Optimization of Poultry Processing Wastewater Treatment by *Chlorella vulgaris*

SYAMIMI AFIQAH BINTI ABDUL GHANI

MASTER OF ENGINEERING SCIENCE

FACULTY OF ENGINEERING AND GREEN

TECHNOLOGY

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By

SYAMIMI AFIQAH BINTI ABDUL GHANI

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ABSTRACT

OPTIMIZATION OF POULTRY PROCESSING WASTEWATER TREATMENT BY *CHLORELLA VULGARIS*

Syamimi Afiqah Binti Abdul Ghani

Poultry processing wastewater that rich with organic carbon source and nutrients is largely produced by poultry processing industry. The application of microalgae methods for poultry processing wastewater treatment offers a more sustainable solution, as the nutrients in the wastewater are assimilated by the microalgae during treatment, leading to nutrient recovery and the production of lipid by-products, contributing to sustainable development goals. This research investigates the use of *C. vulgaris* microalgae for the treatment of poultry processing wastewater under mixotrophic conditions. The study aims to optimize the factor of photoperiod and inoculum size toward the duration of wastewater treatment by *C. vulgaris*. Moreover, the acclimation strategy, different light sources (LED vs. natural sunlight), and harvesting methods were being investigated to confirm the feasibility of microalgae method for wastewater treatment in real application and also for lipid production. The methods involved cultivating *C. vulgaris* in poultry wastewater and assessing its ability to remove key pollutants, such as Chemical Oxygen Demand (COD), ammoniacal nitrogen, nitrate, nitrites, and orthophosphate. The optimal conditions for poultry wastewater treatment were achieved under 12 hours of photoperiod and at 0.13 g/L inoculum size. The microalgae-based method is

feasible for practical application when combined with acclimation strategies, the use of LED grow lights, and the chitosan flocculation method for microalgae harvesting. This research concludes that *C. vulgaris* is an effective and sustainable solution for poultry wastewater treatment and lipid by-product production. The findings demonstrate that the potential of microalgae systems for large-scale applications in wastewater management and bioenergy production, offering an environmentally friendly and cost-effective alternative to traditional treatment methods.

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APPROVAL SHEET

This dissertation/thesis entitled "**OPTIMIZATION OF POULTRY PROCESSING WASTEWATER TREATMENT BY *CHLORELLA VULGARIS***" was prepared by SYAMIMI AFIQAH BINTI ABDUL GHANI and submitted as partial fulfillment of the requirements for the degree of Master of Engineering Science

Approved by:

____
(Associate Prof. Ir. Ts. Dr. TOH PEY YI) Date: 29/1/2026
Supervisor
Department of Chemical Engineering
Faculty of Engineering and Green Technology
Universiti Tunku Abdul Rahman

____
(Dr. CHNG LEE MUEI) Date: 29/1/2026
Co-supervisor
Department of Chemical Engineering
Faculty of Engineering and Green Technology
Universiti Tunku Abdul Rahman

(Dr. LEONG SIM SIONG) Date: 29/1/2026

Co-supervisor

Department of Chemical Engineering

Faculty of Engineering and Green Technology

Universiti Tunku Abdul Rahman

FACULTY OF ENGINEERING AND GREEN TECHNOLOGY
UNIVERSITI TUNKU ABDUL RAHMAN

Date: 29/01/2026

SUBMISSION OF DISSERTATION

It is hereby certified that Syamimi Afiqah Binti Abdul Ghani (ID No: 23AGM01326) has completed this dissertation entitled "OPTIMIZATION OF POULTRY PROCESSING WASTEWATER TREATMENT BY *CHLORELLA VULGARIS*" under the supervision of Ir. Ts. Dr. Toh Pey Yi (Supervisor) from the Department of Chemical Engineering, Faculty of Engineering and Green Technology, Dr. Chng Lee Muei (Co-Supervisor) from the Department of Chemical Engineering, Faculty of Engineering and Green Technology and Dr. Leong Sim Siong (Co-Supervisor) from Department of Chemical Engineering, Faculty of Engineering and Green Technology.

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DECLARATION

I, SYAMIMI AFIQAH BINTI ADBUL GHANI hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

SYAMIMI AFIQAH BINTI ABDUL GHANI

Date 29/01/2026

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LIST OF SYMBOLS AND ABBREAVIATIONS

Abbreviation	
AOM	Algogenic Organic Matter
COD	Chemical Oxygen Demand
BOD	Biochemical Oxygen Demand
UV-Vis	Ultraviolet-Visible
UTAR	Universiti Tunku Abdul Rahman
ROS	Reactive Oxygen Species
DOE	Department of Environment
DOSM	Department of Statistics Malaysia
WW	Wastewater
FER	Fertilizer
AOB	Ammonia-Oxidizing Bacteria
NOB	Nitrite-Oxidizing Bacteria
EPA	Eicosapentaenoic Acid
DHA	Docosahexaenoic Acid
LED	Light Emitting Diode
N2O	Nitrous Oxide
MIDA	Malaysian Investment Development Authority
FEGT	Faculty of Engineering and Green Technology

CHAPTER 1

INTRODUCTION

1.1 Research Background

The population of Malaysia is extremely varied, which is one factor that contributes to the country's extensive range of unique, locally made food products (Poulain et al., 2020). An increase in food production and manufacturing is a result of population growth, and roughly 10% of Malaysia's manufacturing sector output is produced by the food production and processing sector, which is expanding at a rate of roughly 3% annually (MIDA, 2021). This growth in food production is accompanied by a corresponding increase in wastewater generation, particularly from the food processing and livestock sectors. Approximately 56 billion livestock animals are raised and slaughtered each year for human consumption globally, with projections indicating this number could double by 2050 (Koneswaran and Nierenberg, 2008; López-Sánchez et al., 2022). Annually, more than 24 billion tons of livestock wastewater are produced and discharged untreated, contributing significantly to eutrophication (López-Sánchez et al., 2022; Silva-Gálvez et al., 2024). In developing countries, the reasons for wastewater treatment are to preserve the environment and provide alternative water sources to address water scarcity. However, due to a lack of infrastructure, technical and institutional competence, funding, and infrastructure, the discharge of untreated wastewater is a common practise (United Nations World Water Assessment Programme, 2017). When wastewater that has not been treated is released in water bodies, it poses a significant risk to both the human health and aquatic environment (Garg et al., 2022; Singh, Chakraborty and Sehgal, 2023). The growing demand for food, driven by population growth, coupled with inadequate infrastructure in developing countries, makes the issue of untreated livestock wastewater discharge a pressing concern, with serious consequences for both public health and the environment.

Generally, food processing wastewater can be categorized into four main sectors: dairy operations, beverage and bottling, meat, poultry and seafood, and fruit and vegetable (Muro, Riera and Carmen Diaz, 2012; Bharati, Sundaramurthy and Thakur, 2017). The wastewater is characterized by high levels of chemical oxygen demand, biochemical oxygen demand, dissolved and suspended solids, oil, fats, and grease, while generally displaying low toxicity (Li et al., 2019b; Cheruvu et al., 2025). It is produced as a byproduct of various food processing stages such as cleaning, cutting, slicing, washing, frying, salting, coating, and packing. (Kobya et al., 2006; Li et al., 2019b). Similar to other food processing industries, poultry processing requires substantial water usage, with approximately 20 L of water used per chicken slaughtered (Atchala et al., 2025). Poultry processing wastewater from various poultry types such as chickens, turkeys, and ducks is highly pollutant-intensive due to its high COD, BOD, and nitrogen content (Gržinić et al., 2023). Poultry processing accounts for 60% of the meat processing industry in Malaysia, with poultry meat serving as a primary raw material for further processed livestock products (MIDA, 2021). As the most consumed type of meat in Malaysia, poultry enjoys broad acceptance across the country's diverse ethnic groups, with no religious restrictions, unlike beef or pork (Zayadi, 2021). Each poultry bird requires an average of 26.5 litres of water, with the evisceration (7.57 litres) and washing (4.35 litres) stages being the most water-intensive in processing (Cheruvu et al., 2025). These growing environmental concerns emphasize the urgent need for sustainable and cost-effective poultry processing wastewater treatment solutions.

The wastewater treatment system is made up of primary, secondary, and tertiary treatment process (Akbar et al., 2023). In the primary treatment, suspended solids are removed, and the remaining parameters such as COD, BOD, and nutrients are addressed in the secondary treatment, typically using biological methods. The most commonly used microorganisms in wastewater treatment are bacteria, due to their enzymatic diversity and abundance (Nascimento et al., 2018; Singh et al., 2022). In the aerobic process, bacteria biodegrade organic matter in the presence of oxygen, releasing carbon dioxide as a by-product, which contributes to the carbon footprint of the treatment plant (Kirchem et al., 2020). In contrast, the anaerobic process generating methane biogas as a by-product. This process is more energy-efficient than the aerobic method, as it recovers energy from wastewater and reduces aeration energy requirements (Tomei et al., 2021). When biogas is properly collected and stored, the anaerobic process is considered more environmentally friendly; however, the release of methane and carbon dioxide, both potent greenhouse gases, poses risks if not managed properly (Paolini et al., 2018; Jameel et al., 2024). A major challenge in both processes is the management and disposal of the large amounts of sludge produced by bacterial treatment (Guo et al., 2020b; Singh et al., 2022). Given these challenges, wastewater treatment by microalgae offers a more sustainable and efficient method.

From literature review, the microalgae technology is another potential solution to perform the biological treatment onto the wastewater (Morillas-España et al., 2025). The main advantages of this microalgae-based treatment rely on the fact that the continuous exchange of dissolved and gaseous metabolites among the algal populations and aerobic bacteria reduce the high treatment cost due to the elimination of external aeration (Rossi et al., 2020b). The benefits of employing the microalgae method to treat the wastewater is the microalgae method tend to reduce the carbon footprint of the wastewater treatment system and at the same time the generated microalgae biomass can be the biofuel as by-product (Ali et al., 2022). When come to the target of carbon footprint mitigation, microalgae treatment method is outstanding due to the microalgae able to intake the carbon dioxide from atmosphere under light when the autotrophic metabolism is dominant. The net carbon dioxide mitigation is case dependent since the autotrophic and heterotrophic metabolism are varies according to the contribution ratio of the respective metabolism (Park et al., 2021). Hence using microalgae is a naturally good solution for environmental problems such as climate change (Daneshvar et al., 2022). *C. vulgaris* has been widely applied in wastewater treatment studies and shows broad potential across industries such as food production, agriculture, biofuels, aquaculture, cosmetics, and therapeutics (Ru et al., 2020). Microalgal-based treatment processes currently require extended hydraulic retention times (HRTs), usually ranging from 8 to 12 days (de Mendonça et al., 2018; Feng et al., 2024). Table 1.1 below provides detailed information on the optimal inoculum size for different types of wastewaters and their corresponding treatment times.

Table 1.1: Optimized Microalgae Performance on Different Type Of Wastewater

Type of wastewater	Pretreatment	Cultivation medium	Microalgae species	Inoculum size	Nutrient					Optimal day	Cultivation mode/ photoperiod	Reference
					COD (mg/L)	Ammoniacal nitrogen (mg/L)	Nitrate (mg/L)	Nitrite (mg/L)	Ortho-phosphate/ (Phosphate (PHO) (mg/L)			
Poultry slaughterhouse wastewater	Non-sterilized/ diluted filtered	Poultry slaughterhouse wastewater	*Neochloris* sp. SK57	10 vol%	I=2660 F=96.8%			I=600 F=95	OR=3.04 F=79.3	10	Mixotrophic/ 11:12/ sunlight	(Ummalyma et al., 2023)
			Chlorella sp. SL7A		I=2250 F=89%			I=600 F=93.5	OR=3.04 F=64.5			
Aquaculture	Sterilized	Bold basal medium (BBM)	*Chlorella* sp.	30 vol%		F=94.3%			OR=92.2%	14	-	(Nasir et al., 2015)
Domestic wastewater (India)	Raw/ filtered	Nutrient medium	*C. vulgaris*	30 vol%	I=292 F=81.2	I=13.68 F=1.59	I=1.04 F=3.41		PHO=2.37 F=0.43	1	Mixotrophic/sunlight	(Moondra, Jariwala and Christian, 2020)
					I=246 F=92	I=11.17 F=1.63	I=1.85 F=5.6		PHO=2.64 F=0.65	8 hour		
Piggery wastewater	Raw	Piggery wastewater	*Chlorella* sp.	121.62 mg/L (medium concentration)	I=1054	I= <0.5	I=<0.5		PHO=13.67 F=70-80%	10	Mixotrophic / 12:12/ LED light	(Guo et al., 2020a)
Sewage treatment plant wastewater	• Raw • Sterilized	Bold Basal Medium	*Chlorella pyrenoidosa*	10 vol%						21		(Kumari et al., 2021)

*I = Initial; F = Final

Bioremediation using algae is called as phycoremediation. The term *phyco* in Greek means algae and are generally classified into two types i.e., macroalgae and microalgae (Ayele et al., 2021; Priyadharshini et al., 2021). Microalgae are considered to be outstanding bio-remediators because of their excellent tolerant capacity, easy to grow, high binding affinity, large surface area, eco-friendly, re-usable and dead biomass can be used for remediation (Ramakrishnan et al., 2010; Darda, Papalas and Zabaniotou, 2019; Yan et al., 2022). For several years, phycoremediation on different type of wastewater showed a great pollutant removal efficiency such as dairy, piggery, tannery, municipal, aquaculture and more (Toyama et al., 2018; Saranya and Shanthakumar, 2019; Swain, Tiwari and Pandey, 2020; Lee et al., 2021a; Priyadharshini et al., 2021). Several type of wastewater source such as agricultural and municipal had been used as medium to culture microalgae successfully (Khalid et al., 2019; Han et al., 2021). The important factors to be considered to culture microalgae for its biomass and lipid production is the strain selection, cultivation condition such as temperature, light intensity, pH, cultural metabolism and type of wastewater, cultivating systems such as in open or closed pond system (Chowdury et al, 2020). Typically, photosynthesis is carried out by microalgae utilising light as a source of energy, organic carbon and nutrients to produce biomass (Li et al., 2020b; Yaacob et al., 2022). The biomass could potentially be used as feedstock for nutraceuticals, biofuel, animal feed, biochemical, biofertilizer and other output product (Sarma et al., 2021; Siddiki et al., 2022).

As the global economy continues to expand, the challenges associated with the production, treatment, and disposal of wastewater are expected to intensify in the coming years (Singh, Chakraborty and Sehgal, 2023). The environmental and economic advantages of algae position it as a promising solution to sustainably address the increasing demand for wastewater treatment and nutrient removal (Kube et al., 2018; Peter et al., 2021). Freshwater is utilised to meet industrial demands, resulting in the production of significant amounts of wastewater (Kummu et al., 2012; Shrivastava et al., 2022). The poultry industry is one of the generators of wastewater, and there is great potential to use it to produce high-value ingredients, moreover the wastewater can reduce the total production cost for microalgae biomass (Baker et al., 2020; Deng et al., 2021; Franco-Morgado et al., 2023). Therefore, further study onto the microalgae method in secondary wastewater treatment process is indeed important to confirm its feasibility in real industrial application.

1.2 Problem Statement

Microalgae can be cultivated in four primary modes: mixotrophic, heterotrophic, autotrophic, and photoheterotrophic (Zhan, Rong and Wang, 2017; Abreu et al., 2022). Among these, the mixotrophic mode, which utilizes both organic carbon and under light, is considered the most suitable. During the dark period, microalgae will switch to a heterotrophic mode. From literature review, mixotrophic cultivation results in enhanced microalgae growth, biomass production, and lipid accumulation, as well as improve nutrient removal efficiency, making it ideal for a sustainable wastewater treatment and by-product production simultaneously (Zhan, Rong and Wang, 2017; Yun, Kim and Yoon, 2021). The microalgae-based remediation of poultry slaughterhouse effluents is still not widely explored, aside from studies utilizing anaerobic digestion (Ferreira et al., 2018; Viegas, Gouveia and Gonçalves, 2021). Therefore, the potential of microalgae method to treat the poultry wastewater under mixotrophic condition should be investigated.

Microalgae have high efficiency to remove nutrient more than 80% of COD, nitrogen and phosphate (Kusmayadi et al., 2022; Lee et al., 2022; Saidu et al., 2022). Practically, it would generate a very minimal sludge in wastewater treatment. Unlike bacterial-based treatment systems, microalgae effectively remove nitrogen and phosphorus while releasing oxygen, thereby enhancing water quality and contributing to carbon sequestration (Lee et al., 2019). Study by Asadi, Rad and Qaderi (2020) showed that the least duration for phycoremediation on dairy wastewater is 8 days in secondary wastewater. Clearly, it is not conducive microalgae-based wastewater treatment when a large amount of wastewater is discharged. The longer the treatment duration, the larger tank or space required with increase operating cost (Nur and Buma, 2019). Therefore, the duration of wastewater treatment, particularly for moderate to high-strength wastewater like poultry wastewater, needs optimization, as research in this area remains limited.

In a related study, reducing hydraulic retention time (HRT) to 4 days was achieved through genetic modification of microalgae, specifically by overexpressing cytochrome P450 (Xiao et al., 2025). However, this approach can be costly due to the need for genetic alterations and specialized cultivation conditions. There are other aspect that can be look into it to minimise the duration of wastewater treatment process.

Microalgae performance in mixotrophic cultivation is influenced by the photoperiod. Identifying the optimal photoperiod and light intensity is crucial to maximizing the benefits of mixotrophic cultivation, as these are some of the most significant factors in microalgae growth (Patel et al., 2019; Siqueira et al., 2020). Low light intensity promotes protein accumulation, while high light intensity favours carbohydrate and lipid production (Gao et al., 2022b). The photoperiod also affects biomass growth, as microalgae undergo productive photosynthesis to generate photochemical ATP and NADPH during the light phase, and biochemical synthesis of essential growth molecules during the dark phase (Metsoviti et al., 2019; Nikkanen et al., 2021). The duration of light illumination will directly affect the metabolism of microalgae and thus affect the performance of nutrient removal and biomass production from wastewater sample (González-Camejo et al., 2019; Chin et al., 2023; Parveen et al., 2023) and thus affecting the duration of wastewater treatment. Therefore, the effect of photoperiod is important to be investigated in this study.

Besides that, adjusting the initial inoculum concentration of microalgae has been proposed to increase nutrient removal rates and shorten cultivation times (Li et al., 2017). A study from Ummalyma et al. (2023) reported that poultry wastewater treatment with 10% (v/v) microalgae in concentration removed 89% of COD in 10 days. Moreover, studies by Moondra et al. (2020) showed that the nutrients of raw domestic wastewater can be removed by microalgae and achieved up to 92% of nutrient removal efficiency in 24 hr when at 30% (v/v) of microalgae inoculum. Lananan et al. (2014) revealed that at 40% (v/v) microalgae inoculum reduce ammonia and orthophosphate for more than 80% in 3 to 4 days in aquaculture wastewater. However, there is a lack of research on the comparison and optimize the inoculum size of microalgae toward the performance of nutrient removal with respect to time. Further study is needed to explore the performance of microalgae at different inoculation concentrations to improve nutrient removal rates and decrease retention times, especially in poultry wastewater.

In poultry wastewater, high ammonia concentrations and organic loads can inhibit microalgal growth or cause nutrient shock when transfer the lab cultivated microalgae stock into the wastewater for treatment. The implementation of acclimation strategy via preadapts the microalgae into the wastewater during stock cultivation is important to enhance resilience and pollutant removal efficiency (Hu et al., 2019; Rezaei, Akbulut and Sanin, 2019). Acclimation strategy also demonstrating the industrial practice where a portion of biomass generated from the wastewater treatment process will be reintroduced and return to the secondary treatment pond continuously to maintain its cell concentration in secondary wastewater treatment. From literature review, acclimation tends to enhance the growth of microalgal species by increasing biomass production and shortening the lag time, thereby potentially reducing the treatment retention time (Japar, Takriff and Mohd Yasin, 2021). This process is applied in both batch and continuous microalgae wastewater treatments to enhance nutrient removal, with microalgae demonstrating tolerance even to high ammonia concentration (Osundeko et al., 2014; Van Den Hende et al., 2014; Jain, Mishra and Mohanty, 2022; Guo et al., 2025).

Besides that, Lam & Lee (2012) found that outdoor cultivation with a 12-hour light:12-hour dark cycle resulted in higher biomass production (0.37 g/L) compared to indoor 12-hour light:12-hour dark and 24-hour light cycles (0.21 g/L and 0.31 g/L, respectively) in an organic fertilizer waste medium. This study reflects Malaysia's natural conditions, with outdoor cultivation benefiting from local temperature and sunlight exposure. The favourable light exposure for microalgae typically ranges from 12 to 24 hours, as the photosynthesis process is integral to their growth (Gani et al., 2019). The solar light will be the most economical option for the cultivation of microalgae. In Malaysia, although there is around 12 hours of sunlight is available during daytime, however, the unpredictable weather could be a challenge and affect the performance of microalgae method (Imaizumi et al., 2016; Sajadian, Morowvat and Ghasemi, 2018; Muhammad, Abdullah and Julien, 2020; Ahmad et al., 2021a). Given the concern of instability of natural sunlight, the performance of microalgae method under the light source from sunlight and LED grow light is important to be compared in order to confirm the type of light source that is feasible for real application.

Furthermore, the microalgae harvesting method also influences the quality of wastewater after wastewater treatment by microalgae (Huang et al., 2021; Bhatt et al., 2023; Zheng et al., 2023; Thuan et al., 2024). The harvesting of microalgae together with further nutrient removal could enhance the performance of microalgae treatment too (Tan et al., 2019). Therefore, the effectiveness of the acclimation strategy, type of light source, and the microalgae harvesting method is important to be evaluated to confirm the feasibility of microalgae method in real application.

Biological treatment of wastewater typically relies on conventional activated sludge, but this method is not cost-effective due to the increasing energy demand for oxygen supply, which grows in proportion to wastewater strength (Hossain, Cheng and Cord-Ruwisch, 2019). Anaerobic digestion offers a viable alternative to aerobic wastewater treatment technologies, particularly for high-strength wastewater, as it does not require oxygen and generates significantly less excess sludge. While the anaerobic treatment method offers benefits such as biogas production as an energy source, it can result in greenhouse gas emissions if not properly managed (Gao et al., 2022). The discharge of biogas or challenges in gas storage can be overcome by employing the microalgae method, which stores fuel in the form of lipids in biomass and makes handling and storage easier (Siddiki et al., 2022). The potential for microalgae biomass to become a biofuel resource after wastewater treatment makes the microalgae method more advantageous than bacterial-based treatments. Therefore, the biomass productivity and the potential of lipid production is important to be investigated to confirm the feasibility of microalgae method on by-product production.

The selection of microalgal strain is crucial for effective poultry wastewater treatment. *Chlorella* species, particularly *C. vulgaris*, are widely used in wastewater treatment due to their ability to thrive in harsh conditions, rapid growth, and efficient nutrient removal (Nguyen et al., 2022a). Under nutrient limitation and stress, *C. vulgaris* accumulates high amounts of lipids for storage (Sakarika and Kornaros, 2017). Specific strains like *C. vulgaris* have shown strong adaptability to industrial wastewater (Terán Hilares et al., 2021). This makes them promising candidates for poultry wastewater remediation. Research has demonstrated that *C. vulgaris* thrives in nutrient-rich wastewater, tolerates high ammonia concentrations, efficiently removes nitrogen and phosphorus, and exhibits a rapid growth rate, making it a promising agent for both phycoremediation and bioresource recovery (Dai et al., 2023; Farag et al., 2024). These characteristics make *C. vulgaris* ideal for cultivation in wastewater, especially under mixotrophic conditions. In Malaysia, *Chlorella* strains have been shown to produce biodiesel at concentrations ranging from 34.53 to 230.38 mg $L^{-1}d^{-1}$ due to high lipid production (Vello et al., 2014; Cao et al., 2023). Therefore, *C. vulgaris* is employed in this study for wastewater treatment and biomass production.

1.3 Research Objectives

i. To evaluate the nutrient removal efficiency of *C. vulgaris* in poultry processing wastewater following pre-cultivation in a fertilizer medium.

ii. To study the effect of photoperiod and inoculum size of microalgae toward the performance of wastewater treatment.

iii. To evaluate the feasibility of microalgae method in poultry processing wastewater treatment.

