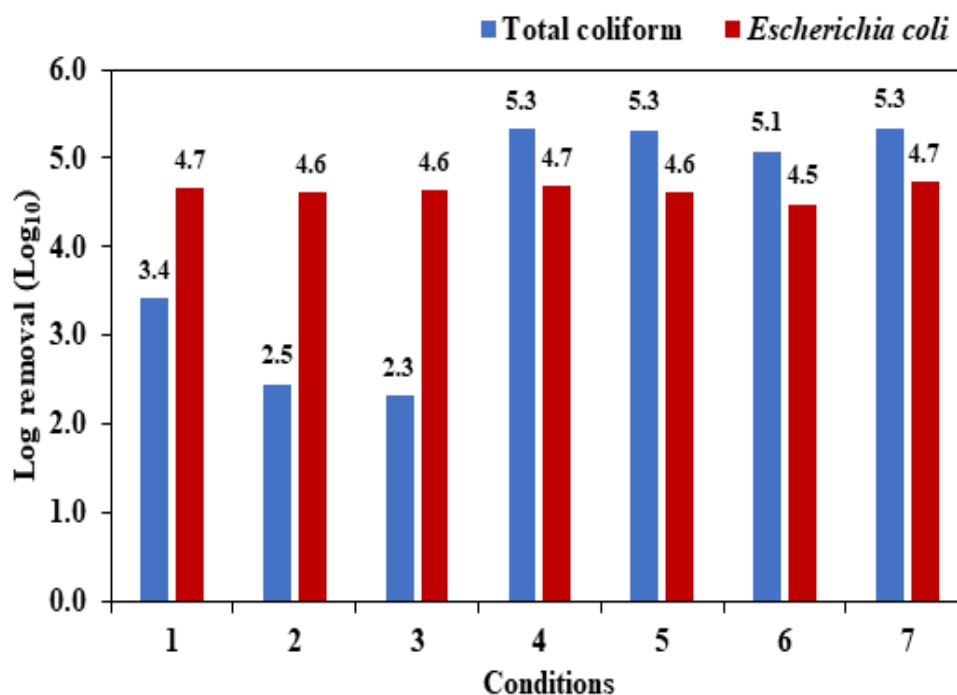


Figure 5: Log removal (Log-Re) for *E. coli* and total coliforms

While the complete Log-Re for *E. coli* cut across the seven conditions, the lowest Log-Re for total coliforms was observed in the cultures with high MAS inoculum proportions. This is probably as a result of the low microalgae biomass growth in these systems, with consequent nutrients availability and reduced secretion of antibacterial compounds (Rani et al., 2021; Delanka-Pedige et al., 2019; Ansa et al., 2012). Additionally, they asserted that operational conditions of cultures viz-a-viz increase in pH and competition with bacteria contribute to inactivation of pathogenic microorganism indicators.

For *E. coli*, high die-off rate is reported in alkaline medium (Liu et al., 2020; Fallowfield et al., 1996). This probably was responsible for the outright Log-Re in the *E. coli* population in all the cultures at D5, the last day of the experiment. The results obtained were comparable with findings from these studies. Importantly, the result showed potential of deploying resultant treated wastewater for non-potable purposes like irrigation and wetting of lawns without posing a risk of contamination by pathogenic organisms, for being in compliance with the World Health Organization standard of 10^3 MPN.100 ml⁻¹ for *E. coli* for this purpose (WHO, 2006).

4.3.6 ***Optimum Inoculum Condition***

Mean difference between the zeroth day and fifth day (D_0 and D_5) was calculated for selected parameters, using 95% confidence interval. From Table 6, the obtained significant variation ($p < 0.05$) further confirmed the previously mentioned optimum biomass productivity, percentage of nutrient removal and recovery for conditions 4, 5, 6 and particularly condition 1. The condition 1 outperformed the remaining conditions with MAS inoculum, and presents the potential for use in an outdoor treatment system for wastewater, and recovery of high-value products. This removal depicts a promising alternative means to providing economically viable resources in an environmentally friendly manner, and as well support the resilience of overburdening natural systems (World Bank, 2020; Chrispim *et al.*, 2020).

However, the typical small size of microalgae (1 - 30 μm), low concentration (0.3–5 g L^{-1}) and negatively charged, slow settling and consequent poor biomass harvesting of microalgae cells posits a serious challenge to biomass recovery. Exploring the industrial recovery methods; filtration, centrifugation and chemical coagulation imposes a high-cost implication, which is presents another challenge. From our study, condition 1 (MAS) has an overriding importance of high biomass recovery through self-settling.

The synergistic interaction of MAS aids the formation of bio-flocculation, and recovery of high quality and nutrient rich biomass by sedimentation under gravity (Zhu *et al.*, 2019). The bio-flocs are made of extracellular polymeric substances (EPS) separately produced by microalgae and bacteria, which are subsequently bonded together and mediate the formation of aggregates of efficient settling characteristics, settling out by gravity (Quijano *et al.*, 2017), thus yielding excellent outcome of biomass harvest.

Table 6: P-value of mean difference for D_0 and D_5 of selected parameters at 95% confidence interval

Parameter	Condition						
	1	2	3	4	5	6	7
Total Alkalinity	0.0177	0.0322	0.0191	0.0320	0.0119	0.0054	0.6058
Productivity	0.0033	0.1475*	0.1711	0.0015	0.0260	0.0067	0.0202
OD₆₈₀	0.0041	0.0041	0.5139	0.0384	0.0001	0.0001	0.0721
TDP	0.0010	0.0217	0.2012	0.0166	0.0077	0.0002	0.0003
T. coliform	0.006	0.1240	0.0522	0.0041	0.0002	0.0003	0.0004

*Values in red font colour are not statistically significant

4.4 Conclusion

This study showed the bioremediation with high-value added products recovery from potential MAS in cultured municipal wastewater under a natural outdoor condition. The condition with the lowest MAS proportion was observed as the best treatment outcome (high microalgae biomass yield, nutrients removal and Log-Re for total coliforms and *E. coli*). Increased proportion of MAS appeared to interfere with photosynthetic process and microalgae cells growth, which adversely affect the overall treatment performance. In general, to derive optimum benefit from the synergy of MAS inoculum for wastewater treatment and recovery of biomolecules, the deployment of appropriate inoculum proportion is germane. However, conducting this experiment in a pilot scale will alleviate concerns around the feasibility for deployment in a real-life scenario.

4.5 References

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5 PILOT SCALE FED-BATCH EXPERIMENT

Hydraulic retention time-effect on the performance of microalgae-activated (MAS) process in municipal wastewater treatment in pilot-scale photobioreactors at outdoor environmental condition

Abstract

This study evaluated the performance of microalgae-activated sludge (MAS) for treating municipal wastewater at different hydraulic retention times (HRTs, 5 and 3 days) in pilot flat panel photobioreactors that were operated in fed-batch under a natural tropical outdoor condition. Results showed that high HRT favoured the performance of MAS with respect to biomass growth, with significantly higher ($p < 0.05$) removal efficiency, 88.0, 79.0, 59.5% for total dissolved phosphorus (TDP), ammonium nitrogen ($\text{NH}_4^+\text{-N}$), chemical oxygen demand ($\text{COD}_{\text{filtered}}$), respectively at 5 days HRT. The average biomass productivity and growth rate for HRTs 5 days and 3 days are $59.41 \pm 12.31 \text{ mg TSS L}^{-1} \text{ d}^{-1}$, $0.08 \pm 0.01 \text{ d}^{-1}$ and $25.56 \pm 11.09 \text{ mg TSS L}^{-1} \text{ d}^{-1}$, $0.03 \pm 0.01 \text{ d}^{-1}$, respectively. The $\log\text{-Re} > 4.0 \text{ CFU mL}^{-1}$ and $> 3.5 \text{ CFU mL}^{-1}$ were achieved for *E. coli* and total coliforms, respectively at both HRTs, and showed the potential for agricultural use for complying with the WHO recommended standard of $10^3 \text{ MPN.100 mL}^{-1}$. Overall, results showed a promising feasibility of outdoor microalgae-based treatment of wastewater cost effectively. However, self-settling efficiency was conversely low 33.0 and 14.0% for HRTs 5 days and 3 days, respectively, and suspiciously impacted by high pH (> 10.0) on bacterial population and the dominance of non-mucilage *Cyanobium* sp. (98%).

Keywords: Bio-flocculation, Microalgae-activated sludge, *Cyanobium* sp., Hydraulic retention time

5.1 Introduction

Across the globe, the human population keeps growing continuously and this raises a concern on the self-sustaining potential of the earth owing to attributable extra-terrestrial exploration and the adverse environmental impacts of the consequent by-products. Putting in perspective, the United Nations' Department of Economic and Social Affairs in 2019 predicted 9.7 billion and 11.2 billion by 2050 and 2100, respectively for human population growth (UN DESA, 2019).

The world population has doubled in the last 48 years, with the continent of Africa having the fastest growth rates (Routley, 2022). By implication, uptake of resources from the earth and discharge of waste (solid, liquid and gas) have concurrently doubled. In effect, phosphorus utilization has been shown would attain its peak between the period 2030 and 2040 in the separate mappings that were conducted by the International Fertilizer Development Centre in 2010 and the United States Geological survey in 2012 (Desmidt et al., 2014; Neset & Cordell, 2011). In connection to this, various authors have predicted different outright depletion of the finite phosphate rock, ranging between 50 to 400 years (Kok et al., 2018), which is likely to have reduced due to continuously increasing demand. This portends hard biting global scarcity effect and food insecurity.