CHAPTER 2

LITERATURE REVIEW

2.1 Characteristics of Microalgae

Microalgae are unicellular photosynthetic microorganisms that play a crucial role in aquatic ecosystems, contributing significantly to global carbon cycling and oxygen production. These microorganisms are highly diverse, with estimates suggesting that there are between 200,000 and several million species, classified into various phyla such as Chlorophyta, Bacillariophyta, and Cyanobacteria (Singh and Saxena, 2015).

Microalgae can grow in a wide range of environmental conditions, from freshwater and marine ecosystems to extreme environments such as hot springs and acidic lakes (Rinaldi et al., 2024). Their remarkable adaptability is attributed to their flexible metabolic pathways, which include phototrophic, heterotrophic, mixotrophic, and photoheterotrophic modes of nutrition. In phototrophic growth, microalgae use sunlight as the primary energy source to fuel their metabolic processes. In contrast, under heterotrophic conditions, they rely on organic carbon sources such as glucose. Mixotrophic microalgae, including *C. vulgaris*, can utilize both inorganic and organic carbon sources, making them particularly suitable for wastewater treatment applications (Ethiraj, Samuel and Indumathi, 2024). This versatility in metabolic modes significantly enhances the practical application of microalgae in various industrial settings.

In addition to their metabolic flexibility, the structural components of microalgae support efficient metabolic activities. The chloroplasts, which contain chlorophyll and various accessory pigments, play a pivotal role in light absorption for photosynthesis, enabling the microalgae to harness light effectively for energy production (Manoj and Manekkathodi, 2021; Tyoker Kukwa and Chetty, 2021). Additionally, microalgal cells contain mitochondria, which are essential for respiration and energy production, a golgi apparatus for intracellular transport, and vacuoles that help with storage and osmoregulation, ensuring cellular stability in diverse environmental conditions. One significant factor that influences microalgal stability is the secretion of algogenic organic matter (AOM), a mixture of polysaccharides and proteins that affects cell aggregation, charge, and flocculation, which ultimately impacts their harvesting potential (Zhou et al., 2024) Furthermore, microalgal cell walls exhibit varying compositions, depending on the species, with some containing cellulose, silica, or polysaccharides, which influence their resistance to environmental stress (Khan, Shin and Kim, 2018; Moreira et al., 2023). This structural resilience enables microalgae to thrive in diverse environments and under challenging conditions, further enhancing their potential for industrial applications.

Microalgae undergo a distinct life cycle comprising six growth phases: lag phase, exponential phase, linear phase, declining relative growth phase, stationary phase, decline phase and death phase (Skifa et al., 2025). Microalgae exhibit a rapid growth rate, with some species growing faster than terrestrial plants, making them highly efficient for biomass production and also thrive in a variety of wastewater types (Bora et al., 2024). During the exponential phase, rapid cell division leads to high biomass accumulation, but as nutrients become limiting, microalgae enter the stationary phase, where growth slows and eventually declines due to resource depletion. The ability of microalgae to rapidly proliferate under favourable conditions makes them an attractive candidate for large-scale biomass production (Hosny, Elshobary and El-Sheekh, 2025). Optimizing growth conditions and integrating technology for high-density cultivation is crucial to overcoming this challenge.

Biochemically, microalgae exhibit high nutritional value, with varying proportions of lipids, proteins, and carbohydrates depending on species and cultivation conditions. Many species, such as *Chlorella sp.* and *Nannochloropsis sp.*, are known for their high lipid content up to 50%, making them potential candidates for biodiesel production (Patle et al., 2021; Rautenberger et al., 2024). The lipid content in microalgae is not only significant for biofuel production but also for omega-3 fatty acid production, which is important for human nutrition (Perdana et al., 2021). The protein content of microalgae can exceed 50% of dry weight, making them suitable for food, health supplement and feed applications (Koyande et al., 2019; Amorim et al., 2021). The amino acid profile of microalgal proteins is another factor to consider, as certain species offer a balanced profile comparable to conventional protein sources.

Microalgae also play a vital role in wastewater treatment due to their ability to remove pollutants such as nitrogen, phosphorus, and heavy metals with removals more than 90% (Liu and Hong, 2021; Salgado et al., 2023). This is achieved through various mechanisms, including the direct uptake of nutrients for growth, adsorption of contaminants onto cell surfaces, and the formation of biofilms that enhance biodegradation (Cheah and Chan, 2021). Certain species, such as *C. vulgaris*, have demonstrated exceptional efficiency in treating industrial wastewater, including wastewater from poultry processing, due to their high nutrient uptake rates and tolerance to varying environmental conditions (Thoré et al., 2021; Abdelfattah et al., 2023).

In conclusion, microalgae are highly adaptable and biologically diverse organisms that can thrive under a variety of environmental conditions. Their rapid growth rates, versatile metabolic processes, and ability to synthesize a wide range of valuable biochemicals make them ideal candidates for numerous applications, including biomass production, biofuel generation, and wastewater treatment.

2.2 Cultivation of Microalgae

Microalgae cultivation plays a crucial role in various industries, including wastewater treatment and lipid production. The choice of cultivation mode significantly influences biomass productivity, nutrient removal efficiency, and metabolic pathways, making it a critical factor in optimizing microalgae-based wastewater remediation (Verma et al., 2020; Abreu et al., 2022). Among the four primary cultivation modes photoautotrophic, heterotrophic, mixotrophic, and photoheterotrophic, mixotrophic cultivation has demonstrated superior adaptability in wastewater treatment due to its ability to utilize both organic and inorganic carbon sources (Cao et al., 2023). This metabolic flexibility enhances both growth efficiency and pollutant removal capacity, making mixotrophic microalgae an attractive candidate for treating nutrient-rich wastewater (Abreu et al., 2012; Mohammad Mirzaie et al., 2016; Verma et al., 2020). In particular, the adaptability of mixotrophic cultivation positions it as a promising approach for treating poultry wastewater, which is characterized by high nutrient and organic loads, overcoming the limitations of conventional treatments. This method is commonly applied in open pond systems and closed photobioreactors, contributing to carbon sequestration and pollutant removal (Daneshvar et al., 2021).

Phototrophic cultivation depends on light and CO_2 but is constrained by factors such as atmospheric CO_2 availability, uneven light penetration, and reliance on stable light cycles. These limitations can reduce microalgal density and promote bacterial growth, which leads to lower biomass as algal cells catabolize their self-produced nutrients (Nirmalakhandan et al., 2019; Cheng et al., 2023). These constraints underline the challenges of using phototrophic cultivation as a standalone method for wastewater treatment, especially in environments with fluctuating light conditions or variable CO_2 levels. When cultivated under phototrophic conditions, lipid content in microalgae ranges from 5% to 68%, depending on species and environmental conditions in outdoor (Khoo et al., 2023). However, since this study focuses on wastewater treatment, this method alone is not suitable for large-scale applications in nutrient-rich wastewater remediation.

Heterotrophic cultivation allows microalgae to grow without light, utilizing organic carbon sources such as glucose or acetate as both an energy and carbon source (Rossi et al., 2024). This approach achieves higher biomass yields than phototrophic systems and enhances lipid accumulation, making it attractive for biofuel production (Perez-Garcia et al., 2011; Yun, Kim and Yoon, 2021). For instance, a study by Tan et al. (2021) demonstrated that *C. vulgaris* achieved its highest lipid yields of only 30% and 16% under heterotrophic and autotrophic conditions, respectively. However, heterotrophic cultivation requires external organic carbon supplementation, which increases operational costs and reduces its sustainability for large-scale wastewater treatment (Rossi et al., 2024). While the heterotrophic growth rate of microalgae can sometimes match or exceed that of mixotrophic growth under specific conditions, mixotrophic microalgae are more resistant to bacterial contamination in wastewater. Their higher bioactivity ensures more effective nutrient removal compared to both heterotrophic and photoautotrophic microalgae (Zhang et al., 2012; Kim et al., 2013; Babaei et al., 2016; Li et al., 2019a; Wu et al., 2024).

Mixotrophic cultivation combines both phototrophic and heterotrophic metabolic pathways, allowing microalgae to simultaneously utilize CO_2 and organic carbon from wastewater (Castillo et al., 2021; You et al., 2021). This dual-metabolism strategy allows microalgae to maintain high biomass productivity even under fluctuating nutrient conditions, making them particularly suitable for wastewater treatment applications (Abreu et al., 2012; Abdelfattah et al., 2023). Unlike heterotrophic systems, where organic carbon supplementation is required, mixotrophic microalgae can assimilate organic waste directly from wastewater and also CO_2, reducing the need for additional carbon inputs while also improving nitrogen and phosphorus removal efficiency (Scapini et al., 2024; Takahashi et al., 2024). Although microalgal nutrient removal has shown promise, the application of microalgae for large-scale nitrogen removal is hindered by the slower growth of microalgae and prolonged treatment processes. High dependency on light availability and CO_2 in autotrophic mode, along with the reliance on organic carbon and CO_2 in heterotrophic mode, limit their application for wastewater treatment. However, the simultaneous performance of autotrophy and heterotrophy in mixotrophic conditions may overcome the challenges posed by both autotrophy and heterotrophy (Nie et al., 2020).

Several studies have demonstrated that mixotrophic microalgae outperform purely phototrophic or heterotrophic cultures in biomass yield, pollutant removal, and lipid accumulation (Mubashar et al., 2021; Patel et al., 2021). For instance, Yan et al. (2024) showed that mixotrophic cultivation of *C. vulgaris* yields 3.48 g/L and exhibits more rapid energy metabolism efficiency, while heterotrophic and autotrophic conditions produce 2.58 g/L and 0.74 g/L, respectively. Microalgae grown under mixotrophic conditions maximize energy conversion via photosynthesis and heterotrophic respiration. A few studies have also shown that the highest lipid accumulation occurs under mixotrophic conditions (Yun, Kim and Yoon, 2021; Anahas, Prasannabalaji and Muralitharan, 2025; Su et al., 2025). Moreover, mixotrophic metabolism enhances CO_2 recycling and carbon assimilation efficiency. Studies have shown that CO_2 released during heterotrophic respiration is reabsorbed for photosynthesis, increasing overall carbon utilization efficiency (Cui et al., 2017; Licata et al., 2025: Abiusi et al., 2020). Another study by Babaei et al. (2018) also proved that mixotrophic conditions resulted in a nitrogen removal efficiency of 87%, while *C. vulgaris* under heterotrophic conditions reduced nitrogen concentration to 22% in ammonium wastewater.

Photoheterotrophic cultivation combines light-driven metabolism with organic carbon utilization (Cheng et al., 2022). While similar to mixotrophic growth, photoheterotrophic cultivation relies primarily on organic carbon supplementation, making it more dependent on controlled conditions than mixotrophic systems. Certain light-regulated metabolic pathways may enhance lipid production, but the requirement for photobioreactors and external organic carbon inputs makes this method less economically viable for large-scale wastewater treatment (Abreu et al., 2022; Abdur Razzak et al., 2024). As a result, photoheterotrophic cultivation is rarely used in wastewater remediation applications, particularly in nutrient-rich effluents such as poultry wastewater.

This study focuses on optimizing mixotrophic conditions for poultry wastewater treatment at the lab scale, with the goal of generating reliable data that could inform future industrial-scale applications. By addressing these challenges, this research aims to contribute to the development of a sustainable, cost-effective microalgal wastewater treatment system tailored to the poultry industry. Before mixotrophic cultivation can be applied at an industrial scale, lab-scale optimization is necessary to assess key performance parameters, including nutrient removal efficiency, biomass yield, and microbial interactions (Novoveská et al., 2023). Studies have shown that mixotrophic microalgae cultivated in controlled laboratory environments can achieve high biomass productivity, but translating these findings to real wastewater conditions requires further investigation (Velásquez-Orta et al., 2024).

Moreover, pilot-scale studies on mixotrophic cultivation in wastewater treatment are limited, with most research conducted at lab-scale conditions using controlled nutrient compositions. This highlights the need for experimental studies that investigate how mixotrophic *C. vulgaris* adapts to the complexity of real poultry wastewater before scaling up to industrial applications. By conducting lab-scale optimization, this study aims to establish fundamental parameters for mixotrophic microalgae performance in poultry wastewater, including evaluating biomass accumulation and pollutant removal efficiency under fluctuating nutrient conditions, assessing the impact of microbial competition and organic carbon variability, and determining the adaptability of mixotrophic metabolism to poultry wastewater composition. These findings will provide critical insights for future industrial applications, ensuring that mixotrophic wastewater treatment systems can be effectively scaled for poultry processing facilities in Malaysia.

2.3 Poultry Processing Wastewater

The poultry industry is the largest contributor among the livestock industry in Malaysia (DOSM, 2017; Zayadi, 2021). The poultry industry is a crucial element of Malaysia’s economy, acting as a key provider of daily food supplies for the population. The Ministry of Agriculture and Food Industry (MAFI) launched National Agrofood Policy 2021-2030 (DAN 2.0) in October 2021. One of the objectives is to increase the poultry’s self-sufficiency level (SSL) to 140.2 % by 2030, an increase from 104.51 % in 2020 (MAFI, 2021). This rapid growth, driven by escalating consumer demand for white meat, results in increased poultry processing, which generates substantial wastewater. Each poultry bird processed results in the production of approximate 26 Litres of wastewater, which 80% from the processing and 20% for cleaning and washing of process equipment (Fatima, Du and Kommalapati, 2021; Zaharia et al., 2024; Cheruvu et al., 2025). Each litre carrying a mean 361.25 mg/L of total nitrogen, highlighting the substantial nutrient burden posed by poultry industry (Yaakob, 2018). According to Department of Veterinary Services (2024), the total number of poultry broilers produced in 2023 was approximately 163 million. This would produce approximate of 4.3 billion litres of wastewater and 1563 tonnes of nitrogen. The U.S. processed 9.54 billion poultry birds in 2022, generating more than 75,000 tons of fatty waste from processing facilities along with 253 billion litres of wastewater (Shanmugam et al., 2025). However, in small scale chicken slaughterhouse generate 88.34 litre per day of wastewater per day, although pollutant concentrations vary depending on each facility's processing scale and operational practices (Abu Bakar, 2019). The composition of wastewater can differ between meat processing plants, influenced by factors such as the plant's size, the specific processes employed, management practices, and local regulatory standards. Hence, managing the resulting wastewater has become an urgent issue. The large volume of wastewater generated not only increases the burden on local water treatment systems but also contributes significantly to environmental pollution. With the high nitrogen concentration in poultry wastewater, this can lead to severe eutrophication in water bodies, affecting aquatic ecosystems.

Poultry slaughtering involves several sequential processes including slaughtering, scalding, defeathering, evisceration, washing, and chilling each of which contributes to the overall volume and pollutant load (Yaakob, 2018; Kanafin et al., 2022). Wastewater from poultry processing plants typically contains high concentrations of total suspended solids, oils and fats, and elevated levels of total carbon, total nitrogen, and total phosphorus. It also presents significant values for chemical oxygen demand and biological oxygen demand, indicating a high organic and nutrient load (Kanafin et al., 2022). Additionally, approximately 35% of the total organic matter in poultry wastewater consists of large floating debris from agglomerated grease and fat, while around 55% is made up of suspended solids containing lipids, proteins, pathogenic microbes, and other contaminants, including fats, oils, grease, suspended solid, pathogenic microorganisms, and emerging pollutants like antibiotics and endocrine-disrupting compounds (Terán Hilares et al., 2021). The contamination primarily arises from blood, stomach and intestinal mucus, organic matter, pathogenic and non-pathogenic microorganisms, and residues of detergents, disinfectants, blood, and manure, which contribute to elevated total phosphorus levels these wastewaters are rich in nutrients such as nitrogen and phosphorus, and contain blood, fats, oil, grease, and proteins contain pathogens also (Bustillo-Lecompte and Mehrvar, 2015; Kanafin et al., 2022; Shanmugam et al., 2025). As highlighted in Table 2.2, the nutrient composition of poultry wastewater particularly with its high COD, nitrogen, and phosphorus levels is significantly indicating the substantial nutrient load that must be managed.

Table 2.1: Nutrient Composition of Food Wastewater from Different Industries

Industry	COD (mg/L)	Total Nitrogen (mg/L)	Total Phosphorus (mg/L)
Meat Processing	527-15,256	60-339	26-76
Dairy Processing	10,000–50,000	14–830	9–280
Food Processing	125–100,580	3–5694	0–351
Slaughterhouse	500-15,900	50-841	25-200

Sources: (Bustillo-Lecompte and Mehrvar, 2015; Njoya, Basitere and Ntwampe, 2019; Fatima, Du and Kommalapati, 2021; Kan et al., 2021; Nayyar et al., 2021; Vaishnav et al., 2023)

Table 2.1 provide variety of nutrient pollutant range in different type of wastewater, however in different industries even in the same categories will have different nutrient pollutant level. Generally, slaughterhouse wastes are animal byproducts that remain unutilized after slaughtering. Hence, poultry processing wastewater falls under slaughterhouse, food, and meat processing categories, as it involves slaughtering poultry and processing its meat, leading to high contamination levels. Such nutrient-rich discharges promote eutrophication in water bodies, leading to algal blooms, oxygen depletion, and ecosystem degradation (Kapsalis and Kalavrouziotis, 2021).

The operations of the poultry meat industry can be divided into two major categories which is production and processing. Poultry production encompasses all activities related to raising live birds, including breeding, hatching, grow-out, feed manufacturing, and waste management (Rothrock and Locatelli, 2019). Processing refers to the steps that transform a live bird into meat products and by-products, such as slaughtering, further processing, rendering, waste management, and conversion into food products (Luján-Rhenals et al., 2016a; 2016b; Baéza, 2020). Slaughterhouses are a part of the food processing industry (Philipp et al., 2021).

In Malaysia, most poultry industries employ halal-certified workers for slaughtering, with the process closely monitored by a Halal Malaysia officer (Suharni, Chew and Mohd Saiful Rizal, 2021). While some companies may not require halal certification, there is a strong demand for halal-certified poultry products in large retail chains across the country. Poultry processing uses approximately 26 litres of water per bird. Scalding and plucking stages in the slaughter of birds represent half of the total water consumption used during the entire process, with scalding alone consuming about 1-1.5 litre per bird (Mead, 2004; Arispón et al., 2023). During stunning by water immersion requires a tank where the water is changed each shift, ensuring the water level remains high enough for the poultry's head to touch the electrically charged water while hanging upside down (Bailone et al., 2022). Water immersion chilling involves submerging carcasses in cold water baths, leading to water absorption and wastewater production (Meiramkulova et al., 2020a). Slaughterhouses consume significant quantities of freshwater primarily for processing and cleaning operations, resulting in the production of wastewater. However, during the bleeding process, poultry blood must not be disposed of in the wastewater system but should instead be contained in a separate area. Blood accounts for approximately 7.4% to 11% of the poultry's total body weight, with only about 2.5% of the blood draining from the carcass after slaughter (Osborne et al., 2024). With the remaining blood, still present, is washed together with contaminated water in further process.

Table 2.1 showed the differences in all nutrients in different wastewater, which show that the food processing wastewater the highest among all in term of all nutrients. Moreover, food industries, including dairy, poultry, alcohol fermentation, edible oil, fruit juice, sugar, slaughterhouse, and cattle (Nayyar et al., 2021). However, sugary-containing industrial wastewaters, commonly from starch, monosodium glutamate, breweries, dairy, and sugar industries, are characterized by extremely high COD ranging from 80,000 to 100,000 mg/L, along with relatively high concentrations of nitrogen and phosphorus (He et al., 2022). The food industry has different of nitrogen source level and phosphorus due to food ingredients, preservatives and additives. For example, potassium sorbate, sodium nitrate, and nitrite are among the most used substances in food processing, particularly for curing meat products. These compounds help control pathogenic and spoilage microorganisms, thereby extending the shelf life of processed meats and other food products. (Niklas et al., 2023; Palhano et al., 2025). In contrast, slaughterhouses typically have high ammonia levels in manure due to the animal diet, as nitrogen in faeces primarily consists of unabsorbed protein and uric acid in poultry or urea in mammals, which are rapidly hydrolyzed to form ammonia through microbial degradation and also meat and blood (Kundu, Debsarkar and Mukherjee, 2013; Meiramkulova et al., 2020b; Sreyvich, Petrus and Purnomo, 2020; Bist et al., 2023). Together food processing wastewater and slaughterhouse wastewater will produce high contamination level wastewater (Manios et al., 2003; Bustillo-Lecompte and Mehrvar, 2017).

2.4 Biological Treatment in Wastewater Treatment

Wastewater treatment is a multi-stage process designed to remove physical, chemical, and biological contaminants from water before discharged or reuse. It is typically categorized into primary, secondary, and tertiary stages (Ding, 2024). Primary treatment involves physical methods such as screening, sedimentation, and flotation to remove suspended solids, oils, and grease and chemical methods such as flocculation and sedimentation method (Sathya et al., 2022). Secondary treatment utilizes biological processes primarily bacterial metabolism to degrade dissolved organic matter and remove nitrogenous compounds. Tertiary treatment, used in regions with stricter effluent quality regulations, serves as the final cleaning process to remove inorganic compounds and enhance effluent quality before reuse, recycling, or discharge into the environment (Rahimi, Modin and Mijakovic, 2020).

Biological wastewater treatment methods are valued for their cost-effectiveness, eco-compatibility, and ability to degrade complex organics without excessive chemical input (Sathya et al., 2023). This system is widely deployed in industrial sectors including poultry processing (Baker et al., 2020). Poultry wastewater exhibits fluctuating nutrient conditions and also high contamination, impacting biological treatment processes. This variability can hinder the growth of microorganisms essential for effective treatment, necessitating adaptive treatment strategies (Tirapanampai et al., 2022; Odone et al., 2024). The organic load in poultry wastewater is significantly high leading to challenges in maintaining optimal treatment conditions.

2.4.1 Bacteria-Based Wastewater Treatment

Bacterial methods have been foundational in biological wastewater treatment due to their cost-effectiveness and efficiency in breaking down organic pollutants (Aragaw, 2021; Thirumalaivasan et al., 2024). These systems rely on microorganisms, primarily bacteria, to decompose organic matter through both aerobic and anaerobic processes.

The conventional treatment method for slaughterhouse wastewater mirrors that of municipal wastewater treatment, comprising primary, secondary, and tertiary stages. During the primary stage, approximately 60-70% of solids and 80-90% of fats are removed, resulting in a reduction of biological oxygen demand (BOD) by 30-35% (Salam et al., 2022). In the secondary phase, biological oxidation which may be aerobic, anaerobic or combination of both are employed to further degrade organic pollutants (Vidal et al., 2019). In tertiary treatment, sedimentation is used to allow suspended solids to settle, with the remaining liquid being disinfected using ultraviolet (UV) treatment or chlorine before discharge into water bodies (Kothari et al., 2024). Anaerobic digestion is often preferred for treating slaughterhouse wastewater due to its lower equipment requirements, but it is often followed by aerobic treatment to remove residual pollutants, as anaerobic treatment alone may not be sufficient for complete pollutant removal (Verstraete et al., 2022; Lawal et al., 2023: dos Santos Pereira et al., 2024).

Anaerobic digestion, which occurs in the absence of oxygen, breaks down organic compounds into methane, CO_2, and water (Induchoodan, Haq and Kalamdhad, 2022). This process can be performed in low-rate systems such as ponds or high-rate reactors, with the effectiveness of anaerobic digestion depending on the type and configuration of the reactor (Van Lier et al., 2015; Baker et al., 2020). Anaerobic digestion is characterized by low sludge production and minimal energy requirements but needs to be combined with other treatments to achieve high performance in nutrient and organic matter removal. Few studies proved that batch reactors with anaerobic digestion followed by other method such as solar photoelectron-Fenton treatment have demonstrated up to 95% removal efficiency, while down-flow expanded granular bed reactors (DEGBR) can achieve over 95% removal during peak performance days (Vidal, Huiliñir and Salazar, 2016; Njoya et al., 2021). Another study by Yao et al. (2023) proved that by using an integrated anaerobic–aerobic treatment approach, up to 99% COD removal was achieved from a high-strength stream, initially containing 20,862 mg COD/L. Similarly suggested by Li et al. (2008), intermittently aerated sequencing batch reactors (SBRs) alternate aeration and mixing phases to achieve efficient nitrogen and phosphorus removal, where ammonium is partially oxidized to nitrite during aeration and reduced to nitrogen gas during mixing, reducing oxygen and COD demands while enhancing biological phosphorus removal.

The four main processes in anaerobic digestion are: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Which these processes break down organic carbon into methane and carbon dioxide and slightly increase the nitrogen in the medium (Induchoodan, Haq and Kalamdhad, 2022). However, the anaerobic ammonia oxidation (anammox) and denitrification bacteria is a promising biological nitrogen removal technology with the benefits of high nitrogen removal efficiency for anaerobic wastewater treatment (Chen, Guo and Li, 2022). A study by Liu et al. (2022) showed that denitrification achieved a 100% removal efficiency of nitrite nitrogen and a 71% removal efficiency of total organic carbon, while anammox demonstrated a 93% removal efficiency of nitrate nitrogen and a 55% removal efficiency of ammonium nitrogen in aqua pond. However, as food wastewater or slaughterhouse characterized as high in nitrogen content hence need to couple with other treatment for high removal efficiency.

In contrast, aerobic digestion utilizes oxygen to decompose organic matter into biomass and carbon dioxide. While aerobic systems are effective at breaking down organic pollutants, they generate large amounts of sludge and require significant energy for aeration (Qamar et al., 2022; Chi et al., 2023). As a result, aerobic treatment is often employed as a post-treatment following anaerobic processes to remove remaining contaminants. Furthermore, despite their effectiveness, recent advancements in aerobic treatment methods for slaughterhouse wastewater have been limited due to these operational costs (dos Santos Pereira et al., 2024; Sravan, Matsakas and Sarkar, 2024). Aerobic systems such as aerobic AS, rotating biological contractors (RBCc) and sequencing batch reactors (SBRs) rely on oxygen for the degradation of organic matter but require large land areas and long retention times, particularly in the case of aerobic treatment (Bustillo-Lecompte and Mehrvar, 2017; Shen et al., 2019). In aerobic systems, nitrifying bacteria such as *Nitrosomonas* and *Nitrobacter* convert ammonia into nitrate and nitrite as part of the nitrogen cycle (Ahmad et al., 2021b). Denitrifying bacteria subsequently convert nitrates into nitrogen gas, removing nitrogen from the effluent. Phosphorus removal is typically facilitated by phosphorus-accumulating organisms (PAOs), which store phosphorus intracellularly (Chen et al., 2021).

However, bacterial systems face several challenges, particularly when treating high-strength effluents like poultry wastewater, which contains high concentrations of ammonia and phosphates. Higher ammonia exerts an inhibitory effect on the activity of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), affecting both their catabolic and anabolic processes, which has been studied extensively as a method for achieving partial nitrification by selectively inhibiting AOB and NOB during aerobic nitrification, and this inhibition is crucial in decoupling AOB and NOB activities (Sun et al., 2021). In addition to ammonia inhibition, bacterial systems also struggle with nitrite accumulation. AOB, with a stronger affinity for oxygen than NOB, utilizes accumulated nitrite as an electron acceptor under oxygen-limited conditions, promoting N_2O emission and conserving oxygen needed for ammonia oxidation to hydroxylamine. High N_2O production was linked to the accumulation of nitrite, which inhibited further nitrification, with free nitric acid generated by nitrite playing a more significant role in N_2O emissions than nitrite itself (Thakur and Medhi, 2019).

However, many wastewater treatment facilities, especially in developing countries, face significant financial constraints, which hinder the adoption of modern treatment technologies and infrastructure. Even when advanced technologies are available, inadequate maintenance can lead to system failures, such as equipment breakdowns and pipeline blockages, which decrease operational efficiency and increase costs (Nunes, 2021; Gulfam-E-Jannat et al., 2023). In this context, aerobic and anaerobic digestion methods remain common choices for wastewater treatment. Yet, in developing countries, the disposal of slaughterhouse effluents presents both environmental and economic challenges. Financial limitations further obstruct the implementation of effective treatment technologies (Bhunia, Bhowmik and Mukherjee, 2022). Moreover, with many slaughterhouses located in rural, unsupervised areas, untreated waste is often discharged into the environment, contributing to increased disease transmission risks (Bhunia, Bhowmik and Mukherjee, 2022).

Bacterial treatment systems are commonly used for wastewater but can result in carbon emissions and excessive sludge. In contrast to conventional treatment method, microalgae especially *C. vulgaris,* provide a better alternative for wastewater treatment by efficiently removing pollutants and producing valuable biomass.

2.4.2 Microalgae-Based Wastewater Treatment

Microalgae-based wastewater treatment has emerged as a sustainable and multifunctional alternative to conventional bacterial systems. Unlike bacteria, which rely on complex nitrification-denitrification pathways and often generate excess sludge, microalgae directly assimilate nitrogen and phosphorus into cellular biomass while capturing CO_2 and producing oxygen through photosynthesis (Yolanda et al., 2025). This biologically integrated mechanism not only reduces operational energy demand but also fosters a symbiotic relationship with coexisting bacteria in consortia systems, where microalgal oxygen production supports bacterial activity, thereby enhancing system stability and resilience (Abate et al., 2024). A study on cultivating *Chlorella variabilis* and *Chlorella sorokiniana* in tannery wastewater demonstrated that using microalgae could reduce energy consumption for tannery wastewater treatment by up to 80% (Nagi et al., 2020). Several microalgae species efficiently remove over 80% of ammonia, nitrate, and total phosphorus from wastewater, storing excess nutrients in their cells during growth for future use in cell synthesis (Amaro et al., 2023; Wang et al., 2024). Microalgae cultures offer an efficient biological treatment solution by using inorganic nitrogen and phosphorus for growth, while also removing heavy metals and toxic organic compounds, thereby preventing secondary pollution (Abdel-Raouf, Al-Homaidan and Ibraheem, 2012).

In contrast to bacterial systems, microalgae do not produce pathogenic sludge. Instead, they generate biomass rich in lipids, proteins, and carbohydrates, which can be valorized into biofuels, animal feed, or biofertilizers (Nishshanka et al., 2023). Given the current global crises in energy, environmental degradation, and economic instability, microalgae biorefineries offer a viable solution for integrated remediation and resource recovery (Russell, Rodriguez and Yaseen, 2022).

However, the efficiency of microalgal treatment systems is highly sensitive to environmental and operational factors such as photoperiod, inoculum size, nutrient ratios, and light intensity. Unlike synthetic media, poultry wastewater presents additional constraints including light attenuation, turbidity, and elevated organic loads, which complicate phototrophic activity and nutrient uptake (Audelia, Ratnaningsih and Tazkiaturrizki, 2023). While microalgae produce oxygen during daylight hours, their respiration at night reduces dissolved oxygen below the critical threshold of 2 mg/L (Ali and Mishra, 2022). Following algal death, microbial decomposition intensifies oxygen depletion and releases previously assimilated nutrients, thereby perpetuating the eutrophication process (Akinnawo, 2023). Hence, an optimal condition of microalgae to perform an efficient wastewater treatment need to be considered.

Moreover, a study by Zhao et al. (2022) found that *Scenedesmus sp.* LX1 exhibited high biomass in sterilized wastewater, while non-sterilized wastewater demonstrated higher pollutant removal rates, achieving COD, nitrogen, ammonium, and phosphorus removal efficiencies of 86.92%, 60.75%, 71.81%, and 96.13%, respectively, along with 44.57% protein accumulation. Microalgae compete with local bacteria for nutrients, but at high inoculum densities, they dominate, and when bacteria and microalgae coexist, bacteria aid in nutrient removal and microalgal growth by improving the nutrient ratio for better assimilation (Nagabalaji et al., 2023). This research evaluates the performance of microalgae under these non-sterile, secondary wastewater conditions to assess its suitability for real-world applications.

A considerable body of literature supports the use of nutrient-rich wastewater for microalgae cultivation, as opposed to synthetic media, highlighting its potential to enhance both the sustainability and economic viability of microalgae production (Chakraborty, Gayen and Bhowmick, 2023). Microalgae assimilate nitrogen, phosphorus, and organic carbon into biomass through photosynthesis and mixotrophy, producing oxygen that supports bacterial co-metabolism while sequestering CO_2. These systems generate no secondary sludge, require less aeration, and produce biomass that can be valorized into biofuels or fertilizers, contributing to circular bioeconomy goals (Sarma et al., 2021)

This study utilizes post-secondary poultry wastewater, in which the organic load is partially reduced, making it an ideal matrix for evaluating advanced biological treatment efficiency under real, high-strength effluent conditions. The investigation specifically compares algae-based systems to traditional bacterial approaches, with the aim of identifying a more cost-effective, nutrient-polishing, and bioenergy-generating solution suited to Malaysia's poultry industry. In summary, microalgae-based systems, particularly those employing *C. vulgaris*, represent a promising, cost-efficient, and ecologically sustainable solution for nutrient-rich, high-strength wastewaters such as those generated in poultry processing.

2.4.2.1 *Chlorella vulgaris* in Wastewater Treatment

Among various microalgal species, *C. vulgaris* emerges as a resilient and fast-growing candidate for wastewater treatment applications (Al-Hammadi and Güngörmüşler, 2024). Its remarkable tolerance to elevated nitrogen concentrations, adaptability under fluctuating light and nutrient conditions, and capacity for lipid synthesis make it particularly suited for integrating pollutant remediation with biomass valorization. The chosen microalgae should demonstrate rapid growth, high nutrient and pollutant removal capacity, and strong adaptability, with *C. vulgaris* showing considerable potential for bioremediation due to its robustness, nutrient removal ability, high growth rate under adverse conditions, and tolerance to heavy metals and toxic compounds in different type of wastewater (Katırcıoğlu

Sınmaz, Erden and Şengil, 2023; Maia et al., 2025). A study by Wei et al. (2024) demonstrated, seven microalgal species were cultivated in vegetable processing wastewater, with *C. vulgaris* FACHB 8 exhibiting the highest adaptability and purification performance, achieving over 92% removal of nitrogen, phosphorus, and carbon. Furthermore, this microalga has metabolic plasticity, being able to grow in autotrophic, heterotrophic, and mixotrophic conditions (Znad et al., 2018; Li et al., 2019a; Sousa et al., 2021). *C. vulgaris,* high ammoniacal nitrogen concentration (110 mg/L), can be toxic to microalgae (Svierzoski et al., 2021). Strategies such as using high initial inoculum, promoting fast growth rates, and acclimating microalgal strains can reduce bacterial contamination by 80%, ensuring sustained growth while minimizing contamination risks in microalgal-based wastewater treatment, and enhancing *Chlorella sp.*'s tolerance to high ammonia levels (up to 1000 mg/L) (Chen et al., 2022a; Sutherland, 2022: Shayesteh et al., 2022).

The tropical climate of Malaysia, with average ambient temperatures near 30°C and consistent approximate 12-hour daylight cycles, creates an ideal environment for cost-effective cultivation of *C. vulgaris*. A study Cardozo et al. (2025) and Siqueira et al. (2020) proved that microalgae show high growth in tropical climates with an optimal range of 20 to 30 degree Celsius. These natural conditions reduce energy requirements for heating and artificial lighting, and the alga's ability to thrive in non-sterile environments further simplifies infrastructure needs by utilizing the indigenous microbial communities present in real wastewater streams.

C. vulgaris metabolic flexibility under mixotrophic growth allows it to assimilate both light and organic substrates simultaneously, accelerating biomass generation and pollutant uptake (Nurul Shafiqa et al., 2021). *C. vulgaris* in poultry wastewater treatment not only enhances ammonium removal and bacterial activity but its biomass can be further be used as fertilizer, feed for animal and biofuel, however conventional wastewater treatments produce high sludge output, with sludge treatment and disposal costs accounting for up to 60% of the total wastewater treatment expenses (Ali et al., 2022).

2.4.3 Operational Parameters Influencing Microalgal Efficiency

Following the characterization of *C. vulgaris* potential in poultry wastewater, it becomes essential to consider the operational factors that govern its treatment performance. Parameters such as light conditions, nutrient content, inoculum concentration, and acclimation protocols directly influence nutrient uptake rates, photosynthetic activity, and biomass yield. These factors collectively determine the physiological stability and scalability of microalgae-based systems.

Unlike conventional treatment technologies, microalgal processes are deeply sensitive to environmental fluctuations. Key physiological responses such as chlorophyll synthesis, antioxidant defense, and carbon metabolism are modulated by light quality, carbon inputs, and stress (González-Vega et al., 2021; Thabet et al., 2023). Thus, optimizing these variables is essential, especially in poultry wastewater with its unique challenges like high turbidity, fluctuating nutrient loads, and dense organic matrices.

Inoculum size plays a particularly critical role during initial cultivation. Low inoculum densities can prolong the lag phase and hinder resilience against ammonia toxicity, whereas excessive densities cause self-shading and reduce light penetration (Li et al., 2017; Abdul-Sani et al., 2024). Achieving an optimal biomass seed concentration is therefore key to initiating stable growth under real wastewater conditions. Tropical climate offers ideal average temperatures (~30°C) and consistent daylight, variability in solar intensity (Cardozo et al., 2025). The opaque nature of poultry effluent often limit effective light delivery. These conditions magnify the influence of small shifts in operational input, underscoring the need for site-specific calibration.

Despite extensive litreature on individual parameters, many existing studies isolate variables such as photoperiod or light intensity in synthetic or sterile media. This reductionist approach neglects the complex, interactive effects seen in real-world effluent systems. Consequently, translating lab-scale findings to industrial applications often yields inconsistent results. This study addresses that gap by evaluating multiple operational parameters in tandem within real, high-strength poultry wastewater. By simultaneously assessing photoperiod, light source, inoculum concentration, and acclimation protocols, it aims to identify synergistic effects that maximize nutrient removal and minimize retention time. Such an integrated strategy is essential for scaling microalgae-based treatment to field conditions while maintaining energy efficiency and economic viability.

2.4.3.1 Photoperiod

Photoperiod the duration of light and dark cycles is a critical factor regulating the photosynthetic behaviour, metabolic balance, and nutrient assimilation capacity of microalgae such as *C. vulgaris*. The photoperiod governs essential cellular processes like carbon fixation, lipid biosynthesis, antioxidative responses, and overall culture stability, especially under nutrient-intensive conditions like poultry wastewater (Choi and Lee, 2012; Nurul Shafiqa et al., 2021) . The light-dark cycle is crucial for microalgae, as photosynthesis occurs in two phases: the light-dependent photochemical phase, where light synthesizes ATP and NADPH, and the dark phase, where these molecules are used to synthesize metabolites (Li et al., 2020a).

Continuous illumination (24:0 light-dark cycle) often accelerates biomass accumulation and nutrient uptake in synthetic systems by maximizing light availability. This maximized light exposure can enhance growth rates, making it effective for systems where rapid biomass production is the primary goal. However, in real-world systems such as poultry wastewater treatment, which involves high nutrient levels, environmental stress, and imbalanced conditions. Without a recovery period during the dark phase, microalgae may experience oxidative stress, with intermittent light-dark cycles help maintain chlorophyll integrity and enhance resilience, improving nutrient removal efficiency (Li et al., 2020b; Nisbett et al., 2023). Experimental data from non-sterile citrus effluent proved that microalgae maintained well under a 12:12 photoperiod achieved 81% nitrogen removal and 86% COD removal, while sustaining biomass productivity (Varela Bonissone et al., 2023). Another study by Ferreira, Machado and Lobo (2021) demonstrated that the 24-hour cycle achieved 67.6% removal for soluble phosphorus, 94.0% for ammoniacal nitrogen, and 63.7% for COD in the treatment of constructed wetlands. Hence, this suggests that the effectiveness of light exposure periods may vary with the characteristics of the wastewater, highlighting the need for tailored photoperiod strategies for different wastewater types.

Despite these advantages, most prior studies on photoperiod optimization rely on sterile or synthetic media, often overlooking the complexities introduced by real poultry effluents. Turbidity, organic load, and microbial interactions in such systems require more robust experimental validation to replicate lab-scale benefits in real-world applications. Accordingly, this study evaluates 12:12 and 24:0 photoperiods under actual field conditions to determine their impact on biomass yield, nutrient removal kinetics, and lipid content. These insights are intended to guide optimization of retention time and treatment efficacy in scalable microalgal systems.

2.4.3.2 Light Source (LED vs. Natural Sunlight)

The selection of an appropriate light source is fundamental to optimizing microalgal growth and nutrient removal in wastewater treatment. Light acts as the primary energy input for phototrophic and mixotrophic systems, influencing pigment synthesis, photosynthetic rate, and metabolic productivity in species like *C. vulgaris* (Esteves et al., 2025b).

Light-emitting diode (LED) systems provide precise control over intensity and wavelength, enabling tailored lighting strategies that can enhance photosynthetic efficiency. a study by (Stevčić, Pulkkinen and Pirhonen, 2019). LED light support microalgae growth and enhance nitrate and orthophosphate removal 96% and 94% respectively from aquaculture system wastewater (Couto et al., 2022). LEDs also offer a consistent light profile regardless of weather, which benefits indoor reactors or high-value production systems. However, they are energy-intensive, with conversion efficiencies of just 6–8% and operational costs estimated at USD 15.19 per kg of dry biomass (Schulze et al., 2016). These constraints often limit their scalability in large-volume, low-margin wastewater applications. Although LED systems typically have a higher initial installation cost, they are more cost-effective in the long run compared to other artificial light sources due to their greater energy efficiency and longer operational lifespan (Sajjadi et al., 2018; Wan Mahari et al., 2024).

Natural sunlight, on the other hand, is abundant and free in equatorial regions like Malaysia. Natural daylight night cycle year-round, not only biologically effective but also operationally feasible in outdoor systems (Sarker and Salam, 2019). Outdoor systems harnessing solar radiation have demonstrated strong performance, achieving COD removal at 91.2% in liquid digestate (Sobolewska, Borowski and Nowicka-Krawczyk, 2023). Sunlight-exposed cultures also exhibit higher chlorophyll-a levels and biomass productivity, owing to full-spectrum illumination and better CO_2 fixation under fluctuating diurnal cycles. However, sunlight availability is affected by cloud cover, seasonal shifts, and wastewater turbidity, which reduce light penetration and system predictability (Jargal and An, 2023). Conversely, sunlight-based systems are well-aligned with large-scale, cost-effective wastewater treatment but require design strategies that compensate for light variability.

Despite the promising results in lab environments, few comparative studies have validated LED vs. sunlight systems under real poultry wastewater conditions. Effluent turbidity, organic particulates, and complex microbial communities introduce optical challenges that are absent in synthetic setups. Therefore, this study investigates both light sources under field conditions to assess their respective impacts on biomass growth, nutrient removal, pigment production, and energy consumption. The goal is to identify a lighting strategy that maximizes treatment efficiency while minimizing operational cost within Malaysia's agro-industrial landscape.

2.4.3.3 Inoculum Size

Inoculum concentration is a key yet often under-optimized variable in microalgae-based wastewater treatment, particularly when operating under the complex and dynamic conditions of high-strength poultry effluents. It directly impacts light availability, nutrient uptake kinetics, and culture stability during the initial growth phase (Almomani, 2020; Krichen et al., 2021; Singh and Mishra, 2022). Overly dense cultures may cause self-shading and hinder light penetration, leading to reduced photosynthetic activity, pH drift, and inefficient nutrient utilization (Saccardo, Bezzo and Sforza, 2022; Yahaya, Yeo and Nandong, 2025). High inoculum size can remove about 80% of biological contaminants in anaerobically digested abattoir effluent and 75% COD removal in high strength wastewater with initial COD approximate 6000 mg/L (Shayesteh et al., 2022; Zhai et al., 2022a). Bacterial competition for nutrients with microalgae has been shown to enhance lipid accumulation, which can be beneficial for biofuel production. However, further research is needed to understand and manage this interaction to prevent the displacement of microalgae over time (Luo et al., 2019).

However, with high optimal microalgae inoculum size have shown benefits during early-stage cultivation by promoting quicker acclimation to wastewater and reducing lag phase before exponential growth begins. This has been observed in poultry wastewater systems where faster stabilization correlates with improved nitrogen removal and biomass productivity (Guo et al., 2020a). Optimal inoculum density must be tailored to the specific effluent matrix, as high-strength and low-strength wastewater require different inoculum sizes. High-strength effluents typically demand longer treatment times (Greses et al., 2022). However, using an optimal high inoculum size can reduce the lag phase, accelerate microalgae growth, and decrease retention time. By adjusting inoculum size, nutrient removal can be faster, potentially reducing treatment time to just a few hours.

2.4.3.4 Acclimation

Acclimation is a critical yet frequently overlooked strategy for enhancing microalgal performance in nutrient-rich, toxic, or anaerobically digested wastewaters. When *C. vulgaris* is directly introduced to unfiltered poultry effluent, the combination of high ammonia, organic acids, and turbidity can trigger osmotic stress, metabolic inhibition, or cell lysis. Gradual exposure over several days has been shown to significantly increase survival, improve metabolic resilience, and accelerate nutrient uptake rates (Osundeko, Davies and Pittman, 2013; Zaini and Kasmuri, 2023). The acclimation process, also known as domestication, involves random genetic transformations over multiple generations, during which microalgae are exposed to high concentrations of wastewater, promoting the development of specific genetic mutations that enable a microalgae strain to adapt and survive in non-optimal conditions, such as those found in wastewater (Collos and Harrison, 2014; Wang et al., 2016; Ayre, Moheimani and Borowitzka, 2017; Jain, Mishra and Mohanty, 2022; Xiao et al., 2025).

During acclimation, the microalgae show enhanced chlorophyll-a synthesis and nitrate reductase activity, contributing to higher photosynthetic efficiency, higher growth rate and biomass productivity, and sustained growth in effluent conditions (Osundeko et al., 2014; Japar, Takriff and Mohd Yasin, 2021). Acclimation also enhances compatibility with other operational parameters such as photoperiod and inoculum size, as acclimation could reduce lag phase in microalgae culture. Microalgae's ability to tolerate high nitrogen levels is influenced by both the strain and the form of nitrogen in the medium. Free ammonia can penetrate algal cells and cause toxic effects, with inhibition typically occurring at concentrations above 61–96 mg/L (Rossi et al., 2020a). *C. vulgaris* is capable of withstanding ammonia concentrations of up to 220 mg/L; however, exceeding this limit results in a decline in cell viability to 61% (Zheng et al., 2019). Despite the toxicity of ammonium hindering microalgae's physiological activities, such as the conversion of ATP to ADP, they generally exhibit higher tolerance to ammonium due to their preference for it as a nitrogen source, with *C. vulgaris* tolerating nearly three times more ammonium (625 mg/L) than ammonia (Chai et al., 2021; López-Sánchez et al., 2022). When *Chlorella sorokiniana* was cultivated on concentrated human urine, ammonium concentrations exceeding 1400 mg/L were found to be inhibitory to the microalgae cells (Tuantet et al., 2014). *Chlorella thermophila* experienced growth inhibition at ammonium concentrations above 600 mg/L, which is typically inhibitory for this species, and fed-batch cultivation was considered a potential method to mitigate ammonium toxicity by controlling nutrient addition and preventing toxic buildup (Jain, Mishra and Mohanty, 2022).

Acclimation enables the development of resilient microalgal strains that can withstand environmental fluctuations, enhance nutrient removal efficiency, minimize the use of fresh water and costly sterilization treatments, and shorten culture times by reducing the lag phase, offering substantial long-term benefits despite its initial time investment.

While acclimation protocols are well-supported in controlled lab settings, their application to real poultry wastewater remains underexplored. Most available studies still rely on synthetic or partially sterilized media, limiting practical relevance. To address this gap, the current study incorporates an acclimation process using poultry wastewater, enabling the microalgae to adapt before the main treatment phase. The goal is to ensure culture robustness, reduce time to exponential growth, and improve nutrient removal kinetics under real-world constraints.