Moreover, increasing water demand and pollution arising from associated wastewater (WW) have been linked to water scarcity, with looming serious global water crisis by 2050 if deliberate containment and management strategies are implemented (Boretti & Rosa, 2019; Goh & Ismail, 2018). As shown from the recent review study by Oviedo et al. (2022) approximately 80% of WW is discharged into the environment without adequate treatment, which seems to accord more weight to this assertion. Also, over 50% of the WW generated from 2000 to 2017, was discharged into the environment without prior treatment (WHO and UNICEF, 2019).

While direct discharge of municipal and industrial WW into the environment without treatment has been noted for environmental pollution, ecosystem obliteration from algae bloom, dissolved oxygen depletion, death of aqua species, and public health epidemics from ingestion of pathogenic microorganism (Akpore & Muchie, 2011; UNESCO, 2017; Ruas et al., 2017; Gonçalves et al., 2017), this practice undermines the economic potential and

prevents the recovery of biomolecule materials present in WW, which can be used as feedstock for different industrial processes.

In the study conducted by Moges et al. (2020) using microalgae-based system to recover nutrients from municipal WW, the resultant nutrient rich biomass and improved effluent quality showed potential for environmental protection and preservation. Specifically, municipal WW has been shown to contain 20% potential of global phosphorus demand and 90% phosphorus demand from animal manure for agricultural purposes if harnessed (Kok et al., 2018), using low-cost decentralized technologies which is operated in close proximity to water, energy, and organic and inorganic matter (Silva et al., 2019). This has shown to be a good substitute to phosphorus fertilizer, and would invariably reduce mining of phosphate rock and accompanying environmental problems.

Since nutrients are unavoidable by-products of the pervasive inadequate treatment of municipal WW, biorefinery treatment technologies such as microalgae-based systems are explored to recover the nutrients and other biomolecules present therein (Barros et al., 2022), and their industrial applicability will incentivize maximum use of the resource materials and ensure increased value-chain production. These nutrients (ammonium, nitrates, phosphorus, lipids and pigments) are concomitantly used up and entrapped in microalgae cellular composition, forming nutrient-rich biomass (Hwang, 2016; Andrade et al., 2021), usable as biofertilizer, biofuel and animal feeds (Liu and Hong, 2021; Dourou et al., 2020; Molineuvo-Salces et al., 2019; Matamoros et al., 2016; Wang et al., 2010).

Industrially, microalgae biomass is harvested with filtration centrifugation, chemical coagulation to overcome the negatively charged imposed poor settling of microalgae cells (Fasaei et al., 2018). Alternatively, as a means to save operational cost, natural and readily available low-cost coagulants have been explored to harvest nutrient-rich microalgae biomass; over 90% recovery with tannin (Teixeira et al., 2021), high percentage recovery was achieved with *Moringa oleifera* (Silva et al., 2020), and chitosan and cationic starches (Matter et al., 2019). Also, pH induced auto-flocculation achieved 94% biomass recovery (Ummalyma et al., 2016), and present the advantage of wide applicability of resultant biomass against the backdrop of biomass contamination with heavy metals from chemical coagulants (Quijano et al., 2017). However, the financial requirements and implications for materials provision (continuous supply), equipment (purchase and maintenance) in a large-scale real-life scenario may rob-off on the sustainability of the process.

To reduce operational cost and improve the sustainability of treatment process, the synergistic interaction of microalgae and activated sludge (MAS) was explored, harnessing the bio-flocculation potential to recover nutrient-rich biomass by sedimentation under gravity (Zhu et al., 2019; Quijano et al., 2017). However, the plethora of studies so far conducted in this regard were carried out under controlled experimental environment largely within the temperate region using photobioreactors and high-rate algae pond (Serejo et al., 2020; Prosenc et al., 2021; Ruas et al., 2017; Ji et al., 2021; Toledo-Cervantes et al., 2019; Buitron & Coronado-Apodaca et al., 2022; Wang et al., 2020; Su et al., 2012).

Following this, the performance of MAS was evaluated under a natural outdoor condition and photoperiod at a lab-scale, to delineate the behavior and best MAS inoculum proportion, for exploration in a pilot flat-panel photobioreactor. Three proportions of MAS inoculums and corresponding controls were tested to determine the best MAS inoculum based on biomass productivity and contaminants removal. Considering the limitations in lab-scale experiment, bordering on operational feasibility and acceptability for commercial utilization, the predetermined MAS inoculum was evaluated in a pilot-scale photobioreactor (50 L) using five- and three-days hydraulic retention times (HRTs), to evaluate treatment effectiveness and resource removal and recovery potential under a natural tropical outdoor condition and photoperiod. This offers the benefits of cost-saving from cost externality tied to treatment in a controlled environment, and enhances the sustainability of the process.

Therefore, the purpose of this experiment was to explore the effect of HRT on the performance of predetermined MAS inoculum in a pilot flat panel photobioreactor, in treating municipal WW in terms of biomass production, and removal of phosphorus, nitrogen, including log-Re for *Escherichia coli* and Total coliforms under natural tropical outdoor condition and photoperiod. This study is anticipated to enhance the implementation of the MAS process for treating municipal wastewater in tropical low-income regions, and also promote the adoption of biocircular economy practices.

5.2 Materials and Methods

5.2.1 *Anaerobically Digested Municipal Wastewater*

The anaerobically digested municipal WW used as culture broth was obtained from an upflow anaerobic sludge blanket (UASB) reactor, Wastewater Treatment Plant (WWTP) located in the district of Bauru City, São Paulo, Brazil. The UASB reactor sits on 6,683.14

squares meters space (22°16'01.0" S, 49°05'12.05" W), and serve a population of between 30,000 and 50,000 inhabitants, at a flow rate of 78L s⁻¹. The operating temperature ranged between 17 - 25 °C at 8 hours hydraulic retention time (HRT). Weekly collection of WW from the UASB using pre-washed 20 L plastic jerricans and subsequently stored inside refrigerator at 4 °C for preservation (Matamoros et al., 2016). The WW was allowed to be at room temperature before it was transferred to the photobioreactors (PBRs). Characterization analysis was carried out twice weekly per collection, after it was filtered through 0.45 µm membrane filter. The average characteristics of the anaerobically digested municipal WW is presented in Table 7.

Table 7: Mean and Standard deviation of municipal wastewater composition

Parameter	Unit	Mean ¹	Standard deviation
pH	-	6.67	0.16
NH ₄ ⁺ -N	mg N L ⁻¹	59.45	3.25
TDP	mgPO ₄ ³⁻ L ⁻¹	6.14	0.49
Chemical Oxygen Demand	mg L ⁻¹	133.64	17.49
Dissolved oxygen	mgO ₂ L ⁻¹	0.89	0.48
Total Suspended Solids	gTSS L ⁻¹	0.06	0.03
Total Alkalinity	mgCaCO ₃ L ⁻¹	368.50	41.81

1

¹n=24

5.2.2 *Experimental Set-up*

The best microalgae-activated sludge (MAS: 0.10 g/L native microalgae and 0.20 g/L activated sludge) proportion from a previously determined lab-scale test of three MAS conditions of different proportions was operated in pilot flat panel photobioreactors in fed-batch (n=2) (Chapter 3). Each flat panel has a total capacity of 64 L (120 cm long, 60 cm high, 10 cm wide), and was operated with 50 L working volume. The MAS culture (reactor 1) was operated alongside a control in reactor 2 (microalgae proportion of in reactor 1) (Fig. 6). The experiment was operated in two HRTs, 5 days (operational HRT for the lab scale experiment) and 3 days in succession. The natural photoperiod ranged from 11.3 to 12.8 and 13.0 to 13.8 hours for 5 days HRT and 3 days HRT, respectively. Daily volumetric exchange with municipal WW of 10 L and 16.7 L without biomass cycling (to determine daily biomass production) was carried out to achieve 5 days HRT and 3 days HRT, respectively.

The ambient temperature and solar irradiance ranged between 16.0 to 26.6 °C, 21.6 to 28.3 °C and 129.63 to 509.64 $\mu\text{mol s}^{-2} \text{s}^{-1}$, 434.24 to 946.13 $\mu\text{mol s}^{-2} \text{s}^{-1}$ for 5 days HRT and 3 days HRT, respectively. Minjiang pump PS 950 with flowrate 1.0 Lmin⁻¹ with sparging tubes were used to achieve thorough mixing during the experiment (Alcántara *et al.*, 2015).

The ambient temperature and light intensity readings were obtained from UNESP Meteorology centre automatic weather forecast station (IPMet) situated at latitude 22°21'27.6" and longitude 49°01'40.8" in Bauru. Obtained data were recorded at 5 minutes intervals, and the solar radiation data were converted from Rs (W m⁻²) to PAR ($\mu\text{mol m}^{-2} \text{s}^{-1}$) using the conversion factor of 2.02, as outlined in Reis & Ribeiro (2020).