2.5 Microalgae Harvesting Method

Harvesting microalgal biomass remains one of the most technically demanding and cost-intensive steps in wastewater-based algal systems, accounting for as much as 20–30% of total operational expenditures. Unlike bacterial flocs, microalgae such as *C. vulgaris* possess small cell sizes (typically 2–10 µm), low densities, and negatively charged surfaces, which prevent natural sedimentation. This necessitates the use of mechanical, gravitational, or chemical separation methods to efficiently recover algal biomass from the culture medium (Ghazvini et al., 2022; Awan et al., 2024). Conventional solid-liquid separation processes are effective in removing suspended organic matter from its soluble fraction. These processes can be gravitational, mechanical, or chemical, involving methods such as sedimentation, filtration, centrifugation, and the use of flocculants and coagulants (Osabutey et al., 2023).

This section evaluates three primary harvesting strategies centrifugation, gravity sedimentation, and flocculation within the context of poultry wastewater treatment. Each method is analyzed based on its feasibility, cost, energy demand, and suitability for field-scale deployment in turbid, nutrient-rich effluents.

2.5.1 Centrifugation

Centrifugation is among the most widely adopted harvesting techniques in laboratory and pilot-scale microalgal studies. It utilizes centrifugal force to separate algal cells from the liquid phase, achieving recovery efficiencies of up to 99% within minutes, even at low biomass concentrations (Najjar and Abu-Shamleh, 2020). Typical rotor speeds range from 3,000 to 10,000 rpm, producing gravitational forces capable of rapid sediment even small, low-density algal cells. This makes centrifugation ideal for applications requiring precise and fast biomass separation, such as pigment extraction or strain isolation. However, the method is energy-intensive rendering it impractical for large-scale wastewater systems (Gerardo et al., 2015; Gupta, Pawar and Pandey, 2019; Bhatt et al., 2024). Another limitation is the mechanical stress imposed during high-speed rotation, which can damage delicate cell walls and release intracellular contents (Yin et al., 2020). This not only complicates downstream processing but also reduces biomass quality, particularly in biofuel applications where lipid integrity is critical.

2.5.2 Gravity Sedimentation

Gravity sedimentation is a low-cost and energy-free method traditionally used in primary wastewater treatment. It operates based on Stokes' Law, relying on density differences between suspended solids and the surrounding liquid to facilitate settling. However, due to their small size, low density (~1.05 g/L), and surface charge, microalgae like *C. vulgaris* exhibit poor natural sedimentation behaviour (Awan et al., 2024).

In poultry wastewater, sedimentation is further challenged by high turbidity and emulsified organic matter, which reduce visibility and alter fluid viscosity. Moreover, the strong negative zeta potential of algal cells (~ –20 to –30 mV) promotes electrostatic repulsion, preventing spontaneous aggregation and long-term settling (Toh et al., 2014). Despite these limitations, gravity sedimentation remains relevant when paired with upstream aggregation techniques such as flocculation or co-precipitation (Zhu, Li and Hiltunen, 2018; Yin et al., 2021). Once aggregated, algal flocs exhibit enhanced settling velocities and can be efficiently recovered using sedimentation tanks or inclined plate clarifiers. These features make it attractive for decentralized or rural operations seeking passive harvesting options.

2.5.3 Flocculation

Flocculation has emerged as the most promising methods for large-scale microalgae harvesting due to its scalability, energy efficiency, and compatibility with wastewater treatment systems. It works by aggregating suspended cells into larger flocs using charged compounds or polymers that neutralize cell surface charges and promote settling (Sharma and Arivalagan, 2021). Suspended culture has been the subject of more in-depth studies compared to attached culture (Wicker et al., 2023). Although attached cultures have demonstrated good nutrient removal and growth in current studies, their maintenance and application costs are significantly higher compared to suspended cultures.

Flocculants can be classified into three categories: inorganic coagulants (e.g., ferric chloride), synthetic polymers (e.g., polyacrylamide), and bioflocculants (e.g., chitosan, starch, microbial EPS). Among these, chitosan a biodegradable cationic polymer derived from chitin offers excellent performance in wastewater systems due to its dual function aggregating algal biomass and facilitating nutrient removal (Das, Ghosh and Pramanik, 2024). The total process efficiency achieved 95% COD, 96% TKN, and 91% TP removal, underscoring the combined effectiveness of chitosan flocculation and microalgal culture (Silva-Gálvez et al., 2024). A study by Li et al. (2022) showed that modified chitosan can adsorb phosphate and ammonium at 99.6% and 48.4% , respectively. Additionally, chitosan has not been commercially adopted due to insufficient scientific validation of its mechanism and efficiency. However, it generates non-toxic, biodegradable sludge with high nutritional value for microalgae culture, is eco-friendly, cost-effective, and produces sludge volumes five times lower than those created by inorganic salts (Kurniawan et al., 2020; Saiyad et al., 2024).

Given the current global crises in energy, environmental degradation, and economic instability, microalgae biorefineries offer a viable solution for integrated remediation and resource recovery (Russell, Rodriguez and Yaseen, 2022). Suspended-biomass reactors, where microalgae move freely, are widely used in industry due to their lower operational costs, whereas immobilized microalgae systems, though less common, have been shown to provide faster and more efficient harvesting of microalgae. (Plöhn et al., 2021; Han, Zhang and Ho, 2023). Hence, this demonstrates that optimizing microalgae-based wastewater treatment could enhance both treatment efficiency and harvesting performance, offering a more sustainable and cost-effective solution. Flocculation with chitosan provides an efficient, eco-friendly method for large-scale microalgae harvesting, offering excellent nutrient removal and low operational costs. Microalgae biorefineries using this approach present a sustainable solution for wastewater treatment and resource recovery, addressing global environmental and economic challenges.

2.6 Microalgae in Biofuel Production

Microalgae have gained substantial attention as a renewable source of biofuel due to their ability to accumulate energy-rich lipids, their fast growth rates, and their capacity to thrive in nutrient-laden wastewater streams. Among candidate species, *C. vulgaris* stands out for its versatility, scalability, and robust metabolic plasticity, making it ideal for integrated wastewater treatment and energy production.

Under nutrient-sufficient conditions, microalgae primarily channels assimilated carbon into growth-oriented pathways producing proteins, carbohydrates, and structural biomass. However, when subjected to nutrient limitation particularly nitrogen or phosphorus stress the alga initiates a shift toward triacylglycerol (TAG) accumulation in the cytoplasm, serving as a metabolic energy reserve (Zhang et al., 2025). This reprogramming is characterized by upregulation of lipid biosynthesis enzymes such as acetyl-CoA carboxylase (ACCase) and diacylglycerol acyltransferase (DGAT), downregulation of nitrogen assimilation enzymes, and morphological transformations in chloroplast and vacuole architecture (Fan et al., 2014; Song et al., 2024).

The production of microalgal lipids is considered a third generation "feedstock" of high interest, as containing up to 30% oil by weight of dry biomass, microalgae can yield nearly 10 times more oil than palm, which is considered the most efficient vegetable oil crop. (Sajjadi et al., 2018). Microalgae produce a variety of lipid-like compounds, with glycerolipids being the most abundant and well-described. Glycerolipids are divided into storage and structural lipids, with triacylglycerols (TAGs) being the primary form of storage lipids. TAGs, composed of fatty acids such as saturated and monounsaturated fatty acids, serve as an energy reserve during stress conditions like nutrient starvation, protecting the photosynthetic machinery and maintaining cellular functions (Morales, Aflalo and Bernard, 2021). These lipids, especially polyunsaturated fatty acids like EPA and DHA, are valuable for nutraceuticals and biofuels. The accumulation of TAGs occurs when there is an energy imbalance in the cell, preventing oxidative damage and maintaining cell integrity. TAGs are inert and store carbon and energy, which can be used for membrane synthesis and metabolism when conditions improve (Yang and Benning, 2018).

However, full-scale deployment of algal biofuel systems remains challenged by inconsistent lipid yields across seasons and wastewater batches, high energy inputs for harvesting, drying, and lipid extraction, and limited access to low-cost photobioreactor infrastructure (Zhang et al., 2014; Bharte and Desai, 2021).

2.6.1 *Chlorella vulgaris* in Biofuel Production

C. vulgaris has earned recognition as one of the most suitable microalgal species for biofuel production due to its rapid growth rate, resilience under varying environmental conditions, and high lipid accumulation potential under stress up to 81% lipid (Thajuddin et al., 2026). Its cultivation versatility across autotrophic, heterotrophic, and mixotrophic modes makes it particularly well-suited for integration into wastewater treatment systems that simultaneously serve as nutrient sources and metabolic stimulants.

The lipids produced under nitrogen or phosphorus starvation dominated by palmitic (C16:0), oleic (C18:1), and linoleic (C18:2) fatty acids conform to international biodiesel standards including ASTM D6751 and EN 14214, making *C. vulgaris* a viable substitute for conventional oil crops (khalaji, 2022). *Chlorella*, *Botryococcus*, *Scenedesmus*, *Dunaliella*, *Neochloris*, and *Nannochloris* are well-known for their ability to remove nutrients from livestock wastewater and their capacity for lipid accumulation. Notably, *Neochloris* and *Nannochloris* can accumulate up to 50% of their total dry weight in lipids under nitrogen starvation conditions (Kadir et al., 2018).

When cultivated in wastewater, *C. vulgaris* performs exceptionally well due to the simultaneous presence of high ammonia and organic carbon, which act both as nutrient resources and metabolic stressors (He et al., 2013; Gao et al., 2018; Xia et al., 2021). This dual effect stimulates both growth and lipid accumulation. These outcomes are often accompanied by enhanced carotenoid levels, indicating sustained photosynthetic activity even under stress (Xi, Kong and Chi, 2021; Sherin, Aswathi and Puthur, 2022).

Mixotrophic cultivation not only increases carbon availability but also improves energy balance within the cell, supporting faster growth and greater biochemical productivity. The inclusion of dark phases in the photoperiod further enables cells to recover from photooxidative stress and regulate nitrogen assimilation. These physiological advantages are particularly relevant in wastewater environments, where environmental stressors often fluctuate and nutrient loads are high (Chen et al., 2022b; Esteves et al., 2024).

Despite these advantages, field-scale implementation still faces several challenges, including variability in lipid yields due to effluent composition, high energy demands for dewatering and lipid extraction, and the capital costs of photobioreactor infrastructure. This study responds to these limitations by testing optimized cultivation strategies including photoperiod design, inoculum adjustment, light source selection, and acclimation within real poultry wastewater systems. The findings aim to confirm *C. vulgaris* as a robust, dual-purpose species capable of delivering both environmental remediation and bioenergy production at scale.

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Research Flowchart

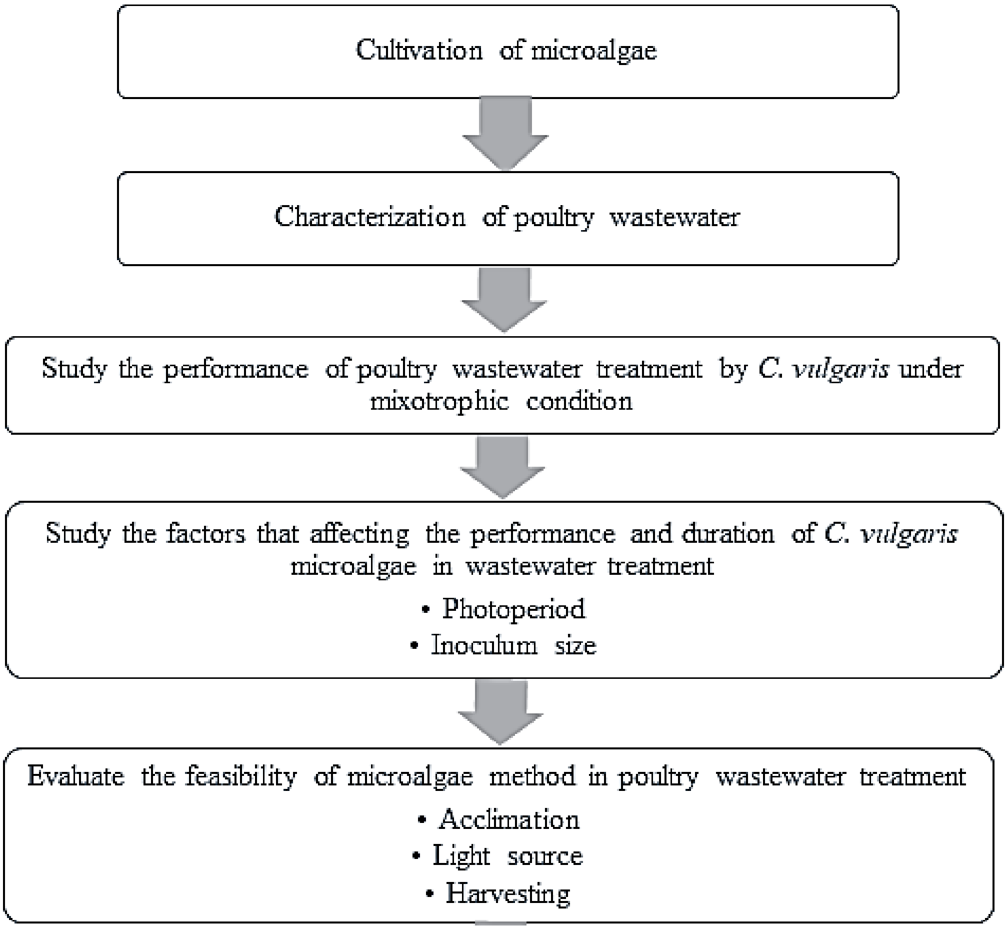

Figure 3. 1: The Research Flowchart

3.2 Materials and Chemicals, and Equipment

Materials/Chemicals	Suppliers/Sources
C. vulgaris Microalgae Strain Culture	Collection of Algae and Protozoa (CCAP)
Chitosan (50,000-190,0000 g/mol)[a]	Sigma-Aldrich
Acetic Acid (99.8 %)[b]	R&M Chemicals
Methanol (D99.9 %)b	Sigma-Aldrich
Chloroform (>99-99.4 %)[b]	R&M Chemicals
Nitrogen, Ammonia	HACH
Nitrate LR Reagent Vial	HACH
Reactive Phosphorus	HACH
COD Reagent Set, HR	HACH
Centrifuge	HERMLE Z326K
Ultrasonic Water Bath	Elma S180H Elmasoic
Ultraviolet-Visible (UV-Vis) spectrophotometer	HACH DR6000
Air pump	Big Boy
Digital Analytical Balance	Sartorius M-pact AX224
Magnetic stirrer plate	2mag MIX15 eco
Magnetic hotplate stirrer	Stuart SB162-3
Oven	Memmert UN110
COD Reactor	HACH DRB 200
Fumehood	IRYAS Laboratory Fumehood

3.3 Cultivation of C*hlorella vulgaris* Stock

The stock culture of *C. vulgaris* was cultivated in a fertilizer-enriched medium, maintaining a water-to-fertilizer volume ratio of 1:0.0005. Initially, 1 litre of the culture was placed in 2-litre bottles and cultivated under LED illumination at 3000 lux, with continuous aeration with flow rate of 4 L/min, for a period of 7 days at room temperature. Following the initial cultivation, the entire 1 litre culture was transferred to a 5 litres bottle, resulting in a final volume of 5 litres, where the culture was maintained for an additional 3 weeks under the same conditions.

Following cultivation, microalgae were harvested using chitosan flocculation. The culture was transferred to a bottle equipped with a magnetic stirrer, and 10 mg/L of chitosan was added into the microalgae sample at a volume ratio of 1:500 of chitosan: microalgae sample together stirred to ensure uniform distribution and effective flocculation. Once complete flocculation occurred, the mixture was allowed to settle. The supernatant was then removed, and the resulting concentrated microalgal paste was collected for subsequent experimental use.

3.4 Cultivation of *Chlorella vulgaris* in Wastewater under Mixotrophic Condition

The inoculum size, corresponding to 0.1 g/L of concentrated microalgae, was inoculated into 250 mL of poultry wastewater, with three replicates for each treatment. The cultures were maintained under continuous LED illumination at 3000 lux and continuous aeration supplied by an air pump using ambient air with flow rate maximum of 4 L/min. Biomass and nutrient evaluation samples were collected from all three replicates at designated intervals throughout the 12-day treatment period. The sampling process was repeated at different time points, with all three replicates harvested for each specific day of sampling.

3.5 The Performance of Wastewater Treatment by *Chlorella vulgaris*

3.5.1 Photoperiod

A total of 0.1 g/L of microalgae concentration was inoculated into 250 mL of poultry wastewater, with three replicates per treatment. Two light conditions were tested: continuous LED illumination at 3000 lux, and a 12-hour light/12-hour dark photoperiod. All other conditions, including continuous aeration and temperature, were maintained as described in Section 3.4. Biomass and nutrient samples were taken at designated time points over the 12-day treatment period, with three replicates collected at each harvest.

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3.5.2 Inoculum Size

A total of 0.13 g/L, 0.25 g/L and 0.34 g/L of C. vulgaris inoculum concentration, which made up by adding 2 ml, 3 ml, and 4 ml of microalgae paste with three replicates for each inoculum mass. The culture was maintained under the same conditions as described in Section 3.4, with 12:12 photoperiod, illumination at 3000 lux and continuous aeration. The cultivation period lasted for 4 days, and daily harvests were performed to collect samples for biomass and nutrient analysis. Three replicates were taken at each harvest to evaluate the growth and nutrient removal efficiency.

3.6 Feasibility Study on *Chlorella vulgaris* in Poultry Wastewater Treatment

3.6.1 Acclimation of Microalgae Stock to Poultry Wastewater

Two distinct pre-culture conditions were employed to prepare the inoculum for microalgae cultivation. Initially, *C. vulgaris* was cultured in a fertilizer nourished medium under continuous illumination (3000 lux) for a period of 7 days. After the pre-culture phase, 1 litre of the culture was transferred into a 5 litres bottle containing 4 litres of poultry processing wastewater, resulting in a final culture volume of 5 litres. Alternatively, another set of cultures was prepared by inoculating 1 litre of *C. vulgaris* into 4 litres of fertilizer nourished medium, with the culture maintained under the same light and temperature conditions for 7 days.

Following the pre-culture phase, 0.1 g/L of microalgae concentration from each culture was transferred into 250 mL of poultry wastewater, with three replicates per condition. The cultures were maintained under a 12-hour light/12-hour dark photoperiod, continuous aeration, and a light intensity of 3000 lux for a period of 4 days. Daily harvesting was conducted throughout the cultivation period to assess biomass production and nutrient removal efficiency, with the primary objective of evaluating the effects of the pre-culture medium on microalgal growth and pollutant removal in poultry wastewater.

3.6.2 Light source

C. vulgaris was cultured for 4 days in wastewater treatment under two different light sources. In the LED treatment, microalgae were exposed to continuous aeration and 3000 lux illumination under a 12-hour photoperiod. In the sunlight treatment, microalgae were cultured outdoor with continuous aeration and natural sunlight for the entire duration. Both treatments were maintained under similar conditions for the 4-days cultivation period.

3.6.3 Harvesting Methods

Microalgae wastewater treatment was conducted for 2 days, followed by harvesting using two methods: flocculation by chitosan and centrifugation, with three replicates for each method. For chitosan flocculation, 20 mg/L of chitosan was added to the sample at volume ratio of 1:500 for chitosan: poultry wastewater under magnetic stirring until flocculation occurred, and the mixture was left to settle for 60 minutes. The supernatant was evaluated for cell separation efficiency.

Cell separation efficiency was determined after 60 minutes of sedimentation. A 3.5 mL sample, collected approximately 1 mm below the liquid surface, was analysed for absorbance at 680 nm using a UV-Vis spectrophotometer. The cell separation efficiency was calculated using Equation 3.1:

$$\text{Cell separation efficiency} = \frac{ABS_o - ABS}{ABS_o - ABS_{centrifuged}} \times 100\% \qquad \text{(Equation3.1)}$$

Where ABS_o is the absorbance intensity of the medium, ABS is the absorbance intensity of the sample, and $ABS_{centrifuged}$ is the absorbance intensity of the centrifuged (clear) medium.

3.7 Measurement of Nutrient Concentrations in Wastewater

3.7.1 Measurement of Ammoniacal Nitrogen Concentration

The amount of ammoniacal nitrogen in the sample was measured using the HACH Method 8155. First, 10 ml of the sample and a blank sample were placed in separate vials. To each vial, ammonia salicylate powder was added, then the vials were shaken and left for 3 minutes to allow the reaction to begin. Afterward, ammonia cyanurate powder was added to each vial, and the vials were shaken again and left to react for 15 minutes. Then, the ammoniacal nitrogen was measured by UV-Vis spectrophotometer.

3.7.2 Measurement of Nitrite Concentration

To measure nitrite levels, Hach Method 8153 was followed using the NitriVer® 2 high-range nitrite reagent powder. First, 10 ml of the sample was added to a test vial, and the nitrite reagent powder was mixed in before sealing the vial. The vial was then shaken and allowed to react for 10 minutes. A blank sample of 10 ml was also prepared. Both the sample and the blank were placed into the UV-Vis spectrophotometer for analysis of the nitrite concentration.

3.7.3 Measurement of Nitrate Concentration

The nitrate content was measured using Hach Method 8039 with NitraVer 6 reagent powder. A 10 ml sample was placed in a test vial, to which the reagent powder was added before sealing the vial. The vial was then shaken for one minute while the UV-Vis spectrophotometer was started. Afterward, the vial was left for 5 minutes to allow the reaction to complete. A blank sample of 10 ml was also prepared, and both the sample and blank were analyzed in the UV-Vis spectrophotometer to determine the nitrate concentration.

3.7.4 Measurement of COD Concentration

The COD concentration was determined by using high range COD Digestion Vials according to HACH Method 8000. The temperature of DRB200 reactor was set to 150°C. To ensure proper mixing, the test vial was gently inverted a few times. Next, 2 mL of the sample was introduced into the vial, which was sealed and inverted again to ensure an even mixture. The vial was then placed into the COD reactor for two hours. Then, the vial was carefully shaken once more before being left to cool to ambient temperature. COD was measured by using UV-Vis spectrophotometer.

3.7.5 Measurement of Orthophosphate Concentration

The ortho-phosphate level was determined using HACH Method 8048 with the Reactive Phosphate TNT Reagent Set. A 5 mL portion of the sample was added to a test vial. This vial was sealed and gently shaken to ensure the contents mixed evenly. The vial was then placed into the UV-Vis spectrophotometer for calibration using the 535 P React. PV TNT program. After calibration, the PhosVer 3 Phosphate Powder Pillow was introduced into the vial and shaken for 20 seconds. The vial was left to react for 2 minutes. Afterward, the orthophosphate was measured by using the uv-spectrophotometer.

3.8 Measurement of Microalgae Biomass

Microalgae biomass was collected by centrifugation from a 250 mL sample. The biomass paste was then placed in an oven and left overnight at 60°C. Afterward, the biomass was weighed using a digital analytical balance and recorded in grams.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Characteristic of Poultry Wastewater

In this study, raw poultry wastewater was freshly collected from an industrial processing plant and characterized for key pollutants. The results are presented in comparison to typical discharge limits referenced from Malaysia's Environmental Quality (Industrial Effluent) Regulations 2009 (DOE, 2009):

Table 4. 1: Poultry Wastewater Composition vs. Discharge Limits

Parameter	**Measured Range (mg/L)**	**Regulatory Limit**	**Compliant**
COD	390 – 1500	200 mg/L	✗
Ammoniacal Nitrogen (NH_3–N)	20 – 52	20 mg/L	✗
Nitrate (NO_3^-)	40 – 120	No limit specified	–
Nitrite (NO_2^-)	200 – 800	No limit specified	–
Orthophosphate (PO_4^{3-})	43 – 123	No clear standard	–

4.2 Performance of *Chlorella vulgaris* in Wastewater Treatment under Mixotrophic Condition

In this study, *C. vulgaris* was cultivated for a period of three weeks to reach the stationary phase, ensuring maximum cell density before harvesting for wastewater treatment. Numerous studies recommend an inoculum size of 10vol% for microalgae, which has been shown to achieve optimal nutrient removal efficiency (Ansari et al., 2017; Elvitriana et al., 2018; Sobolewska et al., 2023; Ummalyma et al., 2023). However, a portion of the concentrated sludge will be recycled back to secondary treatment process in industrial practice to maintain the biomass concentration in wastewater, and thus, this practice will be demonstrated in this study. The chitosan flocculation was employed as a method for harvesting the microalgae from the stock culture. After harvesting, the microalgal biomass was concentrated, and the volume was adjusted to match the required 10% (v/v) of inoculum size for wastewater treatment. It was determined that 0.1 g/L of the biomass concentration was equivalent to a 10% (v/v) inoculum in a 250 mL wastewater volume. This adjustment ensured that the biomass density was sufficient to promote effective nutrient removal without overloading the system with excess culture medium or diluting the wastewater. From litreature review, the chitosan offers a more economical and practical solution compared to centrifugation (Gouveia et al., 2016; Acosta-Ferreira et al., 2020; Kumaran, Singh and Joseph, 2021; Xiao et al., 2025). Therefore, chitosan was selected in this study to harvest *C. vulgaris* from the stock culture and inoculate it into poultry wastewater for treatment evaluation, as discussed in detail in section 2.5.3 of the literature review.

Under mixotrophic conditions, *C. vulgaris* demonstrated an increase in biomass concentration, reaching 1.02 g/L after 12 days (Figure 4.1). As suggested by Park et al. (2024), the supply of light and carbon source together with suitable microalgal strain can significantly enhance biomass production. This is particularly in mixotrophic cultivation systems, where both light and organic and inorganic carbon sources contribute synergistically to accelerated growth. Result shows a steady increase in growth was observed from Day 0 to Day 2, corresponding to the early stages of exponential growth, with biomass concentration rising from 0.2155 g/L to 0.3715 g/L, marking a 72% of increment. From Day 5 to Day 7, the growth rate increased more sharply, marking the continuation microalgae growth. The most substantial biomass accumulation occurred between Days 7 to 12, suggesting a full adaptation of microalgae to the complex wastewater matrix. As the microalgae adapted to these conditions, growth rates improved substantially, indicating the resilience and adaptability of *C. vulgaris* under real wastewater conditions.

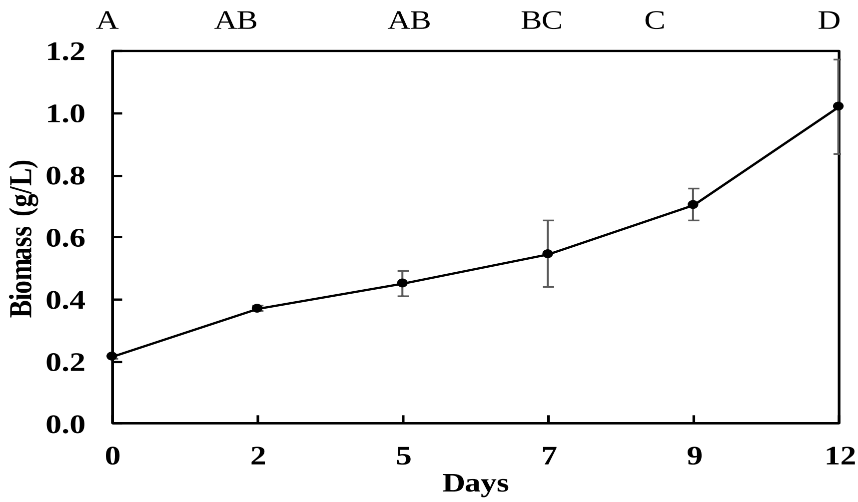

Figure 4. 1: Biomass Growth of *C. vulgaris* under Mixotrophic Conditions. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

When compare with the result of the study by Ummalyma et al. (2023), the biomass concentration of *C. vulgaris* at day 12 in this study was slightly lower than that of *Neochloris sp*. SK57 (1.43 g/L) and *Chlorella sp*. SL7A (1.31 g/L) which were grown in non-sterilized poultry wastewater but under natural sunlight. Natural sunlight offering full-spectrum light and fluctuating intensities is generally known to promote higher microalgal productivity compared to artificial illumination, such as LED grow light, that used in this study (Yustinadiar, Manurung and Suantika, 2020; Fariz-Salinas et al., 2024). Additionally, different source of poultry wastewater containing different content of suspended solids, nutrients, and organic matter that can hinder light penetration and nutrient availability, which may have contributed to different biomass yield with each other's (Chakraborty, Gayen and Bhowmick, 2023; Silva-Gálvez et al., 2024). On the other hand, study by Oliveira et al. (2019) showed that *Scenedesmus obliquus* reached a biomass concentration of 3.8 g/L in raw poultry effluent, which was attributed to the 2 times higher in COD content in the wastewater. This implies that higher COD content provides higher amount of organic carbon source to the microalgae growth and thus promotes higher biomass concentration. Similar condition for the study by Sudhanthiran and Perumalsamy (2022) demonstrated a biomass concentration of 2.43 g/L using *C. vulgaris* in dairy wastewater, which had a higher nutrient concentration than the poultry processing wastewater. Therefore, the *C. vulgaris* was proven can grow in poultry processing wastewater under mixotrophic condition.

In parallel with biomass growth, COD removal was also significant as shown in Figure 4.2. The initial COD concentration of poultry wastewater at 1500 mg/L reduced to 380 mg/L significantly by Day 2, resulting in a 75% removal efficiency. The COD levels stabilized after Day 2, indicating that most of the biodegradable organic matter was consumed during the early growth phase. This pattern demonstrates the effectiveness of mixotrophic cultivation, where the simultaneous utilization of organic and inorganic carbon under light accelerates microalgal growth and organic pollutant degradation. Research by Devi et al. (2022) reported that the COD removal efficiencies of approximately 75.6% under similar conditions, supporting the validity of the results obtained in this study. Organic carbon serves as not only an energy source but also a structural component for cell metabolism, reinforcing the observed linkage between biomass accumulation and COD removal (Wu, Hu and Wang, 2019).

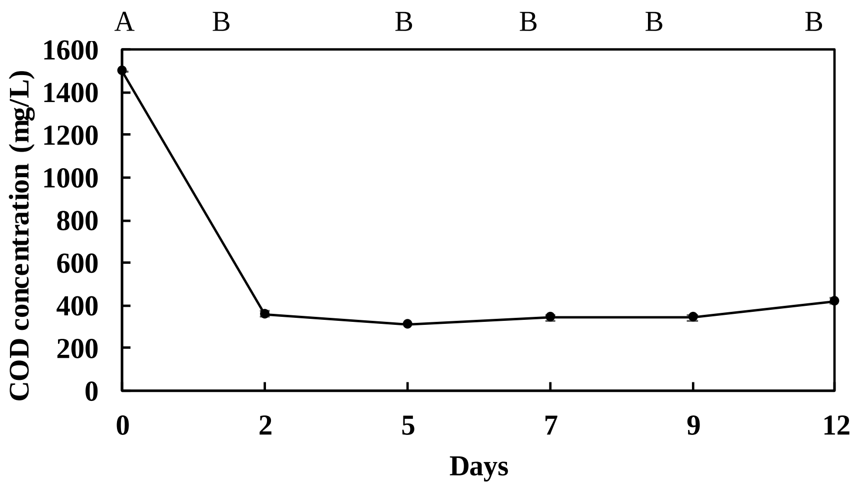

Figure 4. 2: COD Concentration in Wastewater under Mixotrophic Condition. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

The assimilation of nitrogen by microalgae occurs in the form of nitrates, nitrites, and mainly ammonium (Carletti et al., 2024). Nitrogen is one of the most prevalent constituents of microalgae after carbon and plays a crucial role in their metabolism, as it is a fundamental component of proteins, nucleic acids, chlorophyll, and other compounds (Shah et al., 2022; Li et al., 2025). The ammoniacal nitrogen concentration in poultry wastewater as shown in Figure 4.3 initially measured 40.0 mg/L at Day 0, increased slightly to 47.5 mg/L by Day 2. After that, the ammoniacal nitrogen rapidly decreased to 0.8 mg/L by Day 5, achieving nearly 98% removal. The concentration of ammoniacal nitrogen was continued to decline to 0.2 mg/L at Day 7 and eventually reached 0.0 mg/L by Day 9 and Day 12, indicating complete ammonium assimilation by the microalgal cells.

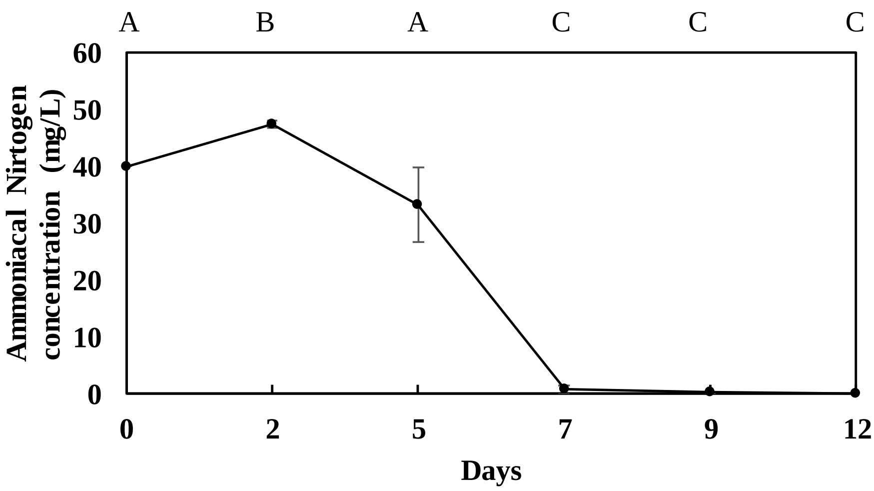

Figure 4. 3: Ammoniacal Nitrogen Concentration in Wastewater under Mixotrophic Condition. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

The initial increment of ammoniacal nitrogen from Day 0 to Day 2 is likely due to the ammoniacal nitrogen released from chitosan, organic matter, and the inoculum (Chan, Salsali and McBean, 2014; Silva et al., 2019). Chitosan, a flocculant used to harvest the microalgae, releases nutrients into the aqueous phase, which are subsequently absorbed by microalgae (Angelo, França and Faez, 2021; Zhu et al., 2021; Shi et al., 2024). The rapid removal of ammonia after Day 2 suggests that *C. vulgaris* entered an active ammonia assimilation phase once adapted to the wastewater, with ammonium being the preferred nitrogen form for growth (Geng et al., 2025; Xiao et al., 2025). This result highlights *C. vulgaris*'s strong ammoniacal nitrogen uptake capacity under mixotrophic conditions, where both heterotrophic and autotrophic metabolisms contribute synergistically to nutrient removal. This result suggests that mixotrophic conditions effectively facilitated ammonium uptake, making *C. vulgaris* an efficient nitrogen removal agent in wastewater treatment systems as discussed in literature review where microalgae can withstand the ammonium level up to 1300 mg/L with certain modification. These findings align with studies like Göncü, Şimşek Uygun and Atakan (2025) who reported high ammonium removal efficiencies in *C. vulgaris* and *Scenedesmus quadricauda*, with removal efficiencies of 83.47% and 80.28%, respectively. In this study, *C. vulgaris* achieved 99.5% of ammoniacal nitrogen removal by Day 7. Treatment of poultry wastewater with *Scenedesmus obliquus* over a 13-day cultivation period resulted in removal efficiencies exceeding 97% for ammonium removal (Oliveira et al., 2019). A study on *Chlorella pyrenoidosa* biofilms reported a 96% removal of ammoniacal nitrogen in mixotrophic biofilms, which is significantly higher than the removal rates in photoautotrophic or heterotrophic biofilms in synthetic wastewater (Wu et al., 2024).

Figure 4.4 showed that the nitrite concentration was initially 500 mg/L at Day 0, and a sharp decrease to 200 mg/L was observed by Day 2, representing a 60% reduction in the early stage of cultivation. Subsequently, the nitrite concentration increased gradually, reaching 250 mg/L on Day 5, 350 mg/L on Day 7, and peaking at 400 mg/L on Day 9 before slightly declining to 350 mg/L at Day 12.

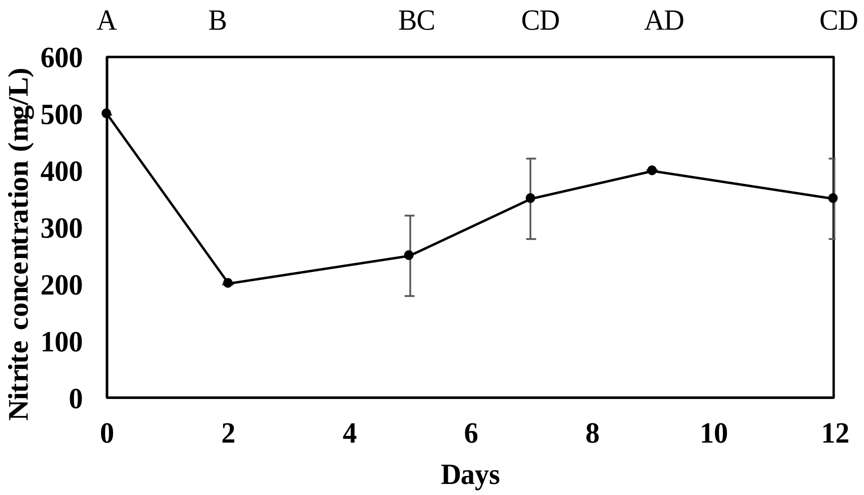

Figure 4. 4: Nitrite Concentration in Wastewater Under Mixotrophic Condition. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$..

The drastic reduction in nitrite concentration from Day 0 to Day 2 suggests that microalgae began actively assimilating nitrite as a nitrogen source early in the process. When compared with Figure 4.3, nitrite was assimilated by *C. vulgaris* before ammonia. The observed increase in biomass production indicates that while nitrite assimilation occurred, there may have been a threshold where excess nitrite started to become a stress factor. High nitrite concentrations can inhibit growth, with ammonium being preferred over nitrate for nitrogen assimilation after Day 2 (González-Camejo et al., 2020; Carletti et al., 2024).

Figure 4.5 showed that the nitrate concentration began at 40.0 mg/L on Day 0 and gradually increased to 65.0 mg/L at Day 5. After Day 5, the nitrate concentration slightly declined to 55.0 mg/L on Day 7 and 50.0 mg/L on Day 9, before rising again to 60.0 mg/L on Day 12.

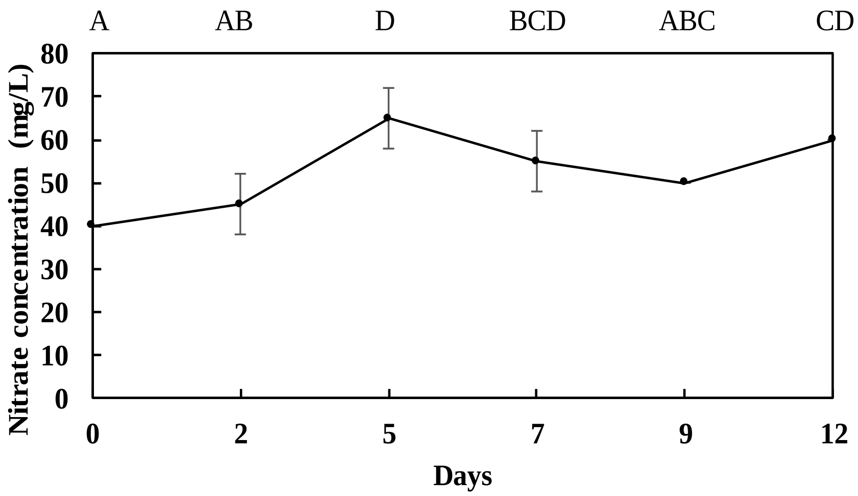

Figure 4. 5: Nitrate Concentration in Wastewater Under Mixotrophic Condition. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

When compare the result of ammoniacal nitrogen and the nitrite, nitrate was less favoured for assimilation. The increment of nitrite and nitrate from Day 2 to Day 5 suggests that nitrite and nitrate might be released from the degradation of organic nitrogen compounds in wastewater. Moreover, the slowdown in biomass growth between Days 2 to 7 (Figure 4.1) may be attributed to the algae adjusting to the high nitrite and nitrate levels. Despite these fluctuations, biomass continued to grow, indicating that the microalgae were resilient to nitrite and nitrate stress and capable of adapting to changing nitrogen sources to ammonia. The nitrite and nitrate enrichment likely caused stress, nutrient imbalance, or photoinhibition that contributed to cell death, which releases the nutrients back into the culture medium (Pandey et al., 2025). Despite the availability of light and carbon sources, nitrate levels remained high throughout the cultivation period due to nutrient imbalance, which prevented effective nitrogen removal and assimilation by *C. vulgaris*. Although nitrate removal is generally preferred over nitrite removal, microalgae typically assimilate ammonia and nitrite only when nitrate levels are low in this study (Kothari et al., 2013). In a study by Taziki, Ahmadzadeh and A. Murry (2016) showed that nitrate and nitrite concentrations of 800 and 150 mg L^{-1} (with ratio 16:3) produced maximum growth rate with 42% and 72% removal respectively. As this indicated a better nutrient removal efficiency the nitrate must be in higher ratio than nitrite while in this study the ratio nitrate to nitrite is 2:25. These findings indicate that nitrate uptake by *C. vulgaris* was limited under the given conditions, likely due to the preferential use of ammonium as the nitrogen source. Nitrite assimilation peaked on Day 2, after which was followed by ammoniacal nitrogen assimilation by *C. vulgaris* from Day 5 to Day 9. This suggests that microalgae favoured one nitrogen source at a time. This study showed that *C. vulgaris* achieved nearly 100% ammoniacal nitrogen removal from poultry wastewater by Day 12 highlights the efficiency of *C. vulgaris* in nitrogen removal, especially in mixotrophic cultivation.

Figure 4.6 showed that the orthophosphate concentration was 123.0 mg/L at the beginning of the cultivation (Day 0). An increase in concentration was observed reached to a peak value of 166.5 mg/L at Day 7. However, after Day 5, orthophosphate levels decreased significantly to 105.5 mg/L on Day 9 and 95.5 mg/L on Day 12.

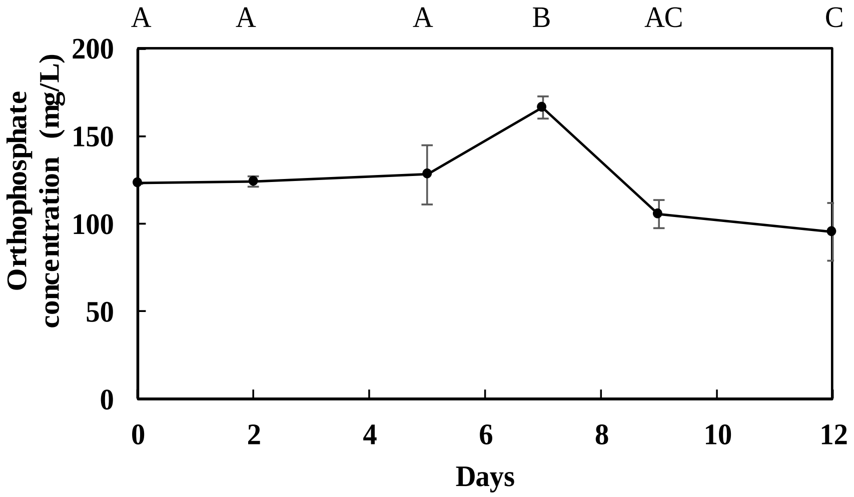

Figure 4. 6: Orthophosphate Concentration in Wastewater Under Mixotrophic Condition. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

Phosphorus compounds, such as polyphosphate and organophosphate, undergo hydrolysis to break down into orthophosphate and free phosphates, making them accessible for microorganisms (Özgür and Göncü, 2023). Phosphorus is essential for the synthesis of DNA, RNA, structural lipids (phospholipids), and compounds like adenosine triphosphate (ATP), which are involved in energy metabolism (Yaakob et al., 2021). Normally, Inorganic phosphorus typically exists in various forms, including orthophosphate, pyrophosphate, and polyphosphate. Orthophosphate is widely considered the primary form of phosphorus utilized by microalgae during growth (Cao et al., 2024). Orthophosphate serves as a key indicator of phosphorus removal and uptake by microalgae, being absorbed into the cells for metabolic use, with its decrease reflecting assimilation (Wei, 2024).

A peak value of 166.5 mg/L was recorded on Day 6, likely due to the release of bound phosphorus from organic matter decomposition and photoinhibition, which may cause microalgae apoptosis and nutrient release into the medium (Wu et al., 2021; Gao and Erdner, 2022; Lu et al., 2022). Despite the fluctuations, the final orthophosphate concentration at Day 12 reflects an approximate 22% reduction compared to the initial value. Orthophosphate uptake by *C. vulgaris* may have been constrained by nutrient imbalance in the wastewater. In a study by Zhai et al. (2022b) indicated mixing starch wastewater can remove 62.00% of total nitrogen (TN), 76.47% of total phosphorus (TP), with total nitrogen to total phosphorus is 13:1. This also indicates that a balanced nutrient ratio is important. Nutrient imbalance can also induce microalgae to accumulate storage compounds like carbohydrates and lipids as a strategy for survival (Gupta and Marchetti, 2024). Somehow the orthophosphate removal hindered due to high nitrite concentration in medium (Saito, Brdjanovic and van Loosdrecht, 2004). *C. vulgaris* treatment on household wastewater with no orthophosphate removal was due to imbalance nutrient ratio in wastewater (Göncü, Şimşek Uygun and Atakan, 2025) which is similar result is obtained from this study. It is concluded that the important point of this study is that nutrient balanced is important for maximum nutrient removal. In comparison to other cultivation modes, mixotrophic conditions provide supplementation of illumination and nutrients, which are important for biomass production as they are directly linked to orthophosphate assimilation (Sirajo and Zaini, 2023). Wu et al. (2021) showed that mixotrophic cultivation of *C. vulgaris* demonstrated superior phosphorus removal and transformation capacity compared to photoautotrophic and heterotrophic modes. After glucose supplementation in the mid-phase, it achieved complete orthophosphate removal and converted 79.32% of total phosphorus into intracellular organic forms, outperforming other modes in both efficiency and metabolic assimilation. These results suggest that mixotrophic conditions, when carefully balanced, offer a highly efficient approach for phosphorus removal in wastewater treatment systems.

This study highlights the *C. vulgaris* grow well in the poultry wastewater under mixotrophic condition and manage to remove the COD and ammoniacal nitrogen from the wastewater.

4.3 Performance Study on *Chlorella vulgaris* in Wastewater Treatment

4.3.1 Photoperiod

This section investigates how different durations of light exposure affect the performance and duration of *C. vulgaris* in treating poultry wastewater. Two light cycles: a 12:12 (L/D) cycle and a 24:0 L cycle were compared. The main objective of this experiment was to evaluate how variations in light exposure impact biomass growth and the efficiency of nutrient removal, specifically for Chemical Oxygen Demand (COD), ammoniacal nitrogen, orthophosphate, nitrate and nitrite.

The biomass data in Figure 4.7 clearly illustrates how microalgae growth varies between the 12:12 light cycle and continuous 24:0 light exposure, especially when considering the growth phases. For the 12:12 cycle, the biomass starts at 0.22 g/L and increase to 0.44 g/L by Day 9, followed by a decrease to 0.38 g/L at Day 12. For the 24:0 cycle, the biomass started at the same initial biomass of 0.22 g/L and increase to 0.82 g/L by Day 12.