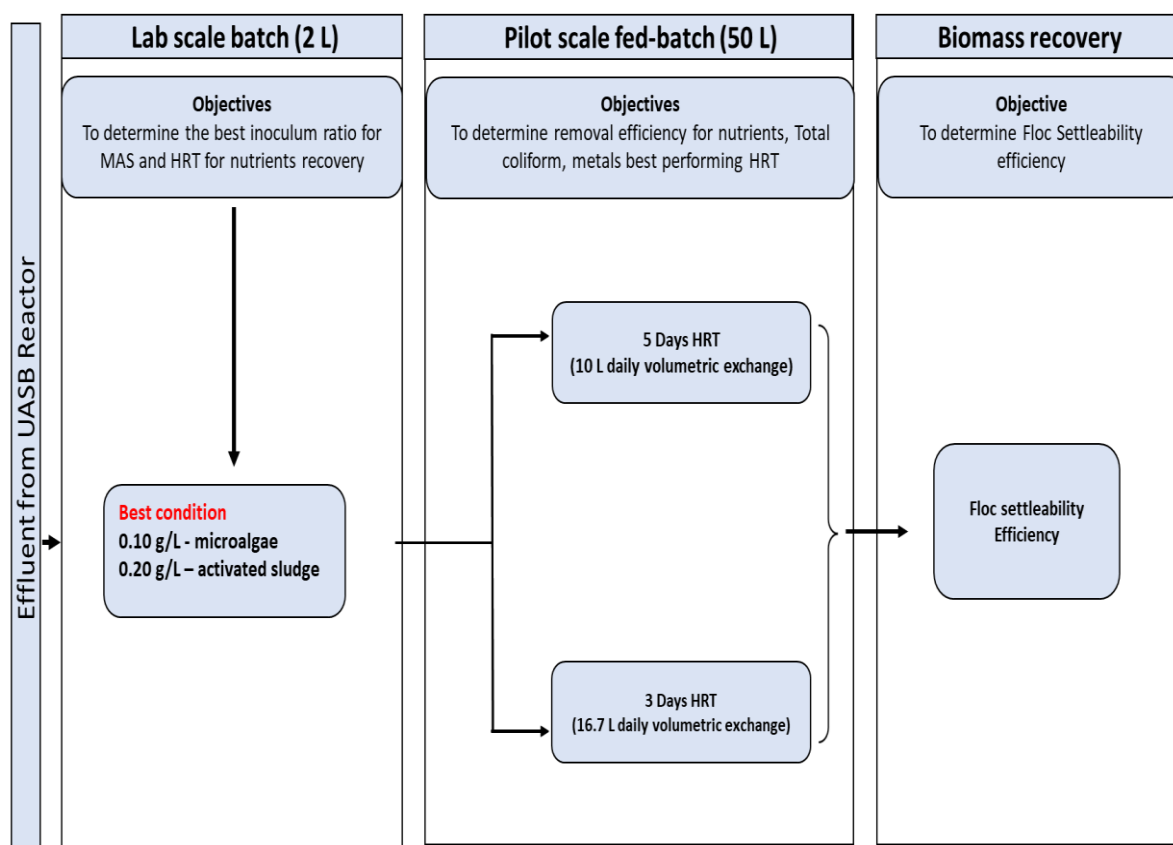


Figure 6: Framework of experimental procedure: The laboratory scale batch experiment was performed separately in previous test, explained in chapter 3.

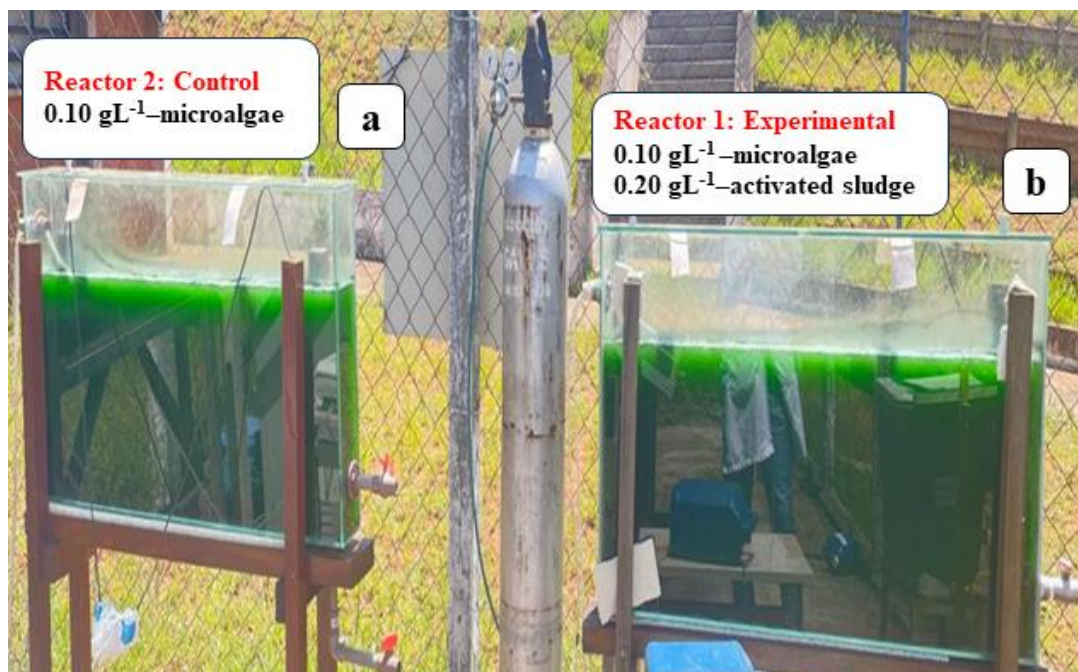


Figure 7: Set-up of pilot Flat panel photobioreactors (a) Control flat panel containing microalgae (b) Experiment flat panel containing mix-culture of microalgae and activated sludge

5.2.3 *Microalgae and Activated sludge Inoculum*

Native microalgae bioprospecting grown in UASB anaerobically digested municipal WW was used as culture inoculum. Both photobioreactors (experimental – R1 and control – R2) were each inoculated with 822 mL of microalgae at 6.08 g L^{-1} of total suspended solids (TSS). Microalgae inoculum is a mix community of *Chlorella* sp. (65.4%), *Cyanobium* (13.6%), *Desmosdesmus* (8.1%), *Chlamydomonas* (7.4%) and *Scenedesmus* (5.4%) being the predominant specie. The microalgae were morphologically identified at the genus level based on specialized studies, using microalgae databases (Guity & Guiry, 2023) and identification keys (Bicudo & Mnenzes, 2017). Samples of 2 mL were collected fortnightly and fixed with formalin at a concentration of 5% (Bicudo & Mnenzes, 2017). The activated sludge was obtained from a WWTP located at Botucatu City, São Paulo, Brazil. The experimental photobioreactor (R1) was inoculated with 558 mL of activated bacteria at 17.92 g L^{-1} of TSS. The activated sludge was incubated in a 10 L polyethylene reactor at a temperature range of 24 to 30 °C under light: dark photoperiod of 12:12 hour at $154 \pm 8 \text{ mmol m}^{-2} \text{ s}^{-1}$ for 14 days, with daily wastewater (WW) exchange (gradually increased the operational municipal anaerobic WW in mixture with the WW from Botucatu WWTP), for

acclimatization prior to photobioreactor inoculation. Also, Minjiang pump PS 950 with flowrate 1.0 Lmin⁻¹ was used for continuous stirring and oxygen supply.

5.2.4 **Biomass Recovery**

At the end of the fed-batch experiment, representative samples were collected from the photobioreactors. 1 L of the culture was measured in Imhoff cones (duplicate) and mixed together gently to determine settleability. The samples were taken at 10 cm below the culture surface of the Imhoff cone with the aid of pipette every 20 mins, for 4 hours, to assess the progress of settling (Su et al., 2011; Trygar, 2013; Zhao et al., 2019). Stop watch was used for timing the sampling at intervals and optical density measurement was determined by Spectrophotometer at 750 nm (NANOCOLOR UV/VIS II, Germany).

5.2.5 **Calculations**

Biomass recovery efficiency (%), biomass productivity (mg TSS L⁻¹ d⁻¹), growth rate (d⁻¹), pathogenic organism log-Re (CFU 100 mL⁻¹) were calculated by the Equations 1 to 5, respectively:

$$\text{Biomass separation efficiency (\%)} = \frac{(OD_{750i} - OD_{750f})}{OD_{750i}} \times 100 \quad 1$$

$$\text{Biomass productivity (mg TSS L}^{-1} \text{ d}^{-1}) = \frac{X_{TSS}}{RT} \quad 2$$

$$\text{Growth rate (d}^{-1}) = \text{Log}\left(\frac{X_{GR}}{RT}\right) \quad 3$$

$$\text{Total coliforms and } Escherichia coli \text{ log-Re (CFU 100 mL}^{-1}) = \text{Log} \frac{CFU_i}{CFU_f} \quad 4$$

Where OD_{750i} and OD_{750f} are the initial and final optical density readings (nm), X_{TSS} is the concentration of total suspended solids in dry weight (mg TSS L⁻¹), RT is the biomass retention time (d), X_{GR} is mean growth rate, CFU_i is the pathogenic organism concentration for influent (CFU 100 mL⁻¹), CFU_f is the pathogenic organism concentration for effluent (CFU 100 mL⁻¹).