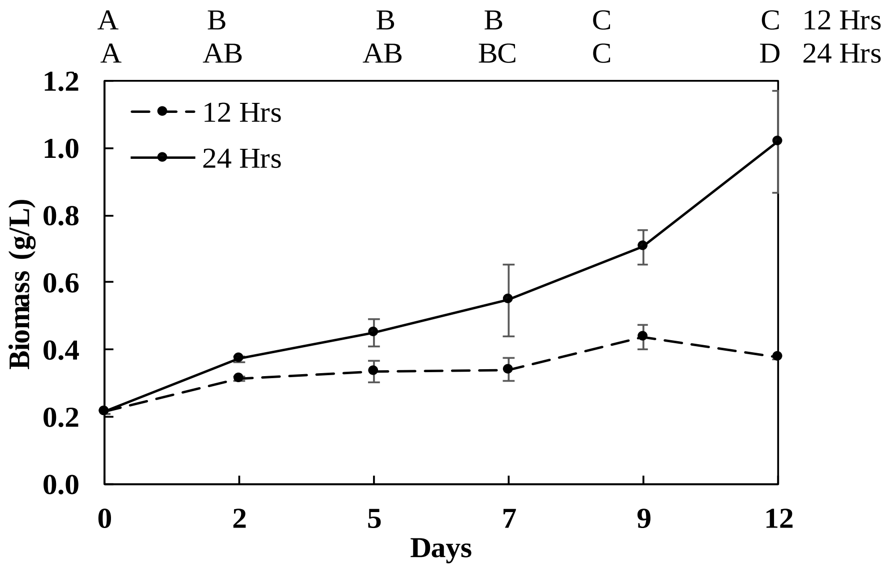

Figure 4. 7: Biomass Concentration of *C. vulgaris* in 12:12 and 24:0 Photoperiod. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

The 12:12 cycle showed a slower microalgae growth rate compared to the 24:0 cycle. In 24:0 cycle, the absence of the dark cycle allows for continuous photosynthesis and energy production, which accelerates growth (Esteves et al., 2025a). The 12:12 cycle showed slower growth rate under controlled light conditions. According to Rani and Ojha (2021) *Chlorella sorokiniana* cultivated in real tertiary wastewater exhibited markedly different biomass productivity under varying photoperiods, reaching 548 mg/L/day under continuous illumination (24:0), while under a 12:12 h light/dark cycle, it decreased to 319 mg/L/day. The reduced biomass under the 12:12 cycle was attributed to limited light availability, which restricted the photosynthetic and energy generation, as during the dark phase, the algae rely on respiration, consuming energy reserves without contributing to biomass synthesis. This alternating cycle may reduce the overall productivity of *C. vulgaris*, particularly in wastewater treatment, where continuous growth is beneficial. This study also noted growth oscillations in the 12:12 cycle biomass increased during the light phase but decreased during darkness, indicating net biomass loss during night hours. This comparison illustrates how extended light exposure enhances algal growth kinetics, while the alternating light-dark regime inherently limits net biomass accumulation due to recurring metabolic shifts and energy expenditure during non-productive dark intervals. For example, a similar study on the green microalga *Dunaliella salina* found that longer light periods led to accelerated growth rates and higher cell densities (Xu, Ibrahim and Harvey, 2016). Moreover, study by Muys et al. (2024) further support these findings, reporting a 50% increase in productivity of both *C. vulgaris* and *Limnospira indica* under continuous illumination.

Conversely, the prolonged photoperiod condition may result in a lower microalgal biomass due to the possible photo-inhibition phenomenon (Serrano-Bermúdez, Montenegro-Ruíz and Godoy-Silva, 2020). A study had reported lower cell concentrations in *Nannochloropsis sp*. when the photoperiod cycle was extended to 24:0h from 18:6h light/dark cycles, attributing to the growth photoinhibition effect (Sirisuk et al., 2018). Illumination intensity plays a crucial role in microalgal biomass production, not only by providing energy for photosynthesis but also by regulating nutrient absorption.

Microalgae require an optimal light intensity range for efficient growth, as both excessively high and low intensities can negatively affect their physiology low light intensity limits photons for photosynthesis, reducing nitrogen metabolism and growth, while excessive intensity leads to photoinhibition (Chen et al., 2023b; Li et al., 2024b; Meng et al., 2019). Balancing light intensity is key to optimizing microalgal productivity and nutrient uptake. The light intensity range typically applied in microalgae cultivation has been reported to vary between 1000 and 8000 lux, corresponding to an equivalent of 15 to 150 μmol m^{-2} s^{-1} (Devi and Parthiban, 2020). The study by Yaqoubnejad, Aminirad and Taghavijeloudar (2021) highlights that *C. sorokiniana pa.91* achieves a maximum biomass concentration of 1.61 g/L under 4000 Lux with a 12:12 photoperiod, while 3000 Lux also yields significant growth (1.41 g/L), demonstrating that moderate light intensities (3000–4000 Lux) optimize biomass production without photo-inhibition. While 4000 Lux provides the highest biomass, 3000

Lux offers a cost-effective alternative with lower energy consumption, making it suitable for large-scale industrial applications. In this study, the light cycle of 24:0 with intensity at 3000 lux promote high growth rate of *C. vulgaris* in the poultry wastewater.

Figure 4.8 demonstrates the impact of photoperiod onto the chemical oxygen demand (COD) removal efficiency by *C. vulgaris*. Initially, the COD concentration was at 1500 mg/L for both the 12:12 and 24:0 photoperiods, and both COD decreased rapidly to approximately 380 mg/L for the 12:12 exposure and 360 mg/L for the 24:0 cycle. There was no significant difference between the two photoperiods on COD removal.

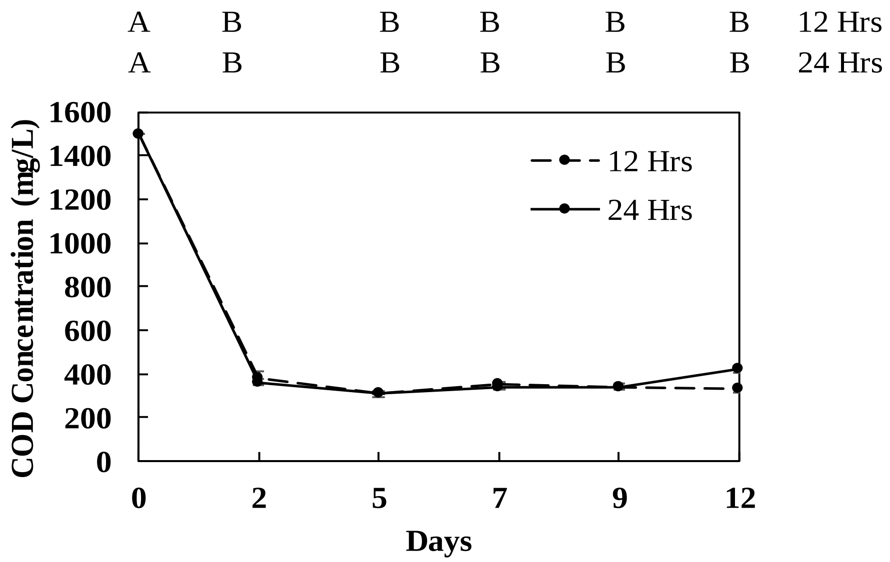

Figure 4. 8: COD Concentration in Wastewater under 12:12 and 24:0 Photoperiod. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

Notably, towards the end of the study, the COD concentration slightly increased under the 24:0 exposure, while the 12:12 exposure maintained its removal efficiency. This might be due to self-shading effect by the high concentration of microalgae when in the 24:0 cycle, where the death microalgae cells contribute to the increase in COD (Lee et al., 2019). A comparable COD removal trend was reported by Leong et al. (2022) where both the 12:12 and 24:0 exposures resulted in a rapid reduction in COD during the first two days, accounting for up to 88% of the total removal in municipal wastewater treated with *C. vulgaris*. After this initial drop, the COD concentrations remained relatively stable until Day 14, with final COD removal efficiencies of 92.6% for the 12:12 photoperiod and 95.0% for the 24:0 cycle, indicating only a minor difference between the two. This aligns with the present study's findings. Therefore, the 12:12 cycle offers a more cost-effective and sustainable solution, particularly for large-scale wastewater treatment systems, as it reduces energy consumption without compromising COD removal efficiency.

Figure 4.9 showed the removal of ammoniacal nitrogen under the 12:12 and 24:0 photoperiods. Initial ammoniacal nitrogen concentrations were at 40.0 mg/L for both treatments. For the 12:12 cycle, ammoniacal nitrogen removal occurred steadily, with a significant 98% reduction by Day 9 and 100% reduction by day 12, which indicating steady metabolic assimilation. Conversely, under the 24:0 photoperiod, ammoniacal nitrogen removal showed an initial increase in concentration by Day 2 (to 47.5 mg/L) and by Day 12, 100% removal of ammonium was achieved by Day 12, which is similar to the 12:12 photoperiod condition.

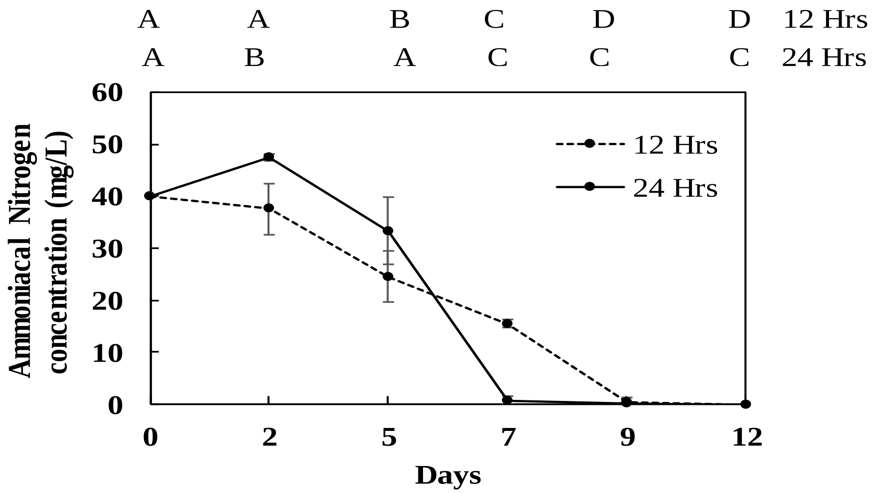

Figure 4. 9: Ammoniacal Nitrogen Concentration in Wastewater under 12:12 and 24:0 Photoperiod. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

The 12:12 light-dark cycles support optimal metabolic conditions, including the regeneration of key intermediates necessary for ammonium assimilation (Akizuki, Cuevas-Rodríguez and Toda, 2021). For the 24:0 photoperiod, an initial increase in ammonium concentration on Day 2 is likely due to metabolic stress under continuous light supply and due to chitosan release nutrient in wastewater. Alternating light/dark cycle can avoid the photodamage in microalgae (Straka and Rittmann, 2018; Gao et al., 2023). Although the continuous light exposure in 24:0 cycle initially caused delays, the system was ultimately able to overcome short-term stress and effectively remove ammoniacal nitrogen after Day 2 and at faster removal rate than 12:12 cycle due to the rapid increased biomass growth.

Hence both condition shows that the ammoniacal nitrogen can be removed with similar efficiency at the end of day suggesting extending photoperiod doesn't enhance the ammoniacal nitrogen removal. Similar study by Ferreira, Machado and Lobo (2021) showed that no significant difference between ammonium removal in 12:12 and 24:0 photoperiod in toilet raw effluent. In the study by Gao et al. (2022a) showed that the ammoniacal nitrogen removal from cocoon wastewater was 93.59% under a 12:12 photoperiod and 91.13% under 24:0, indicating that longer light exposure did not significantly affect ammoniacal nitrogen removal.

Under the 12:12 cycle, *C. vulgaris* exhibited rapid and consistent nitrite removal as shown in Figure 4.10. By Day 5, the nitrite concentration dropped from 500 mg/L to 200 mg/L, which representing a 60% removal efficiency. From Day 7 to Day 12, the concentration stabilized between 250 and 300 mg/L, which maintaining a 50% removal efficiency by the end of the study. In contrast, the 24:0 cycle exhibited more rapid nitrite removal than the 12:12 cycle, with nitrite concentrations dropping from 500 mg/L to 200 mg/L by Day 2, which reflecting a 60% removal efficiency. However, the nitrite concentration began to increase and reaching 400 mg/L by Day 9, ultimately stabilizing at 350 mg/L by Day 12. This led to a final removal efficiency of only 30%, which reflecting a significant decline.

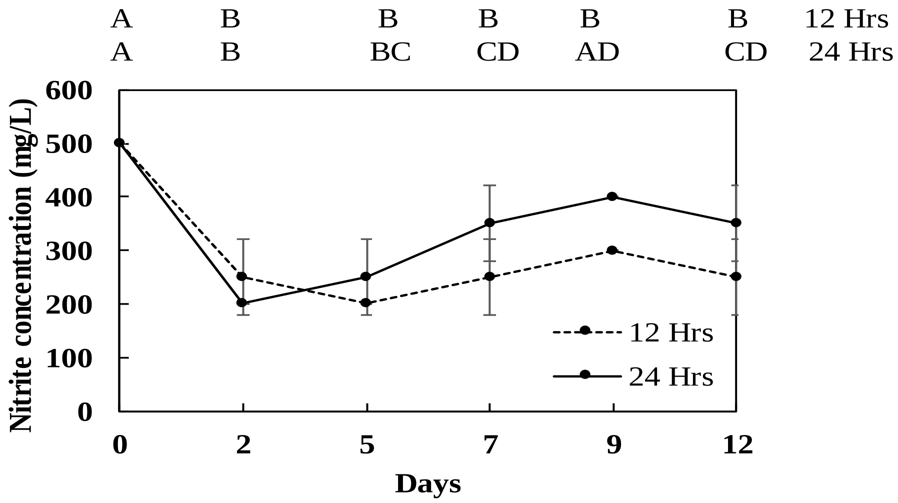

Figure 4. 10: Nitrite Concentration in Wastewater under 12:12 and 24:0 Photoperiod. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

The more rapid nitrite removal by 24:0 cycle than that of the 12:12 cycle was due to the higher microalgae growth rate at 24:0 cycle tend to assimilate more nitrite at first 2 days. Moreover, the nitrite assimilation is favoured under light condition and thus promotes higher nitrite assimilation at 24:0 cycle (Harris, 2009).

High nitrite concentrations in this study can hinder algal growth by damaging the photosystem II (PSII), though *Chlorella* strains can tolerate elevated nitrite levels, maintain photosynthetic efficiency and excreting excess nitrite as a protective response (Chen and Wang, 2020; Su, 2021). Excessive nitrite can lead to algae toxicity by inhibiting photosynthesis, and algal cells release excess nitrite and ammonium when their assimilation capacity is surpassed to avoid toxicity from high intracellular concentrations (Azuara and Aparicio, 1983; Quesada, Gómez and Fernández, 1998; Su, 2021). Therefore, the nitrite content in poultry wastewater of both cycles are in same trend, where the nitrite concentration are increase after the rapid assimilation stage.

The initial nitrate concentration was at 40 mg/L for both 12:12 and 24:0 photoperiods as shown in Figure 4.11. Under the 12:12 photoperiod, nitrate concentration slightly decreased to 35 mg/L by Day 2 and achieving 13% removal efficiency. However, the nitrate concentration further increased to 65 mg/L at Day 12. Under the 24:0 cycle, nitrate concentrations increased from 40 mg/L to 65 mg/L at Day 5 and then fluctuating between 50 mg/L and 60 mg/L after Day 5.

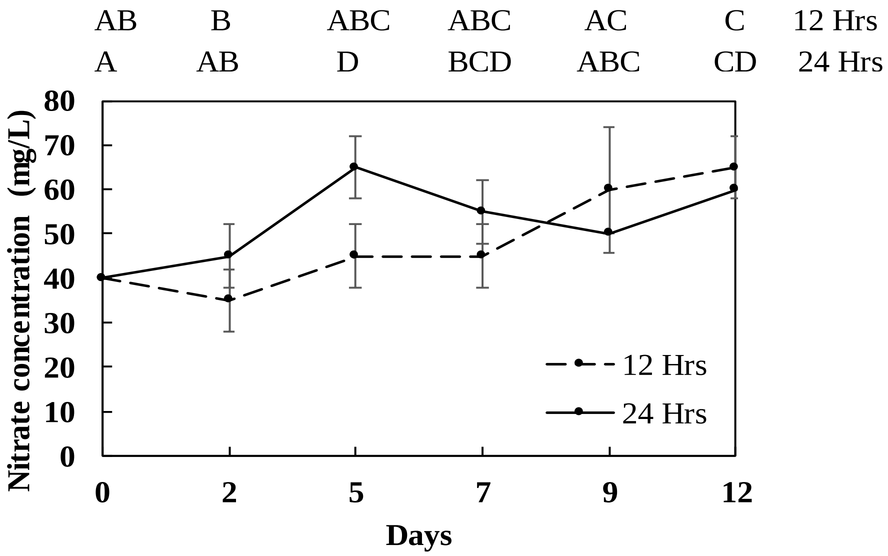

Figure 4. 11: Nitrate Concentration in Wastewater under 12:12 and 24:0 Photoperiod. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

Both photoperiod cycle shown an increment on the nitrate content in wastewater but the release of nitrate into the wastewater at 24:0 cycle is higher than the 12:12 cycle. Continuous light exposure at 24:0 cycle promoted biomass growth, but the absence of a dark phase limited the algae's ability to undergo metabolic recovery. This lack of a recovery phase induced metabolic stress, contributing to the nitrate accumulation observed in the medium. Alternating light/dark cycle can avoid the photodamage or photoinhibition in microalgae (Straka and Rittmann, 2018; Gao et al., 2023). The nitrate release observed during this phase might have been caused by cell stress and cell death, leading to the release of stored nitrate into the medium (Zuppini, Gerotto and Baldan, 2010; Abu-Ghosh et al., 2015; Elisabeth, Rayen and Behnam, 2021).

In overall, nitrogen assimilation is heavily dependent on the photosynthetic activity of microalgae, and as light exposure increases, photoinhibition impairs nitrogen assimilation (Farahdiba et al., 2020; Lu et al., 2024).

The orthophosphate removal trends at different photoperiod were shown in Figure 4.12. Initial orthophosphate concentrations were identical across both photoperiod treatments (123.0 mg/L), yet the pathways and efficiencies of phosphorus uptake diverged significantly yielding only 4% removal under a 12:12 cycle and a notably higher 22% under 24:0 cycle by Day 12. Both cycle showed an increase of orthophosphate concentration at day 2 and day 7 for 12:12 and 24:0 cycle respectively.

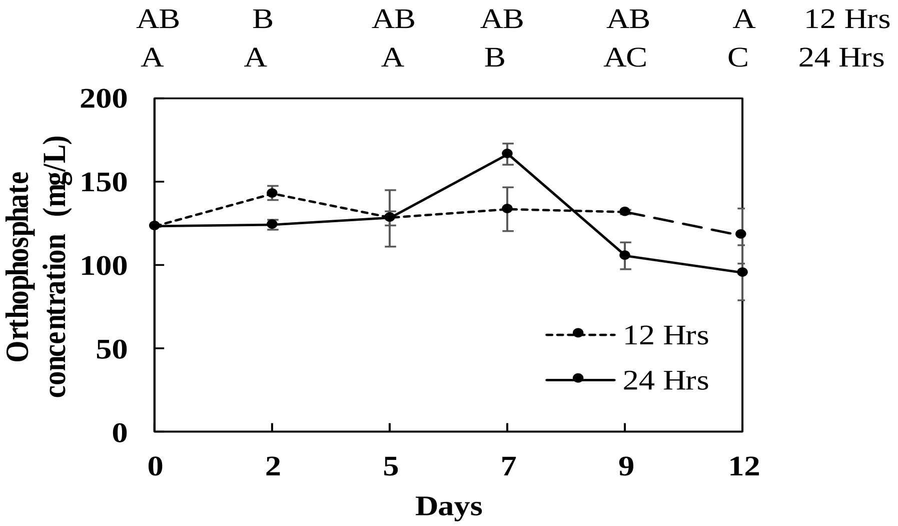

Figure 4. 12: Orthophosphate Concentration in Wastewater under 12:12 and 24:0 Photoperiod. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

The phenomena of orthophosphate release at Day 7 under 24:0 cycle is more significant than that of the 12:12 cycle at Day 2 is mainly due to the photoinhibition or self-shading from 24:0 cycle cause cell death and release the nutrients back to the medium. Whereas the high microalgae growth and cell concentration magnify the amount of release at 24:0 cycle.

The higher orthophosphate removal in 24:0 cycle than that in 12:12 cycle at Day 12 is mainly due to the longer light illumination promote higher orthophosphate assimilation, which further magnify by high microalgae growth and cell concentration at 24:0 cycle. In *Chlorella*, phosphorus uptake depends on ATP, which is mainly produced during the light phase through photosynthesis. When the light-driven ATP production (photophosphorylation) is blocked, the cell reduces its phosphate-related activity, showing that energy from light is essential for effective phosphorus assimilation (Xie et al., 2022). Therefore, phosphorus removal was more efficient under the 24:0 photoperiod, with a 22% reduction by Day 12, driven by continuous biomass growth and active assimilation during the light phase.

In conclusion, the photoperiod of 12 hour is sufficient for the poultry wastewater by *C. vulgaris* for the efficient removal of COD at day 2 while generating biomass as by-product for lipid extraction. The following study was carried out at 12 hr of photoperiod.

4.3.2 Inoculum Size

The biomass growth data over a 5-day period demonstrates distinct patterns across the different inoculum sizes tested at 0.13 g/L, 0.25 g/L and 0.34 g/L which made up by adding 2 ml, 3 ml, and 4 ml of microalgae paste into 250 ml of culture media respectively, as shown in Figure 4.13. All treatments exhibit increasing biomass concentration over time but with clear differences in growth rate. The 4 ml inoculum was at highest growth rate at the first 2 days and then followed by 2 ml inoculum. The grow rate of 4 ml inoculum reduced and enter stationary stage after day 2 and reach to a similar overall biomass grow from Day 0 to Day 4 with the 0.13 g/L inoculum condition.

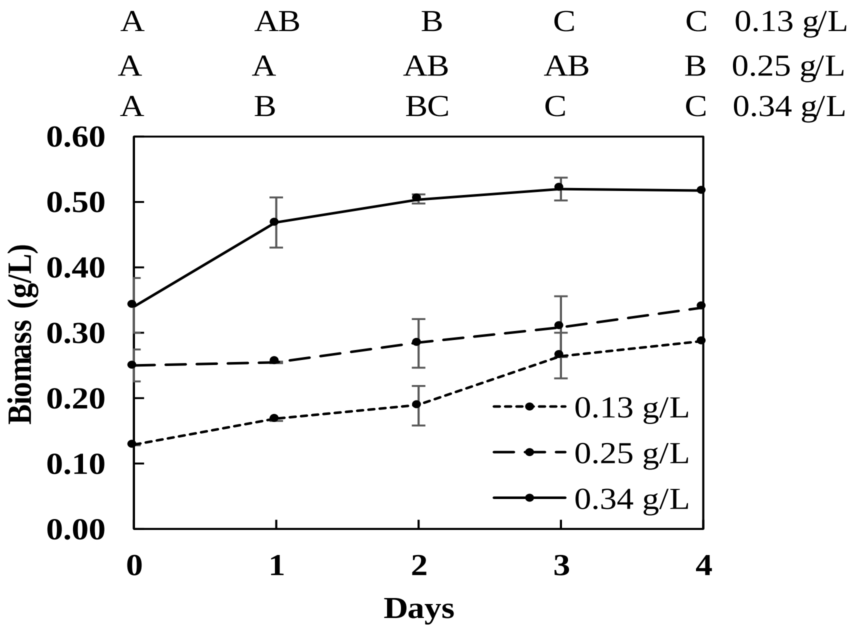

Figure 4. 13: Biomass growth of *C. vulgaris* in Wastewater under Inoculum Size of 0.13, 0.25 and 0.34 g/L. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

The 0.34 g/L inoculum presents a distinct pattern, starting with the highest initial biomass (0.3407 g/L) and rapidly accumulating biomass during the first two days, reaching 0.5042 g/L by Day 2. However, growth substantially slows thereafter, plateauing at approximately 0.5169 g/L by Day 4, a total biomass increases of 52%. The 0.34 g/L inoculum exhibited early growth saturation and then reaching stationary stage sooner than other inoculum size likely due to self-shading, nutrient diffusion limitation in concentrated culture, and crowding effects due to limiting space factors indicating that this inoculum size was excessive (Nasir et al., 2015; Chen, Chang and Chang, 2016; Gupta, Marchetti and Wasewar, 2024; Li et al., 2021). Biomass productivity initially increases with inoculum density, but beyond an optimal concentration, the increment on culture viscosity and reduced dissolved oxygen levels in culture that negatively impact metabolite synthesis (Cheng et al., 2018; Ahamefule et al., 2025).

These observed trends in growth across inoculum sizes are consistent with research by Hazman et al. (2018) who studied microalgal cultivation for palm oil mill effluent treatment using inoculum sizes of 5 %, 10 %, and 15 %. Their results similarly showed that moderate inoculum concentrations promote higher growth efficiency and nutrient removal, whereas higher inoculum densities (15 %) can suppress growth due to self-shading and nutrient competition. This correspondence supports the interpretation that the 0.13 g/L of inoculum concentration in this study represents a more optimal cell density for optimal growth rate and resource utilization.