5.2.6 **Analytical Methods**

The pH level (4500-HB, APHA, 2017), dissolved oxygen (DO, Starter 400D Probe Metre, Ohaus) and temperature were evaluated daily for both HRTs (5 and 3 days). Microalgae growth pattern in cultures was assessed twice weekly (although daily evaluation was done for the first ten days of the experiment during the 5 days HRT) with optical density 680 nm wavelength (OD680nm) (NANOCOLOR UV/VIS II Spectrophotometer), and TSS (2540 D,

APHA, 2017) and cell count (cell mL⁻¹) (Optical microscope (Leica microsystems, D. 35578, Wetzlar, Germany). Monitoring of nutrients removal (4500-P, 4500- NH₄⁺-N, 8507 HACH - NO₂⁻-N, 8039 HACH - NO₃⁻-N, Spectrophotometer NANOCOLOR UV/VIS II, APHA, 2017), chemical oxygen demand (5220 D, Spectrophotometer NANOCOLOR UV/VIS II, APHA, 2017), total Alkalinity (Titration potentiometric with acid sulfuric) was carried out twice weekly. The removal of total coliforms and *E. coli* was determined twice weekly using Chromocult Coliform Agar (Pour Plate 9215B, Merck KGaA, Germany, APHA, 2017).

The analysed nutrients, dissolved nitrate, nitrite, NH₄⁺ and phosphate, were determined according to APHA 2017. Samples were first filtered through 1.2 µm glass fiber pore diameter membrane filter and then through 0.45 µm pore diameter cellulose acetate membrane, to determine the dissolved nutrients. Additionally, total suspended solids gravimetric analysis for productivity (dry weight) represented the biomass in culture medium.

5.2.7 **Data Analysis**

The effects of the two HRTs on biomass yield, nutrients uptake, Log-Re and biomass recovery were assessed for t-test using IBM SPSS Statistics software version 22.0 with 95% confidence interval. Linear correlation relationship between microalgae biomass yield (dry weight) and optical density was evaluated with Pearson's correlation scatter plot.

5.3 **Results and Discussion**

5.3.1 **Effect of hydraulic retention time on Microalgae cell growth**

Microalgae cell growth was evaluated for a period of 84 days, and compared two hydraulic retention times (HRTs), 5 days HRT (53 days, Fig. 8) and 3 days HRT (31 days, Fig. 9), in semi-continuous feeding regime in succession. The difference in cultivation period was based on the difference in initial biomass concentration and the stability of the systems. The cell growth varied significantly ($p = 0.0074$) between the HRTs, taking into focus the exponential growth at 5 days HRT and the stability at 3 days HRT.

For cultivation during the 5 days HRT, lag phase was not observed (Fig. 8a), as the microalgae inoculum may have acclimatized with the biochemical characteristics of the municipal WW which was used for their cultivation. The growth pattern observed was in two levels (first, Day 0 – D0 to day 28 – D28, and day 29 – D29 to day day 52 - D52), with

corresponding microalgae cell density 9.4×10^7 to 3.4×10^8 cell.mL⁻¹ and 2.3×10^9 to 5.9×10^9 cell.mL⁻¹, respectively. The microalgae cell growth remained relatively steady until D28, hence the separate visualization to understand the growth trend (Figs. 7a and b).

From D31, exponential growth was evident (Fig. 8b), although with intermittent rise and fall in cell density but with an overall rise in growth. The experimental (R1) has a similar growth pattern with the control (R2). The steady growth from D0 to D28 could be as a result of competition for nutrients by the species (*Scenedesmus* sp. *Chlorella* sp. and *Cyanobium* sp.) identified in the mixed native microalgae community used as inoculum. Thereafter, a sharp hike in cell growth was observed which corresponded with the transitive dominance of *Cyanobium* sp. onward. In this study, the achieved microalgae cell density was higher comparable with the findings of Vella et al. (2019), that cultivated *T. weissflogii* in inclined pilot tubular photobioreactors outdoor all year round in, including the summer period, using seawater enriched medium (f/2 medium). This phase of the study was carried out during spring (from tail end of September to mid-November, 2022) and the outcome showed a viable outdoor production of nutrient-rich microalgae cell biomass.

During the experiment, the ambient temperature ranged from 17.3 to 26.3 °C, and 29.0 to 43.6 °C for the internal temperature of reactors (Fig. 8c) for R1 and R2 (difference in values was usually ≤ 1 °C). This shows that the ambient temperature range is considerably suitable for microalgae cultivation (Dolganyuk *et al.*, 2020; Carneiro *et al.*, 2020). The light intensity varied from 165.18 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (winter) to 506.42 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (spring) (Fig. 8d).

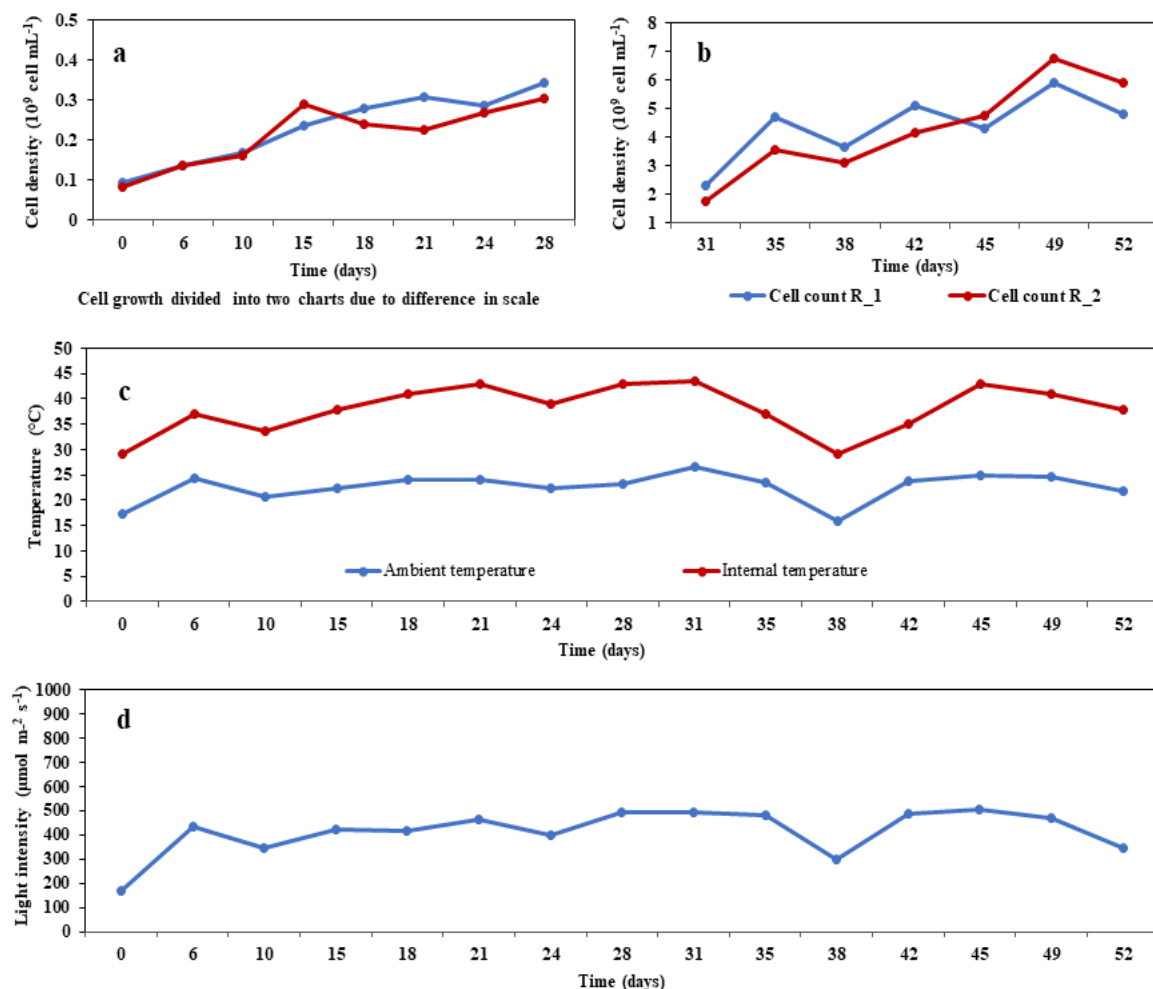


Figure 8: Microalgae cell growth and environmental conditions during 5 days HRT (a & b) cell count (c) daily average ambient and reactors' temperature (d) daily average light intensity from solar radiation

On the other hand, the 3 days HRT spanned through spring and the early start of summer period (mid-November to December, 2023). This is reflected in the difference in light intensity values, which is significantly higher than those recorded during 5 days HRT ($p < 0.05$). The light intensity ranged from 500.41 to 907.55 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 9c). Similarly, the ambient temperature and the internal temperature which ranged from 21.6 to 26.8 $^{\circ}\text{C}$ and 31 to 42 $^{\circ}\text{C}$ (Fig. 9b), respectively, varied significantly ($p = 0.047$).

Although, the adequacy of light intensity range recorded at this experimental phase has been established for exponential growth of microalgae cells (Kliphuis *et al.*, 2010; Carneiro *et al.*, 2020), there was no corresponding growth increase in cellular biomass. As previously indicated, cellular growth between 5- and 3-days HRTs varied significantly, with higher cell growth recorded for the former.

The switch from 5 days HRT to 3 days HTR by increasing the daily volumetric exchange from 10 L to 16.7 L resulted in destabilization of growth trend and later a decline in trend as shown in Fig. 8a, despite the relatively high light intensity. Microalgae cell growth declined to $1.16 \times 10^9 \text{ cell.mL}^{-1}$ and later peaked at $3.86 \times 10^9 \text{ cell.mL}^{-1}$ which is comparatively lower in the end. The decline in microalgae cell production may be as a result of the increased volumetric exchange attributable to lower HRT, which is liable to overdose of possible inhibitory substances in WW that can invariably impact on microalgae cells (Beydes & Karapinar, 2018). Similarly, this was also established in a distant study conducted by Kim et al. (2014) where increasing HRT produced corresponding increase in microalgae cells in High Rate Algal Pond (HRAP).

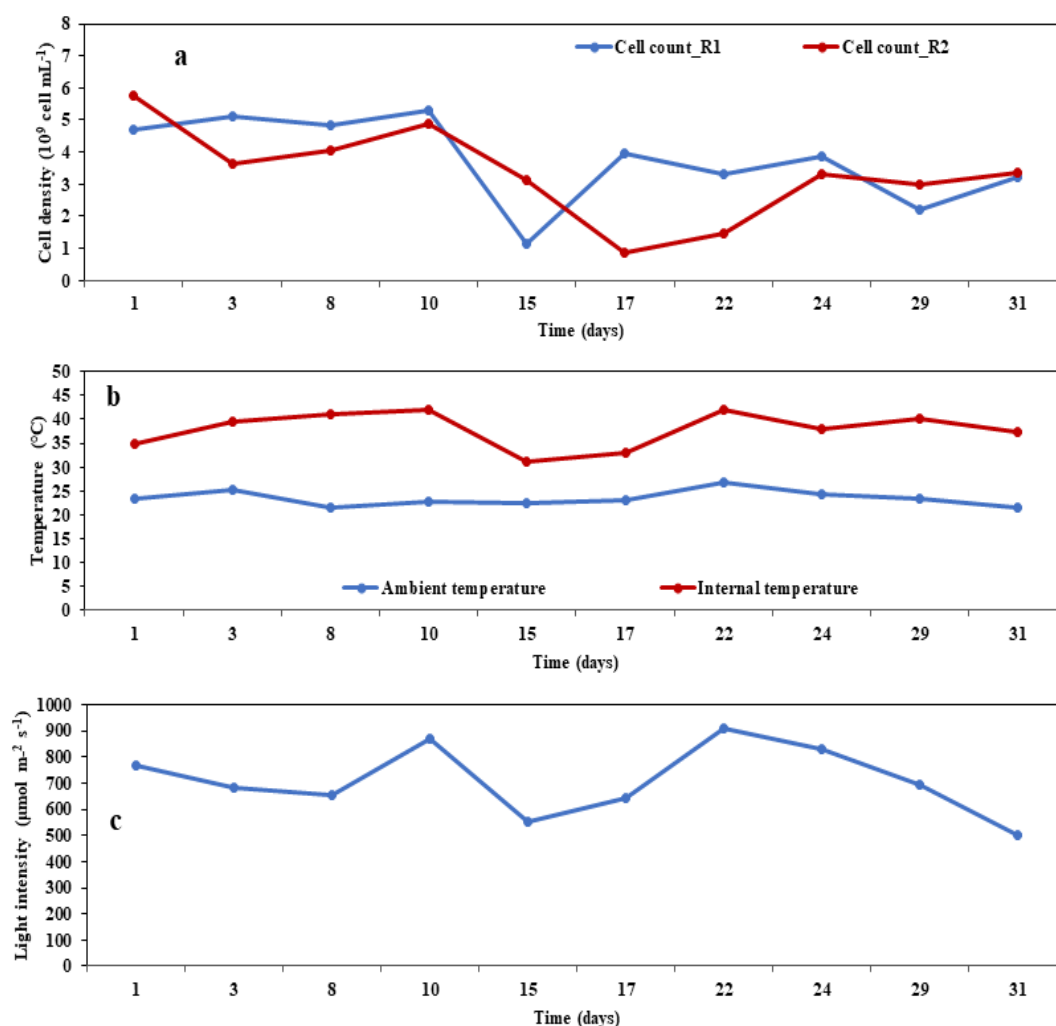


Figure 9: Microalgae cell growth and environmental conditions during 3 days HRT (a) cell count (b) daily average ambient and reactors' temperature (d) daily average light intensity from solar radiation

The estimation of microalgae cell growth and density were also estimated using gravimetric (Lage et al., 2021) and spectrophotometric measurement (680 nm wavelength) (Casagli *et al.*, 2021) of the cultures at set intervals. The concentrations of total suspended solids (g TSS L⁻¹) and values of optical density at 680 nm (OD680) for 5 days and 3 days HRT varied significantly ($p < 0.05$), but conversely for R1 and R2 in the same HRT ($p > 0.05$). Growth trend showed similarity between TSS (Fig. 10a) and OD680 (Fig. 10b) for the 5 days HRT, with a strong positive relationship ($r^2 = 0.86$ for R1 and $r^2 = 0.92$ for R2, Fig. 10c), which is comparable with the finding reported in Komolafe et al. (2014).

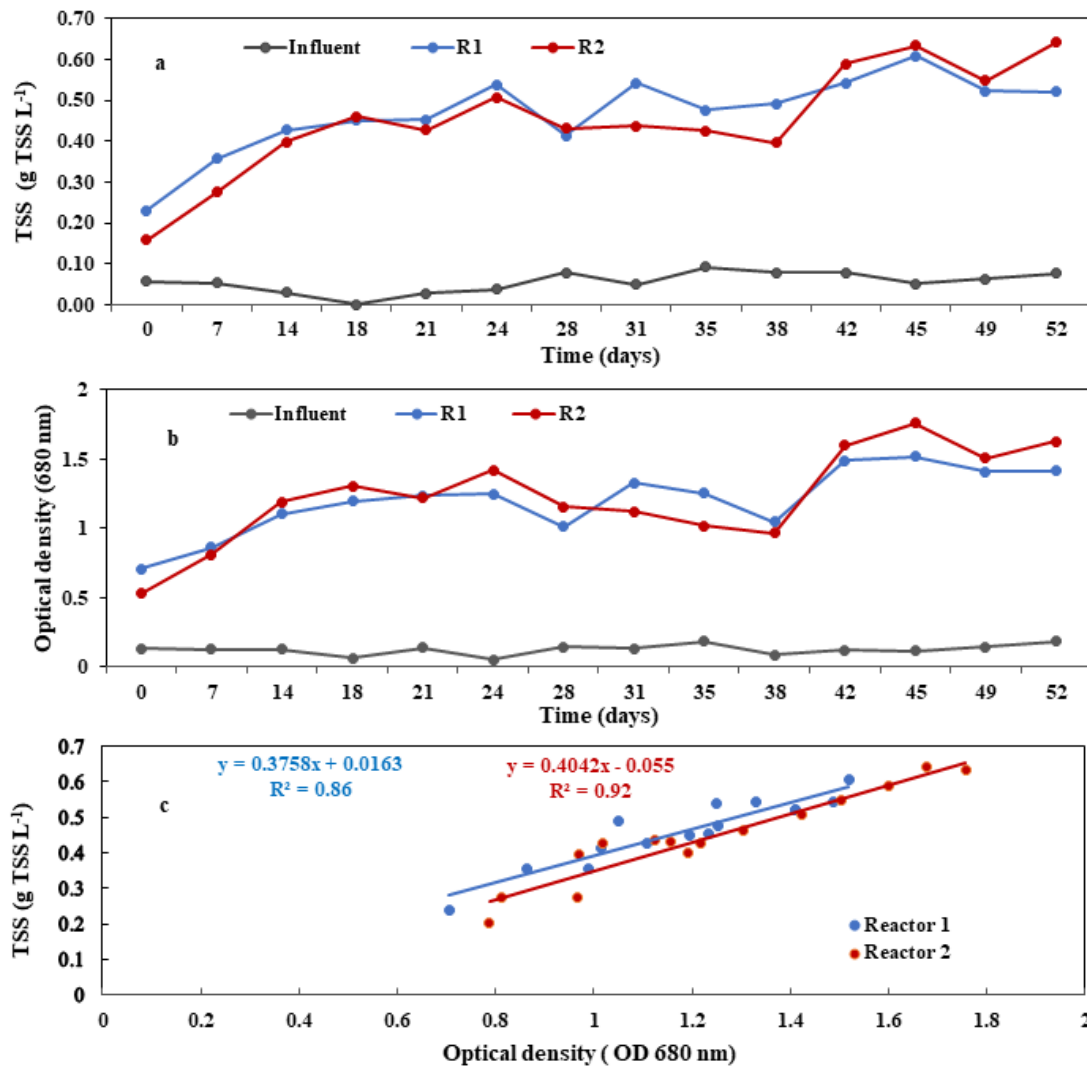


Figure 10: Estimation of microalgae cell growth with total suspended solids (TSS) dry weight and optical density (OD) 680 nm (a - c) TSS, OD 680 nm and scatter plot correlation relationship between TSS and OD 680 nm, respectively for 5 days HRT

Likewise, the experimental culture of 3 days HRT cultivation showed a strong positive relationship between TSS and OD680 for the experimental reactor (R1) ($r^2 = 0.87$, Fig. 11c), and conversely for the control (R2) ($r^2 = 0.34$). The disagreeing values between TSS and OD680, as shown in Figs. 11a and 11b, could be as a result of microalgae cell rupture (coincided with an observance colour change to light-green, indicating microalgae cell death and low TSS), but with probable detectable intra-cellular pigment released into the culture (high OD680), and hence liable to cause a weak correlation.