The result of COD concentration profiles by different inoculum size over the 5-day experiment was shown in Figure 4.14. The 0.13 g/L inoculum showed the higher COD removal at Day 2, which reaching 84% efficiency, and followed by 0.25 g/L and 0.34 g/L inoculum size.

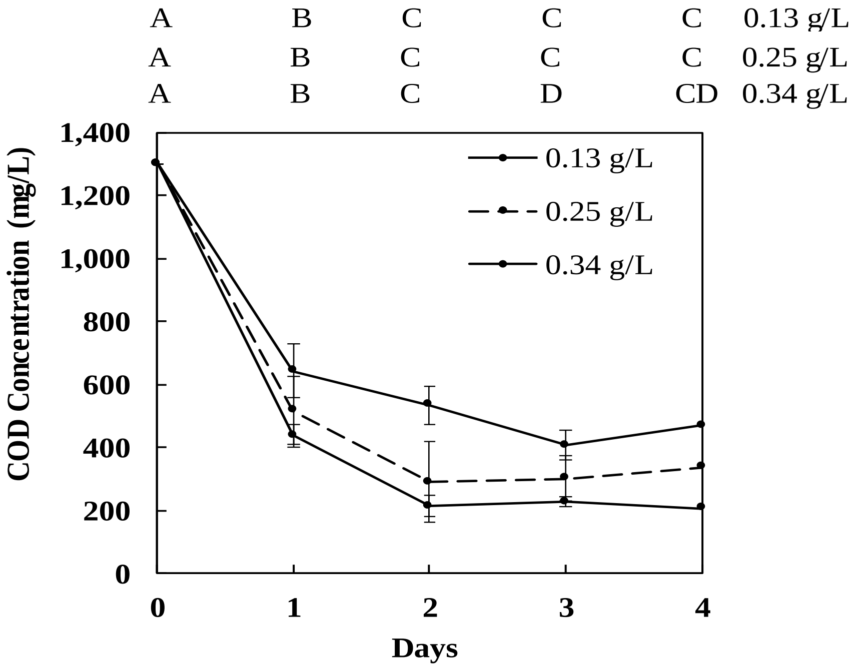

Figure 4. 14: COD Concentration in Wastewater under Inoculum Size Of 0.13, 0.25 and 0.34 g/L. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

The result indicates that the 0.13 g/L inoculum system maintained a favourable biomass concentration and promoted COD degradation. In comparison to the study by Lee et al. (2019) where COD initially decreased by approximately 82% at Day 2 with 5% inoculum size but rebounded due to metabolic plateau. The study by Aksu et al. (2021) use poultry wastewater showed the highest removal at 76% with 12% inoculum size for 15 days. Hence, the 84% of COD removal efficiency at Day 2 with 0.13 g/L inoculum in this study outperformed the others.

The 0.34 g/L inoculum exhibited the weakest COD removal performance. This pattern suggests the system experienced early metabolic strain or saturation due to high cell density in culture media, which likely faced oxygen depletion or self-shading and suppressed the COD reduction (Jeong and Jang, 2021; Condor et al., 2024). The rebound in COD for 0.25 g/L and 0.34 g/L inoculum condition at day 4 suggests that the system may have encountered the accumulation of organic compounds resulting from natural cellular excretion and cell lysis when under nutrient stress and self-shading conditions (Grobbelaar, 2009; Roy, Bera and Mohanty, 2021; Zhou et al., 2024a). Result highlights the importance of optimizing inoculum density not just for biomass yield but also for achieving efficient and sustained pollutant degradation in microalgae-based wastewater systems.

Figure 4.15 showed the performance of different inoculum size on the ammoniacal nitrogen removal was in similar trend with the COD removal. The 0.13 g/L inoculum group achieved the most effective and consistent ammoniacal nitrogen removal compared to the other inoculum size. The 0.13 g/L inoculum achieved 27% of ammoniacal nitrogen removal at Day 2 and 48% at Day 4. High cell inoculum size retarded the cell growth due to self-shading effect and the limitation in nutrient diffusion across the crowded culture, and thus, lowers cell growth promoted lower demand for nutrient assimilation (Bohutskyi et al., 2016; Zhang et al., 2023).

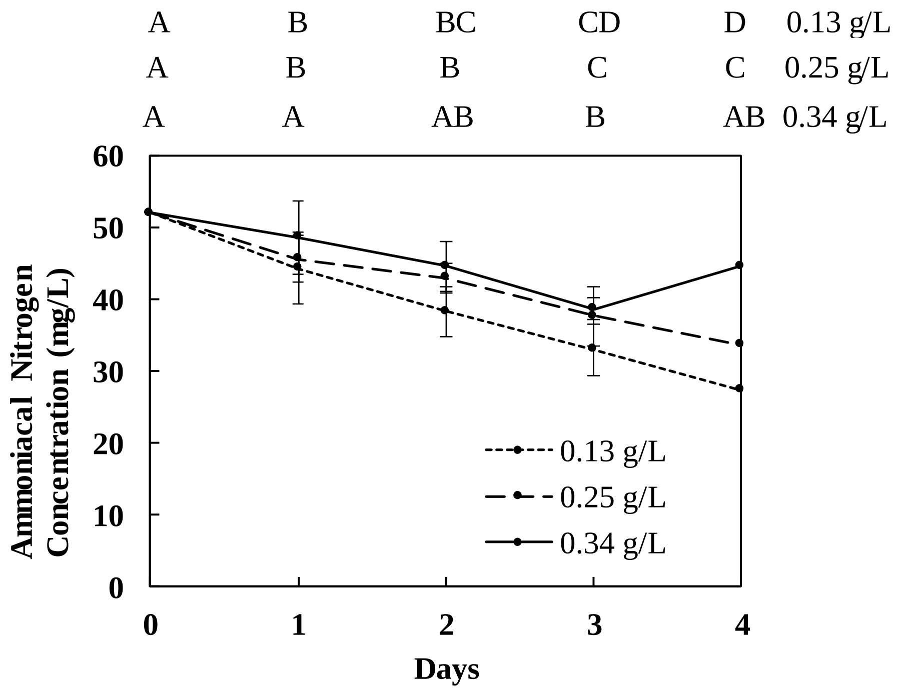

Figure 4. 15: Ammoniacal Nitrogen Concentration in Wastewater under Inoculum Size Of 0.13, 0.25 And 0.34 g/L. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

Similar trends go to the nitrite and nitrate removal as shown in Figure 4.16 and 4.17 respectively, where the nitrite and nitrate removal efficiency achieved 39% and 53% respectively at Day 2 when with 0.13 g/L inoculum. The release of nitrate at the first day when at 0.34 g/L inoculum (Figure 4.17) indicated the cell lysis due to self-shading that caused nutrient released back to culture medium is significant when at very high inoculum size.

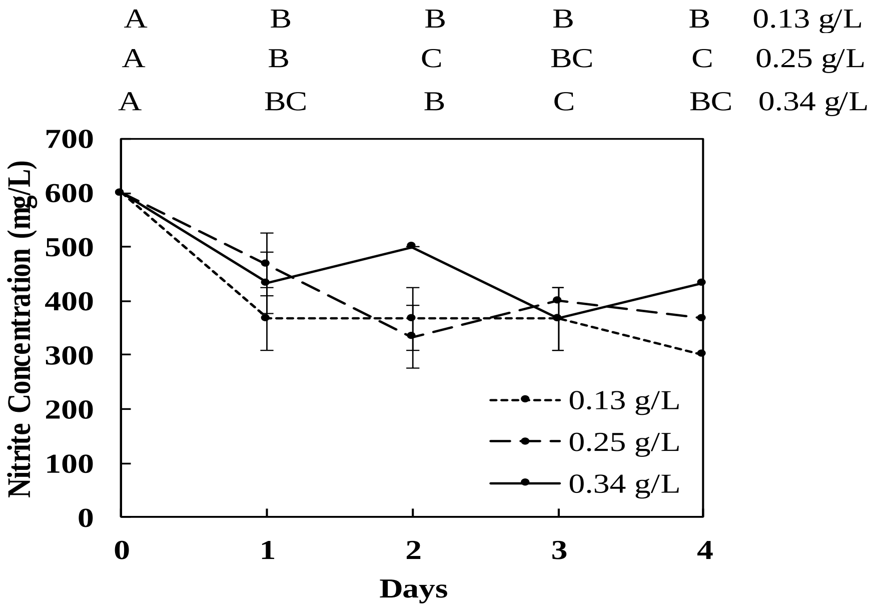

Figure 4. 16: Nitrite Concentration in Wastewater under Inoculum Size Of 0.13, 0.25 And 0.34 g/L. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

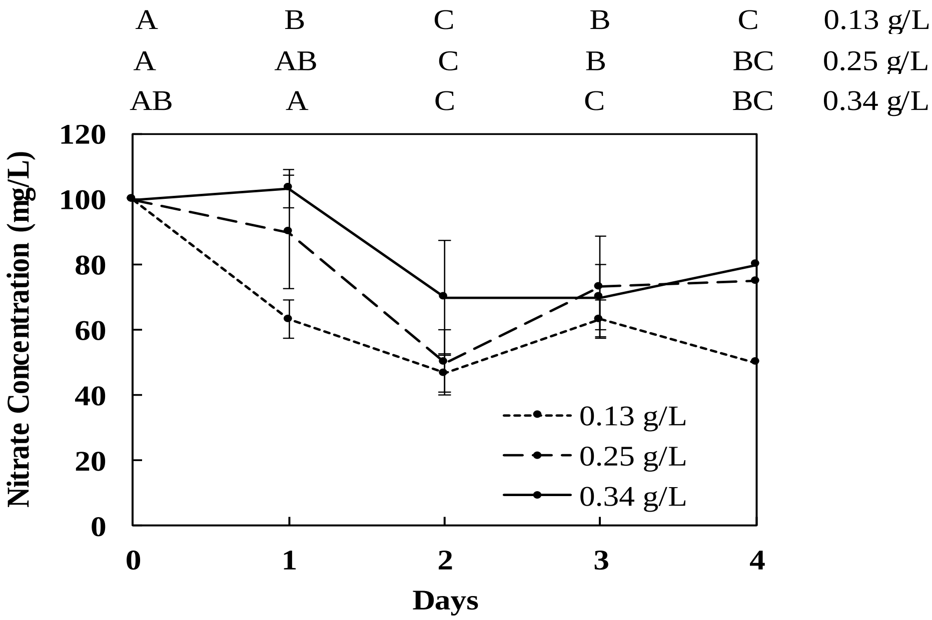

Figure 4. 17: Nitrate Concentration in Wastewater under Inoculum Size Of 0.13, 0.25 And 0.34 g/L. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

Study by Wang et al. (2023) demonstrated that in *Chattonella subsalsa*, nitrate reductase (NR) activity is strongly influenced by light availability, with NR expression increasing under illuminated conditions and decreasing sharply in darkness. The study confirmed that NR relies on reducing equivalents generated through photosynthetic electron transport, making it highly sensitive to conditions that limit light-driven energy production. This mechanistic link explains how reduced light availability such as from self-shading in dense algal cultures can suppress NR activity and impair nitrogen assimilation. These findings align with the nitrate rebound observed in the high inoculum groups of the present study, where dense biomass likely restricted light penetration, leading to insufficient NR activation and inefficient nitrogen uptake.

Figure 4.18 showed that the orthophosphate by *C. vulgaris* at different inoculum size is not significant and can be conclude as no significant orthophosphate assimilation by *C. vulgaris* from the poultry wastewater when at 12 hr photoperiod regardless the inoculum size.

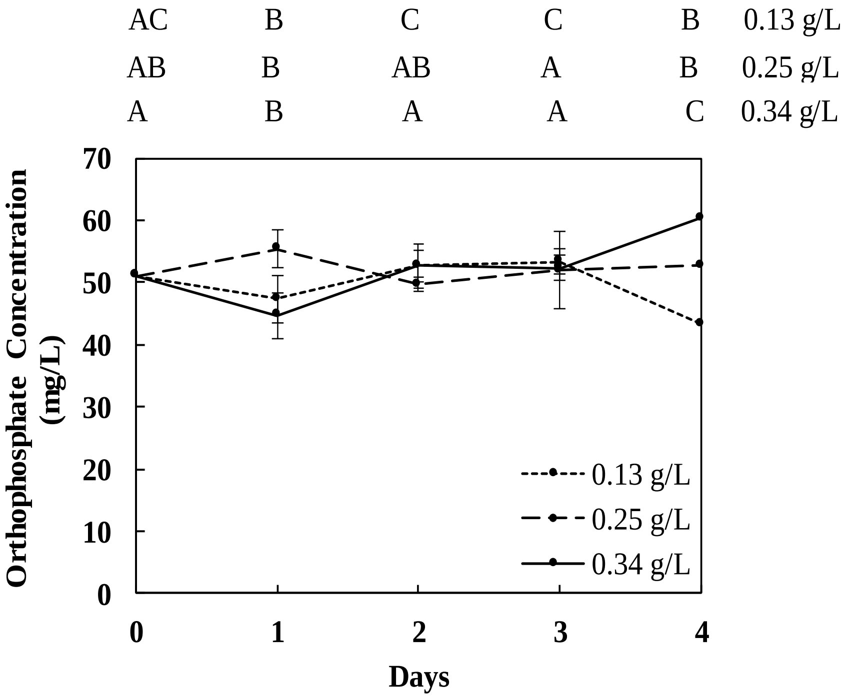

Figure 4. 18: Orthophosphate Concentration in Wastewater under Inoculum Size Of 0.13, 0.25 And 0.34 g/L. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

In conclusion, inoculum size at 0.13 g/L outperformed the others and achieved the removal efficiently at 84% of COD, 27% of ammoniacal nitrogen, 39% of nitrite, and 53% of nitrate from the poultry wastewater at Day 2.

4.4 Feasibility Study on the Poultry Wastewater Treatment by *Chlorella vulgaris*

In this study, the effectiveness of the acclimation strategy, type of light source, and the microalgae harvesting method toward the performance of wastewater treatment by microalgae is important to be evaluated to confirm the feasibility of microalgae method in real application.

4.4.1 Acclimation of Microalgae Stock to Poultry Wastewater

In lab scale, the microalgae stock that will be transferred to the wastewater for treatment study is cultivated in fertilizer enriching medium due to the stable nutrient content manage to control the growth of the microalgae. However, when the microalgae method is being implemented in real industrial usage, the microalgae biomass collected from the secondary treatment process will be returned to the secondary treatment process again to maintain the inoculum size in the wastewater to maintain the treatment effectiveness, which is similar with the concept of microalgae acclimation in wastewater. Therefore, the effect of the acclimation strategy is important to be investigated in order to confirm the feasibility of microalgae treatment method in real application.

This study evaluates how the acclimation or pre-adaptation of *C. vulgaris* microalgae stock affects the performance of *C. vulgaris* in poultry wastewater treatment. The *C. vulgaris* was pre-cultivated in fertilizer medium (FER) and also the poultry wastewater (WW) before introduced to the wastewater for treatment under 12 hour photoperiod. Figure 4.19 showed that the growth of *C. vulgaris* in poultry wastewater at FER condition exhibited significantly higher biomass accumulation than the WW condition. The biomass is increasing from 0.1812 g/L to 0.3395 g/L by Day 4 when at FER condition, which marking an 87% of increment. In comparison, the biomass in WW condition rose from 0.2036 g/L to 0.2917 g/L during the same period, with a 43% of increment in biomass.

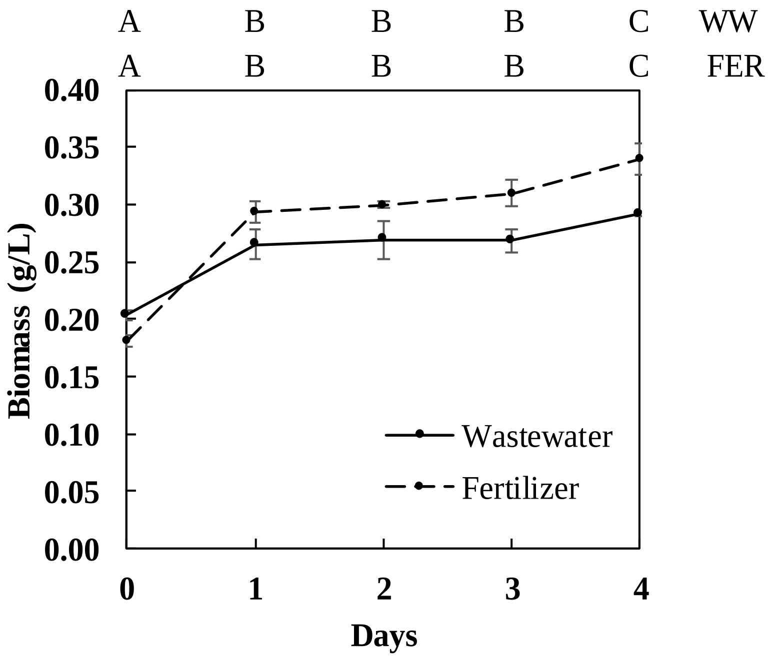

Figure 4. 19: Biomass Concentration of *C. vulgaris* in Wastewater with Acclimated in Wastewater and Preculture in Fertilizer. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

Figure 4.20 showed that the *C. vulgaris* microalgae that pre-cultivated from WW condition promoted a better COD removal performance than that of the FER condition in poultry wastewater treatment. By day 3, the WW condition achieved 73% of COD removal and maintained through day 4, whereas the COD removal percentage in FER condition achieved 60% at Day 4. When compared to the microalgae growth in Figure 4.19, the result showed that the microalgae in FER condition tend to exhibit higher cell growth but assimilate lesser COD from wastewater, while the performance of WW condition was in vice-versa.

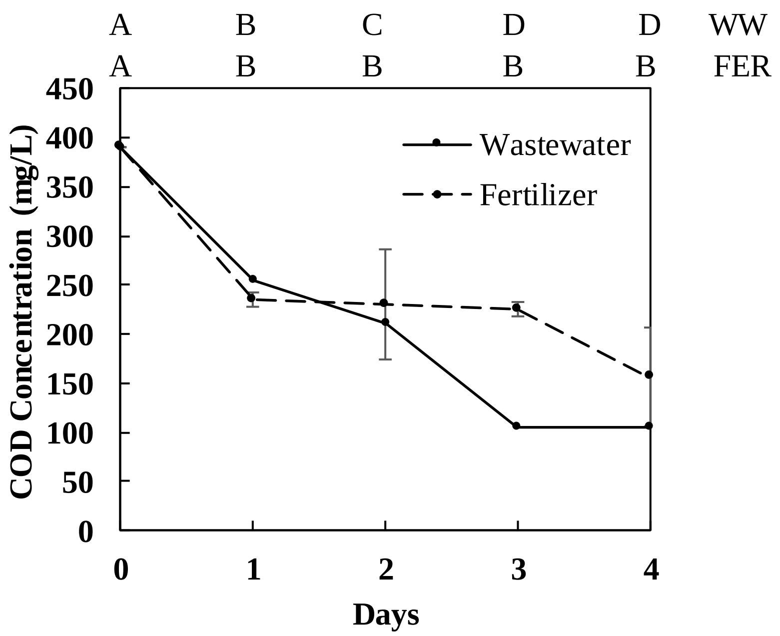

Figure 4. 20: COD Concentration in Wastewater with *C. vulgaris* Acclimated in Wastewater and Preculture in Fertilizer. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

Microalgae growth is closely regulated by both carbon assimilation and nutrient availability. The microalgae from FER condition exhibited a higher growth but assimilate lesser COD indicated that the microalgae that pre-cultivated in fertilizer enriched medium, that is FER condition, is less likely to assimilate the organic carbon from poultry wastewater after transferred to the wastewater from the clear fertilizer enriched medium. The microalgae were get used to assimilate the inorganic carbon source when pre-cultivated in fertilizer enriched medium without COD. However, the microalgae that pre-cultivated in poultry wastewater exhibited a higher tendency to assimilate the organic carbon in poultry wastewater during the wastewater treatment although with a lower growth rate. These findings suggest that acclimation to real wastewater likely enhanced the metabolic flexibility of the microalgae, enabling them to better adapt to the diverse mixture of organic substrates in the post-inoculation environment. In contrast to the fertilizer medium, which may have conditioned the cells to a more limited nutrient profile, wastewater pre-acclimation seems to have primed the microalgae for more efficient COD degradation following reinoculation.

Figure 4.21 showed the ammoniacal nitrogen removal efficiency by *C. vulgaris* that grown under two different preconditioning environments: wastewater (WW) and a fertilizer medium (FER). Initially, both conditions began with the same ammoniacal nitrogen concentration of 20 mg/L. The performance of ammoniacal nitrogen removal in WW condition is more efficient than the FER condition. The removal of ammoniacal nitrogen achieved 100% at Day 3 in WW condition, while the assimilation of ammoniacal nitrogen in FER condition experienced a lag phase in the first day of wastewater treatment and then activated and achieved 100% removal at Day 4.

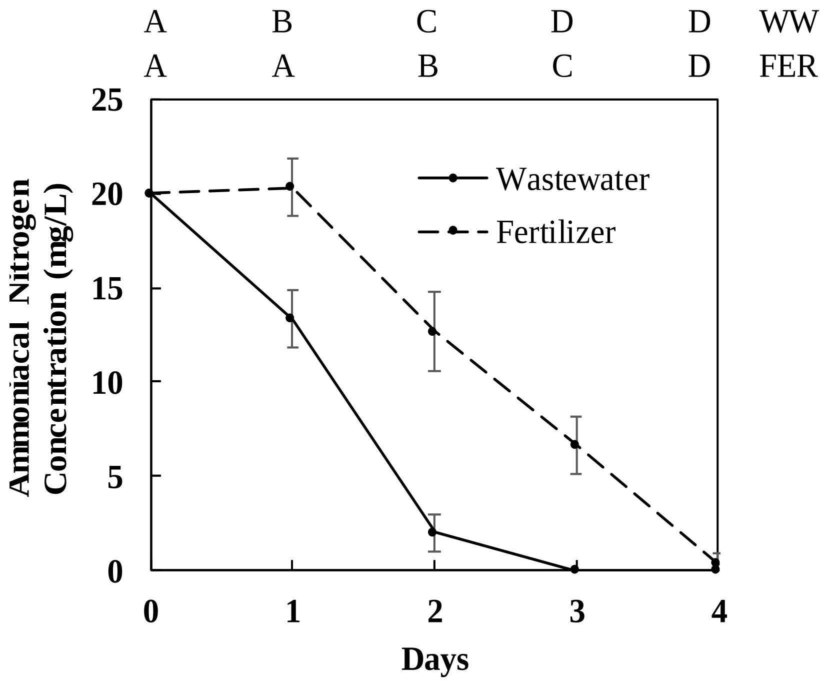

Figure 4. 21: Ammoniacal Nitrogen Concentration in Wastewater with *C. vulgaris* Acclimated in Wastewater and Preculture in Fertilizer. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

The microalgae in WW condition displayed a remarkable ability to assimilate ammoniacal nitrogen, indicating that pre-exposure to the more complex nutrient environment of wastewater likely induced metabolic flexibility. Microalgae show enhanced stress tolerance and pollutant removal efficiency due to improved metabolic adaptability and oxidative stress resistance (Osundeko et al., 2014; Pathak et al, 2021; Zhang et al., 2022; Barra and Greco, 2023). This highlights the crucial role of wastewater acclimation in preparing algae for more efficient nutrient uptake and detoxification.

For the microalgae in FER condition, the slower initial removal of ammoniacal nitrogen could be attributed to the stable, controlled nutrient conditions in the fertilizer medium, which likely did not induce the same stress response seen in the wastewater-acclimated cultures. Preconditioning in a stable medium like fertilizer may not have primed the algae for the same immediate response to ammonium nitrogen assimilation as in wastewater, where nutrient fluctuations and microbial stress are more prevalent. These results align with findings by Katayama et al. (2020) who demonstrated that ammonium tolerance in marine microalgae can be acquired through acclimation, allowing them to thrive at ammonium concentrations up to 180 mg/L. This acclimation process is crucial for enhancing nutrient removal efficiency in nutrient-rich wastewaters, such as poultry wastewater.