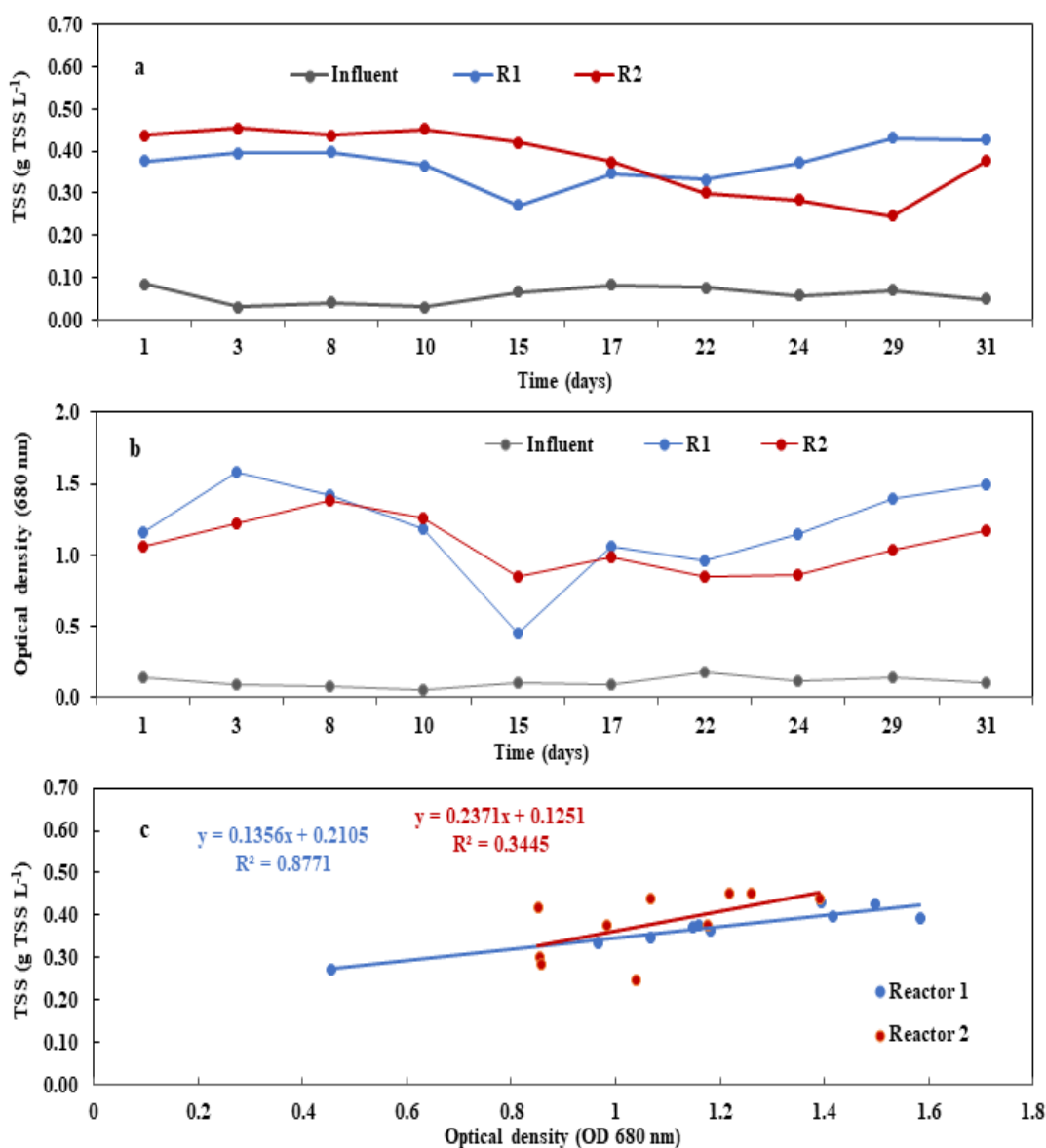


Figure 11: Estimation of microalgae cell growth with total suspended solids (TSS) dry weight and optical density (OD) 680 nm (a - c) TSS, OD 680 nm and scatter plot correlation relationship between TSS and OD 680 nm, respectively for 3 days HRT

Notably, rise and fall in growth trend was observed, which was more prominent on D28, D29 and D38 for 5 days HRT. This is a reflection of the continuously changing atmospheric weather condition. Nonetheless, the growth level achieved is similar to the result obtained in Slompo et al. (2020) that worked with the same reactor but different WW, with productivity and growth rate 54.51 and 63.91 mg TSS L⁻¹ d⁻¹ and 0.08 and 0.09 d⁻¹, respectively (Table 8), despite being conducted in a controlled environment. This clearly displays a promising feasibility of outdoor microalgae-based treatment of wastewater, with minimal resources. Moreover, similarly to what was observed during microalgae cell count (cell.mL⁻¹) after the successive transition to 3 days HRT, progressive decline was observed, although with a sign of recovery afterwards, which may be attributed to increased volumetric exchange (low HRT) (Beydes & Karapinar, 2018; Kim et al., 2014), even with a relatively more favourable environmental weather condition.

5.3.2 Removal efficiency of COD, Nutrients and Alkalinity

The nutrients (phosphorus – P, Nitrogen – N) and the carbonaceous compounds (COD and alkalinity) embedded in the anaerobically nutrient-rich municipal wastewater are taken up for microalgae cell growth and biomass production during photosynthesis, leading to their removal from the wastewater. This is typical of microalgae-based photobioreactors as a promising technology for nutrient cycling and wastewater circular economy.

The removal efficiency of total dissolved phosphorus (TDP), Ammonium Nitrogen (NH₄⁺-N), chemical oxygen demand (COD_{filtered}) and alkalinity at the tested HRTs is presented in Table 8. It was found that the effects of HRT on TDP, NH₄⁺-N, COD_{filtered} removal varied significantly ($p < 0.05$) while no significant difference ($p > 0.05$) was observed for alkalinity uptake at the tested HRTs. As evidently shown in Table 8, MAS cultivation at 5 days HRT out-performed the 3 days HRT counterpart in terms of removal efficiency of the aforementioned compounds. The reduction in removal efficiency with decreasing HRT was comparable with the finding reported in Toledo-Cervantes et al. (2019).

Table 8: Average dissolve oxygen concentration, pH value, removal efficiency of COD_{filtered}, Total Alkalinity, NH₄⁺-N, TDP, biomass productivity, growth rate and settleability for the 5 days and 3 days HRTs

Environmental condition	Unit	5 days HRT		3 days HRT	
		R1	R2	R1	R2
pH	-	10.30±0.03	10.27±2.43	10.60±0.03	10.63±0.03
DO	mg O ₂ L ⁻¹	7.59±0.10	7.57±0.09	8.33±0.09	8.28±0.06
Total Alkalinity	%	66.49±3.40	56.42±5.34	63.26±6.20	49.70±10.35
COD _{filtered}	%	59.52±3.50	57.68±4.23	38.46±6.52	35.36±4.65
NH ₄ ⁺ -N	%	79.00±5.00	74.00±7.00	68.00±5.00	59.00±10.00
TDP	%	88.00±6.00	89.00±5.00	70.00±10.00	67.00±10.00
Productivity	mg TSS L ⁻¹ d ⁻¹	59.41±12.31	63.91±16.45	25.56±11.09	16.11±8.82
Growth	d ⁻¹	0.08±0.01	0.09±0.01	0.03±0.01	0.02±0.01
Settleability	%	33±4.42	23±2.79	14±2.27	14±2.01

TDP, NH₄⁺-N and COD indicate the dissolved proportion in the solution as samples were filtered through 0.45 µm membrane pore size prior to determination

Also, although with the same HRT, Serejo et al. (2020) showed that increasing volumetric culture broth in form of feeding wastewater reduces removal efficiency of MAS treatment systems, just as findings in this study have shown. However, increasing HRT is relative and should be empirically determined for a satisfactory performance, as distant increase may lead to insufficient nutrient loading, with overall adverse consequence on biomass production and nutrients removal.

The removal of nitrogenous compounds ($\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, $\text{NO}_2^-\text{-N}$) from the cultures was considerably by oxidation and subsequent assimilation, including ammonium (Fig. 12). Nitrification was more prominent in R1 for both HRTs, which could be attributable to the activities of nitrifying bacteria that make up the MAS consortia. For 5 days HRT, an apparent decline in $\text{NO}_3^-\text{-N}$ concentration was observed on day 35 and remained steady until the end of the experiment on day 52 (Fig. 12a and b). On the other hand, an abrupt increase in $\text{NO}_3^-\text{-N}$ (nitrification) was observed on transition to 3 days HRT and the level of uptake was inconsistent and comparatively less effective (Fig. 12c and d). Denitrification could be said to be elusive in the reactors considering the average dissolved oxygen concentration $> 7.0 \text{ mg O}_2 \text{ L}^{-1}$ (Table 8), deterring the attainment of anoxic condition, being the preceding requirement for denitrification.

The overriding importance of co-culture of microalgae and activated sludge (MAS) in the uptake of nitrogenous compounds in WW through the combined effects of nitrification and assimilation of nitrate and ammonium was established in Bankston et al. (2020) and Bankston & Higgins (2020). According to Sepehri & Sarrafzadeh (2018), this is achieved in the treatment technology with the aid of autotrophic and heterotrophic nutrient assimilation.

Moreover, the photosynthetic activities of microalgae in the co-culture provides the required amount of dissolved oxygen for oxidative conversion of nitrogenous compounds by nitrifying bacteria (Fallahi et al., 2021), and coupled with direct ammonium assimilation, thereby enabling the removal of nitrogenous compounds in MAS treatment system. These removal mechanisms seem to have played out in the removal of $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ in this study, particularly in R1 and R2 during the 5 days HRT.

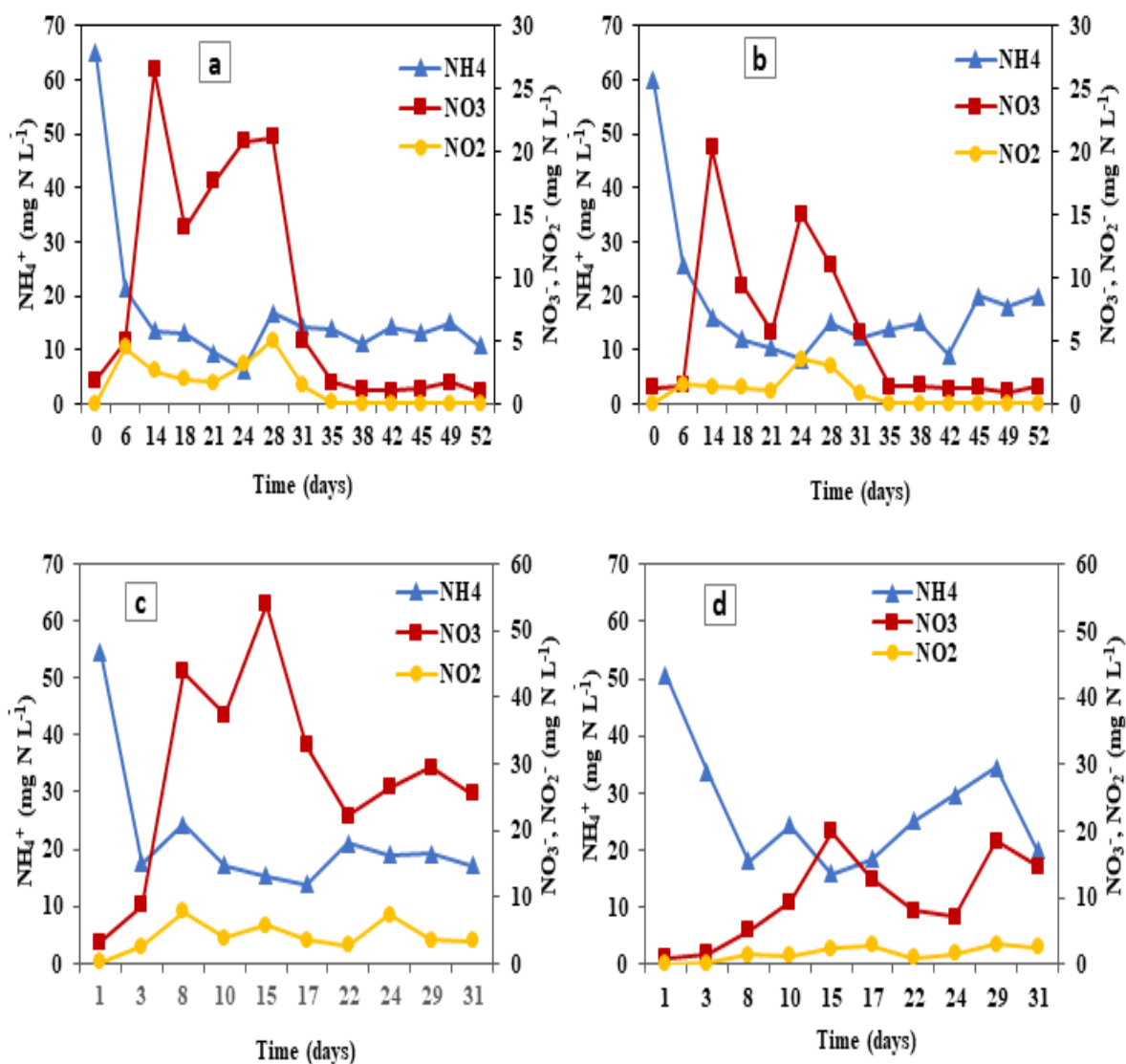


Figure 12: Nitrification and assimilation of nitrogenous compounds (a) Experimental_R1 for 5 days HRT (b) Control_R2 for 5 days HRT (c) Experimental_R1 for 3 days (d) Control_R2 for 3 days HRT

5.3.3 *Escherichia coli* and Total coliforms Removal Assessment

The log-Re removal of *Escherichia coli* (*E. coli*) and total coliforms by MAS in 5 days HRT cultivation (4.54 ± 0.02 , 3.86 ± 0.23 , respectively) showed a consistent pattern with log-Re removal values of 4.39 ± 0.01 (*E. coli*) and 3.64 ± 0.04 (total coliforms) in 3 days HRT cultivation (Fig. 13 a & b). Likewise, the controls manifest similar removal pattern, and showed no significant variation ($p > 0.05$) between the tested HRTs (experimental - R1 and control – R2). However, the slight arithmetic difference (experimental > control) could be as result of relatively speedy nutrients exhaustion owing to competitive uptake by MAS.

The striking impact of microalgae cultivation on the physicochemical condition of culture medium, particularly increase in pH value, which was averagely > 10.0 , and the average level of dissolved oxygen concentration in this study (Table 8), has been linked to inactivation of pathogenic microorganisms like *E. coli* (Ruas et al., 2023; Rani et al., 2021).

Similarly, the study conducted by Liu et al. (2020) reported high *E. coli* mortality and removal in alkaline culture medium. In addition, the removal of pathogenic microorganisms, including *E. coli* and total coliforms have been associated to microalgal toxin secretions (possessing antibacterial and antiviral properties) (Rani et al., 2021; Maghembe et al., 2020; Delanka-Pedige et al., 2019; Ansa et al., 2012), which may be contributory to high log unit reduction (log-Re) of bacterial pathogens reported in this study. However, further study evaluating the removal mechanism of pathogenic organisms with respect to environmental factors (pH, light intensity etc.) and microalgal antibacterial secretion should be carried out.

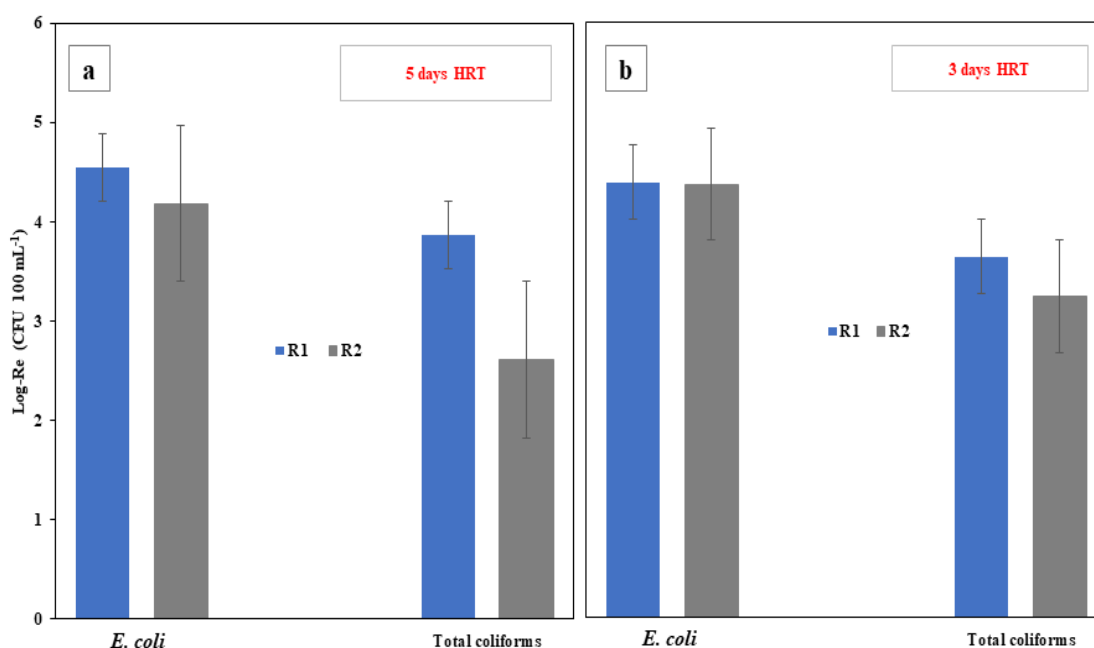


Figure 13: (a) Log-Re for *E. coli* and total coliforms in 5 days HRT (b) Log-Re for *E. coli* and total coliforms in 3 days HRT

Overall, the level of pathogenic microorganism inactivation achieved with this treatment ($9.09 \times 10^{-1} \pm 0.25$ - *E. coli* and $1.84 \times 10^1 \pm 1.83$ - total coliforms in 5 days HRT, and $1.14 \times 10^1 \pm 0.80$ - *E. coli* and $4.78 \times 10^1 \pm 3.11$ - total coliforms in 3 days HRT) demonstrates promising potential for water recovery for non-potable purposes such as

irrigation and lawn wetting, without the risk of contamination and adverse health implication, for being compliant with the World Health Organisation's *E. coli* standard of 10^3 MPN.100mL⁻¹ for this purpose (WHO, 2006).

5.3.4 ***Settleability and Biomass Recovery***

The settleability potential of microalgae and activated sludge (MAS) interaction to harvest nutrient-rich biomass was evaluated under this experimental condition, as a follow-up to the previously established synergistic relationship (Ramanan et al., 2016). The settling was observed for a duration of 4 hours in Imhoff cone under gravity. This is particularly important to ensuring the sustainability of the process, as biomass separation has been adjudged implicated for 20-30% cost in biomass production process (Nazifa et al., 2021; Dassey and Theegala, 2013).

From this study, self-settling biomass recovery efficiency is conversely low (Table 8), although varied significantly ($p = 0.001$) for R1 for the tested HRTs with values 33% and 14 % for 5 days HRT and 3 days HRT, respectively. A similar variation ($p = 0.005$) was noted for R1 and R2 in 5 days HRT. The low microalgae biomass settlement recorded in this study showed poor coalescing influence of bacteria on microalgae floc formation, which is quite in disagreement to findings in previous related studies (Zhu *et al.*, 2019; Quijano *et al.*, 2017; Ramanan et al., 2016; Yan et al., 2022; Zhang et al., 2021; Wang et al., 2022).

During biomass cultivation, change in dominance from *Scenedesmus* sp. to *Cyanobium* sp. was observed with 98% abundance and 2% for *Chlorella* sp., *Desmodesmus* sp. *Scenedesmus* sp. It became prominent on day 28 of the 5 days HRT and was sustained until the end of the experiment, 3 days HRT phase inclusive. The extremely small size of the *Cyanobium* sp. ($< 2 \mu\text{m}$), [matches with the description reported in Pagels et al. (2023)], presented a distinct homogenous characteristic (observed on the reactor and during sampling), and suggests the absence of mucilage secretory compounds like extracellular polymeric substances (EPS) which are responsible for cellular attachment and floc formation (Pagels et al., 2023). This, coupled with the likely effect of high pH value >10.0 on bacterial concentration, may be responsible for the poor microalgae floc formation and low settling outcome.

In previous studies, where high bio-flocculation of microalgae cells and subsequent efficient settling were recorded based on microalgae-bacteria synergy, different microalgae

species were used and operated at a relatively low pH (Beydeş and Karapinar, 2018; Ji et al., 2021; Matamoros et al., 2016; Ruas et al., 2017; Su et al., 2011; Toledo-Cervantes et al., 2019; Wang et al., 2020; Yan et al., 2022). Although, high self-settleability (> 80%) was achieved in a study conducted by Serejo et al. (2020) having similar average pH value (>10.0) and used *Scenedesmus* sp. dominated microalgae consortium in HRAP installed outdoor.

Based on this, the suspected contributory conditions to the low self-settling recorded in this study may be the influence of high pH on bacterial population and EPS concentration and the non-mucilage *Cyanobium* dominated homogenous culture. Therefore, future studies should explore the relationship between these factors for operational feasibility.

5.3.5 **Perspective for Future Study**

The different bacterial strains (activated sludge, nitrifying activated sludge, anaerobic ammonium oxidizing bacteria, aerobic ammonium oxidizing bacteria, anammox sludge, anammox granules), manifest different chemical characteristics (protein, polysaccharide, humic acid and zeta potential) and by extension varying EPS sorption performances, due to the varying environmental conditions (Zhao et al., 2023). Furthermore, bacterial population and sorption removal performance of EPS with respect to certain environmental contaminants were reportedly affected by operational pH (Oberoi et al., 2019; Cao et al., 2019) and temperature (More et al., 2014) of the treatment systems. These presuppose that an ideal operational condition is essential to deriving the overall benefits of microalgae and sludge bacteria co-culture in wastewater treatment.

Though, it has been shown that sludge bacteria and microalgae co-culture provides a cost-effective means of high-quality microalgae biomass recovery from cultures, through EPS mediated bioflocculation and subsequent sedimentation under gravity. Here, low self-settling of biomass (though varied significantly between tested the HRTs: 5 days and 3 days), were recorded. The pH of the reactors (>10.0) and temperature (29.0 to 43.6 °C) were the suspected reasons that might have affected the bacterial population and EPS chemical compositions (not investigated in this study), and coupled with the predominant non-mucilage small sized *Cyanobium* with homogenous characteristic culture.

Thus, this study is foundational for future studies to evaluate the responses of sludge bacteria and EPS chemical composition to environmental factors, bioflocculation potential

bacteria sludge with respect to protein and polysaccharide ratio, and self-settling of *Cyanobium*.

5.4 Conclusion

The performance of microalgae and activated sludge (MAS) for treating municipal wastewater at different hydraulic retention times (HRTs) under a natural tropical outdoor condition were investigated in pilot flat panel photobioreactors that were operated in fed-batch. It was shown that MAS' performances at 5 days HRT outweighed those of 3 days HRT, in terms of biomass productivity and removal efficiency of nutrients which differed significantly, even higher average light intensity and ambient temperature (due to different weather periods and conditions) at the latter phase. It can be stated that the contribution of MAS assimilations on the removal of chemical oxygen demand, total phosphorus and nitrogenous compounds was influenced by HRT. However, similar log-Re of pathogen was achieved at both HRTs. Notably, a change in dominance from *Chlorella* sp. to *Cyanobium* sp. (98%) was observed during the 5 days HRT cultivation and remained throughout the experimental period, including 3 days HRT phase. Consequently, low self-settling of microalgae biomass was conversely recorded, suspiciously influenced by the distinct non-mucilage characteristic of the extremely small sized cells ($< 2 \mu\text{m}$), and high pH on bacterial populations. Therefore, future research could explore the impact of pH and axenic *Cyanobium* sp. on MAS bio-flocculation potential and settling.

5.5 References

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6 CONCLUSION, BENEFITS AND RECOMMENDATIONS

6.1 General Conclusion

This study showed the bioremediation of microalgae-activated sludge (MAS) potential for municipal wastewater treatment under a natural outdoor condition and photoperiod. The MAS proportions were evaluated for microalgae biomass yield, nutrients removal and Log-Re for total coliform and *E. coli*. The condition with the lowest MAS proportion was observed as the best treatment outcome, as increased proportion of MAS appeared to interfere with photosynthetic process and microalgae cells growth. The best MAS proportion was subsequently tested in flat panel photobioreactors at hydraulic retention times (HRTs) 5 days and 3 days under the same environmental conditions, to alleviate concerns around the feasibility for deployment in a real-life scenario. It was shown that MAS' performances at 5 days HRT outweighed those of 3 days HRT, in terms of biomass productivity and removal efficiency of nutrients which differed significantly, even higher average light intensity and ambient temperature (due to different weather periods and conditions) at the latter phase. It can be stated that the contribution of MAS assimilations on the removal of chemical oxygen demand, total phosphorus and nitrogenous compounds is influenced by HRT. However, similar log-Re of pathogen was achieved at both HRTs. Notably, a change in dominance from *Chlorella* sp. to *Cyanobium* sp. (98%) was observed during the 5 days HRT cultivation and remained throughout the experimental period, including 3 days HRT phase. Consequently, low self-settling of microalgae biomass was conversely recorded, suspiciously influenced by the distinct non-mucilage characteristic of the extremely small sized cells ($< 2 \mu\text{m}$), and high pH on bacterial populations. Therefore, future research could explore the impact of pH and axenic *Cyanobium* sp. on MAS bio-flocculation potential and settling.

6.2 Benefits of the Study

- Bioremediation potential and utilization of treated effluent for non-potable;
- Feasibility of application in Low Middle Income Countries (LMICs) with good weather condition;
- Provision of empirical evidence for appropriate MAS inoculum. Optimum performance with the appropriate proportion of MAS inoculum.
- Advancement of wastewater bioeconomy cycle.

- Offers foundational premise for future studies to evaluate responses of sludge bacteria and EPS chemical composition to environmental factors

6.3 Recommendations

The study findings are foundational for future studies to evaluate the responses of sludge bacteria and EPS chemical composition to environmental factors, bioflocculation potential with respect to protein and polysaccharide ratio, and self-settling of *Cyanobium*-bacteria co-culture, due to the varying chemical composition of different sludge bacteria and liability to environmental conditions.