In conclusion, the study illustrates that the preconditioning medium significantly influences the ammoniacal nitrogen removal capabilities of *C. vulgaris* and providing faster and more efficient pollutant removal compared to fertilizer preconditioning.

Figure 4.22 showed the performance of nitrite removal by *C. vulgaris* when in FER and WW condition. The nitrite assimilation by microalgae in both condition were in similar trends, which both condition exhibited nitrite release into wastewater in the first 2 days and then started to be removed by microalgae and achieved 22% and 44% of nitrite removal at Day 4 in FER and WW condition respectively.

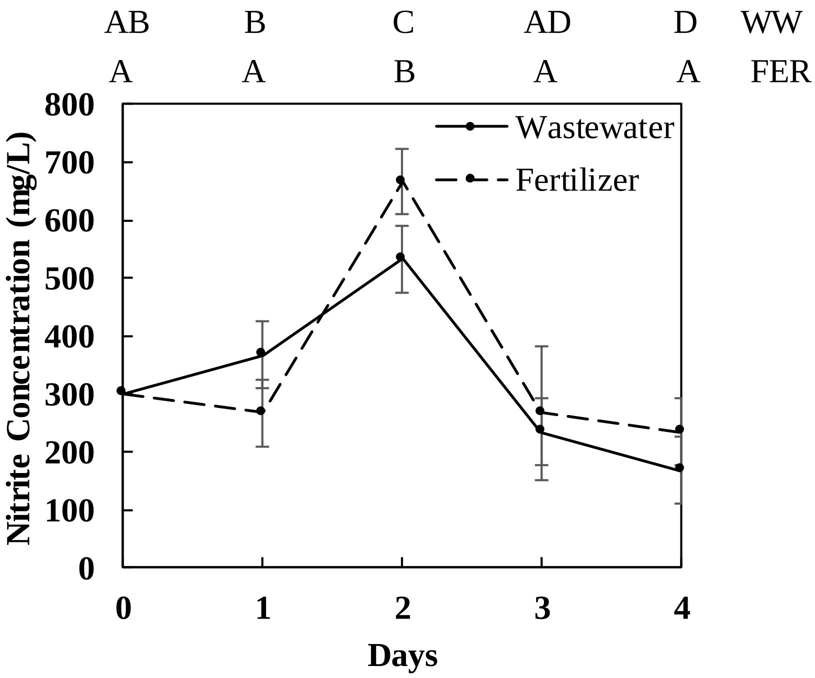

Figure 4. 22: Nitrite Concentration in Wastewater with *C. vulgaris* Acclimated in Wastewater and Preculture in Fertilizer. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

The nitrite release into the wastewater in the first 2 days is mainly from the degradation of organic carbon in poultry wastewater by *C. vulgaris*. The removal of nitrite is not significant if compared to the initial concentration at Day 0 for both conditions, but nitrite removal by microalgae in WW condition is slightly higher than the FER condition likely due to prior acclimation in wastewater.

Figure 4.23 showed the performance of nitrate removal by *C. vulgaris* when in FER and WW condition. There is no nitrate assimilation by microalgae in both condition but exhibited nitrate release into wastewater. The nitrate release in WW condition is higher than the FER condition. This finding is in line with the performance of COD removal as shown in Figure 4.20 where the microalgae in WW condition degrade more organic carbon in wastewater and thus lead to the release of more nitrate nutrients into the poultry wastewater compared to the FER condition.

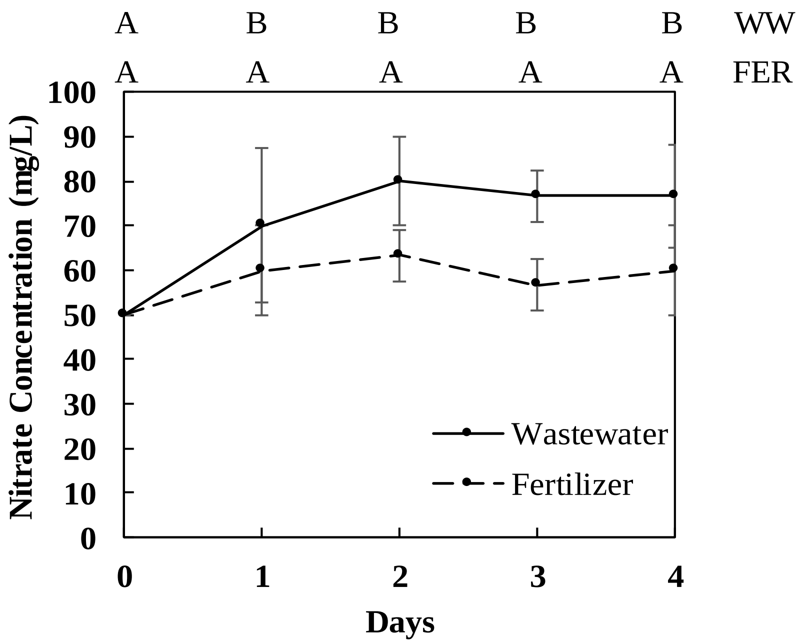

Figure 4. 23: Nitrate Concentration in Wastewater with *C. vulgaris* Acclimated in Wastewater and Preculture in Fertilizer. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

Importantly, the C:N ratio in this study (~1:1) may have contributed to the observed nitrate assimilation behaviour. Studies have shown that low C:N conditions can limit nitrate reductase activity, as insufficient carbon reduces the supply of reductants (NAD(P)H) and energy needed for nitrate assimilation and also the assimilation could be suppressed by ammonium (Xia and Gao, 2005; Wang, Bouchard and Coyne, 2018; Cecchin et al., 2021; Chen et al., 2025). This somehow contributed to elevation of nitrite in wastewater. This study showed that the imbalance of nutrients content in wastewater that inhibits the nutrient assimilation could not be overcome by the acclimation strategy.

Figure 4.24 showed the performance of orthophosphate removal by *C. vulgaris* when in FER and WW condition. The orthophosphate assimilation by microalgae in both condition were in similar trends, which both condition exhibited lag phase initially followed by slight orthophosphate release into wastewater and then the assimilation started and achieved 12% and 27% of orthophosphate removal at Day 4 in FER and WW condition respectively. The removal of orthophosphate is not significant in both conditions, but the orthophosphate removal by microalgae in WW condition is slightly faster and higher than the FER condition likely due to prior acclimation in wastewater.

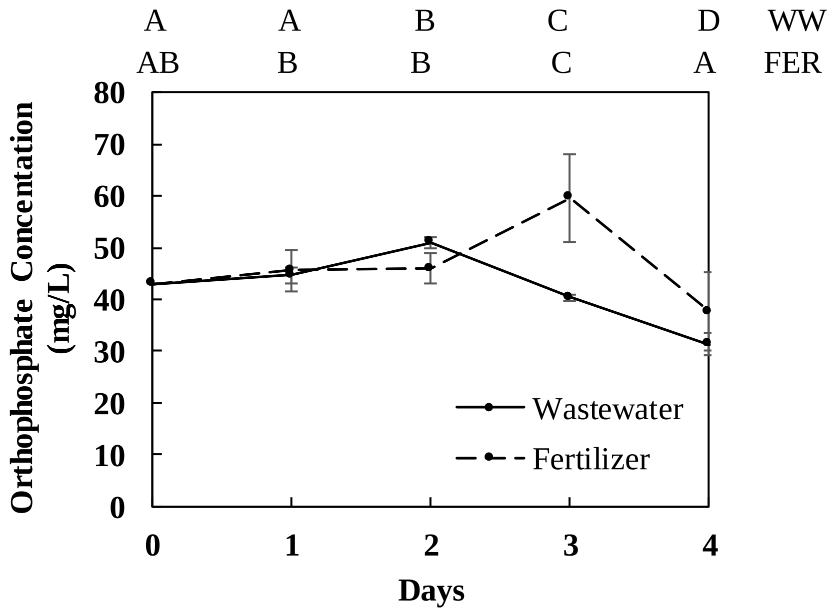

Figure 4. 24: Orthophosphate Concentration in Wastewater with *C. vulgaris* Acclimated in Wastewater and Preculture in Fertilizer. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

In conclusion, the WW-acclimated cultures exhibited more efficient COD removal and ammoniacal nitrogen assimilation at Day 2 compared to the FER condition. These findings highlight the critical role of acclimation strategies for optimizing nutrient removal in microalgal systems, especially in wastewater treatment environments where nutrient dynamics are more complex and variable. Moreover, this finding offers valuable insight to confirm that the application of microalgae treatment method in real application by continuous recycle back the concentrated microalgae biomass to the secondary wastewater treatment process is feasible.

4.4.2 Light source

The growing of microalgae under sunlight is recognised as a cost-effective approach compared to the artificial illumination, such as LED grow light. This study was carried out to investigate the light source that is feasible for real application and able to maintain the performance of wastewater treatment. Figure 4.25 showed the performance of the growth of *C. vulgaris* when in different light source, which is the LED grow light and sunlight. The growth of *C. vulgaris* under LED grow light is higher than that of the sunlight. Under LED grow light, the biomass demonstrated consistent growth across all time points. Starting at 0.3511 g/L on Day 0, the biomass increased to 0.71 g/L at Day 4, which marking a 102% of increment from the initial cell density. In contrast, the growth trend under sunlight was far less consistent and the growth was not significant. The biomass on Day 0 at 0.35 g/L reached 0.40 g/L at Day 4, which resulted in a final 14% of increment compared to the initial cell density.

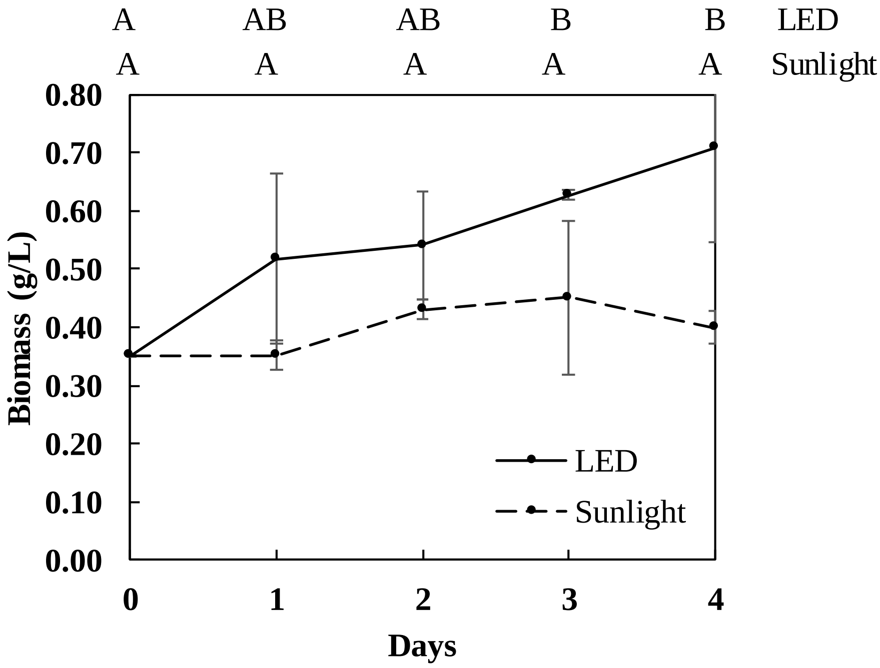

Figure 4. 25: Biomass Concentration of *C. vulgaris* in Wastewater under LED Light and Sunlight. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

The high and consistent growth rate of microalgae under LED grow light indicates that the controlled and constant light exposure provided by the LED grow light fostered optimal conditions for photosynthesis, thereby supporting sustained microalgae growth (Hwang and Maier, 2019; Porto et al., 2022). On the other hand, the fluctuations on the biomass concentration under natural sunlight are likely attributed to the variable light conditions. Sunlight exposure can be interrupted by factors such as clouds, rain, or other weather phenomena, which limit the microalgae access to consistent light for photosynthesis, and thus, affect the growth of microalgae. This finding was supported by Nguyen et al. (2022b) where the biomass productivity under artificial lighting conditions was approximately 27% higher than that under natural lighting conditions during the operation period, as natural lighting cycles are influenced by weather and daily solar irradiance variations, leading to unstable microalgae growth. Moreover, light intensity plays a crucial role in achieving high biomass production in microalgae. Studies have demonstrated that an intensity greater than 3000 lux is optimal for microalgae growth (Přibyl, Cepák and Zachleder, 2012; Mandotra et al., 2016; Jui et al., 2024). In this study, the LED growth light is more likely to provide the necessary light intensity, allowing the microalgae to efficiently convert carbon dioxide into organic compounds like carbohydrates and proteins, which are vital for both biomass accumulation and nutrient removal (Khan, Shin and Kim, 2018). In contrast, the natural sunlight's intensity, which fluctuates throughout the day and varies depending on weather, is less reliable for consistent growth. Although sunlight intensity can occasionally exceed 2000 μmol photons m^{-2} s^{-1}, photoinhibition of microalgae growth occurs when light intensity surpasses 250 μmol photons m^{-2} s^{-1} (Nishi et al., 2022). However, during afternoon is the usual high light intensity (Wan Razali and Pandhal, 2023). When light intensity exceeds this threshold, the rate at which light is captured surpasses the rate of photosynthesis, causing excess energy to be lost through non-photochemical quenching (NPQ), dissipated as heat or emitted as fluorescence, rather than being used for photosynthesis. NPQ helps microalgae protect themselves from the stress of high light intensities and can induce the production of toxic reactive oxygen species (ROS) (Goss and Lepetit, 2015). After light exposure, microalgae require a period of dark recovery, during which NPQ components relax, allowing excess light energy to stabilize. However, following intense light exposure, some NPQ components may not fully relax, indicating potential photodamage or the need for further recovery time (Herdean et al., 2023). The fluctuating light intensity of sunlight often leads to periods of light saturation, where microalgae absorb more light than they can process. This results in inefficiencies in energy conversion and limits the microalgae ability to grow and perform vital processes.

One of the major challenges in using solar light for microalgae cultivation is its variability, which is influenced by day-night cycles and shifting diurnal light patterns (McGee et al., 2020; Pritam et al., 2024). This results in limited periods each day with sufficient light intensity for optimal photosynthetic activity, depending on the geographical location and time of the year (Wild, 1951; Ahmad and Tiwari, 2011; Vialet-Chabrand et al., 2017; Nishi et al., 2022; Sung, Lee and Sim, 2022). In the absence of adequate light, microalgae cells must rely on their internal energy reserves, a process called respiration, which leads to a reduction in cell biomass. Extended periods of unfavourable weather, such as clouds or rain, can even result in culture crash when sunlight is the sole energy source (Benemann and Tillett, 1987; Nwoba et al., 2020; Lee et al., 2021b).

These findings suggest that LED lighting could offer a more reliable and efficient approach for growing microalgae in wastewater treatment and biomass production systems, especially where light availability is inconsistent.

Figure 4.26 showed the performance of the COD removal by *C. vulgaris* when in different light source, which is the LED grow light and sunlight. The microalgae tend to remove the COD more effectively under the

LED grow light compared to the sunlight. Under controlled LED light conditions, the COD concentration started at 580 mg/L on Day 0 and showed a consistent decline over the next four days and reaching 59 mg/L by Day 4, which corresponding to a 90% of COD removal efficiency. When under natural sunlight, the COD starting at 580 mg/L on Day 0 had decreased to 179 mg/L at Day 4, which yielding a final COD removal efficiency of 69%.

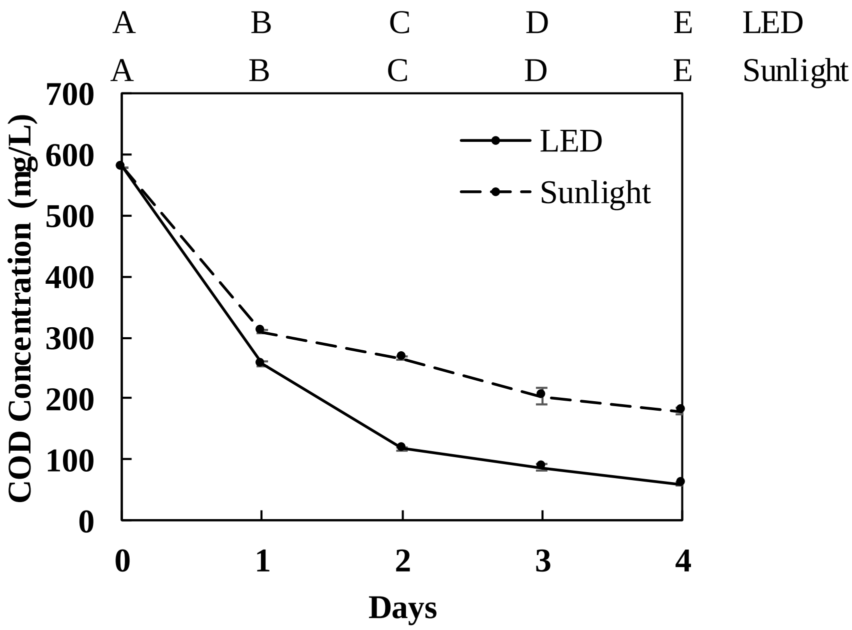

Figure 4. 26: COD Concentration in Wastewater under LED Light and Sunlight. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

The trends in COD removal efficiency are closely linked to the growth conditions of the microalgae. The continuous and stable light intensity under LED conditions enabled *C. vulgaris* to maintain steady growth and efficiently break down organic matter, leading to consistently higher removal efficiencies. In contrast, the intermittent and fluctuating light conditions under sunlight led to less predictable biomass growth and, consequently, slower and less consistent COD removal. These findings highlight the potential advantages of using LED light systems for microalgae-based wastewater treatment, offering a more reliable and efficient method for achieving higher and consistent COD removal compared to natural sunlight, especially in large-scale applications where stable performance is critical.

Figure 4.27 showed the performance of the ammoniacal nitrogen removal by *C. vulgaris* when in different light source, which is the LED grow light and sunlight. The microalgae tend to remove the ammoniacal nitrogen faster and more effectively under the LED grow light compare to the sunlight. Under LED light, microalgae showed a steady decline in ammonia levels, achieving near-total removal (99%) by Day 4, starting from an initial concentration of 47 mg/L. In contrast, under sunlight, although the removal started at the same concentration of 47 mg/L, the progress was slower. By Day 4, the concentration remained relatively higher at 23 mg/L, with removal efficiencies of 52%.

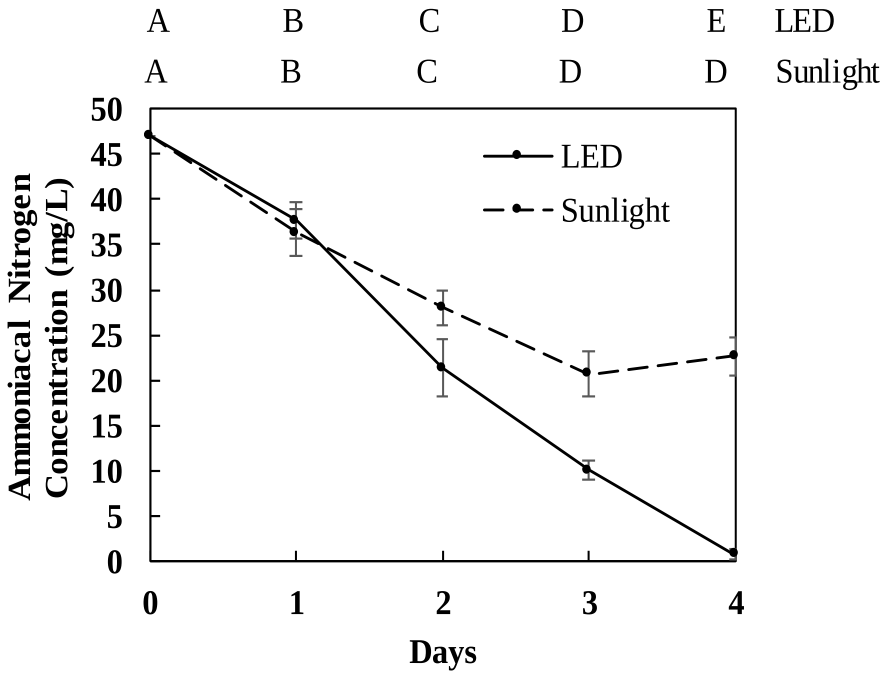

Figure 4. 27: Ammoniacal Nitrogen Concentration in Wastewater under LED Light and Sunlight. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

The variability in sunlight intensity, due to factors like weather and time of day, could be a key reason why microalgae under sunlight conditions showed lower biomass growth and thus lead to lower COD removal and also less consistent ammoniacal nitrogen removal. Sunlight fluctuates not only in intensity but also in the spectrum of light, which can hinder the efficiency of photosynthesis and thus the overall nitrogen assimilation process. Most of the nitrogen removal took place during the light hours (Voltolina, 2005). High photosynthetic efficiency can only be achieved under optimal irradiance and controlled light conditions, ensuring that the majority of absorbed photons are effectively utilized by the culture. The fluctuating irradiances might introduce strong irradiation that exceeding the photosynthetic capacity and casing the cell damage, and thus, more metabolic energy needed to repair the cell caused by photodamage (Benedetti et al., 2018). Hence explained why microalgae growth and ammoniacal nitrogen removal is lesser under sunlight compared to LED grow light. The usage of LED grow light could boost the microalgae growth and hence promote fast assimilation of the ammoniacal nitrogen, which shown in a study by Muqhrey et al. (2024) with 98% of ammoniacal nitrogen removal.

Figure 4.28 showed the performance of the nitrite removal by *C. vulgaris* when in different light source, which is the LED grow light and sunlight. The nitrite removal under LED grow light reach to the highest at Day 2, where the nitrite concentration reduced from 700 mg/L to 400 mg/L and achieved 43% of reduction. The nitrite released back to the wastewater after Day 2. When under sunlight condition, the nitrite was released to wastewater at the first 2 days and then followed by nitrite assimilation and achieved 29% reduction from the initial nitrite concentration.

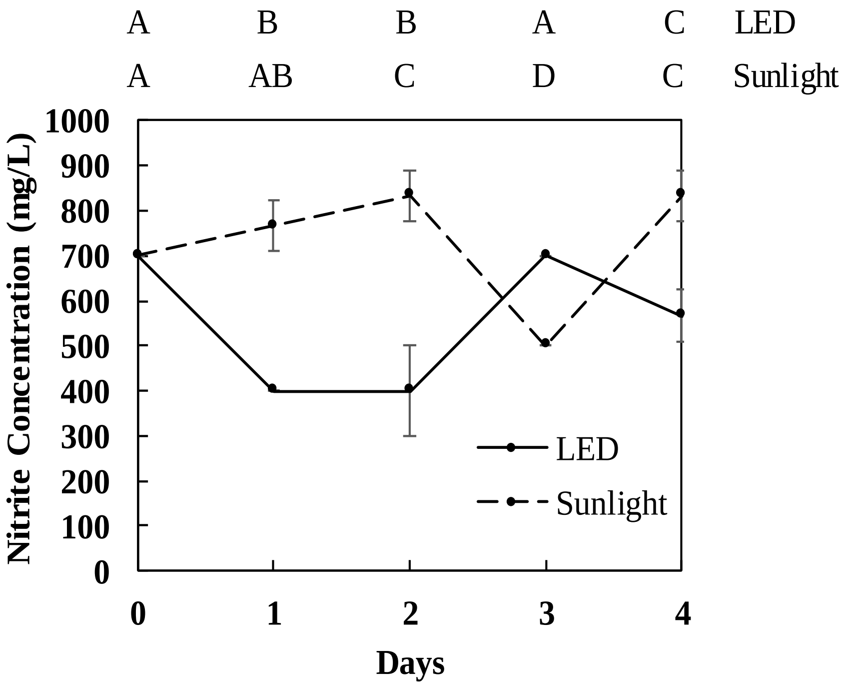

Figure 4. 28: Nitrite Concentration in Wastewater under LED Light and Sunlight. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

The result indicates that LED grow light consistently provided better overall performance. On Day 1 and Day 2, the efficiency under LED was 43%, indicating significant nitrite removal due to rapid growth of microalgae promote nitrite assimilation. However, as the experiment continued, the nitrite removal efficiency decreased when the biomass concentration is high due to the self-shading phenomena tends to cause cell death and lysis. In comparison, the inconsistent sunlight supply induced an unstable and inefficient nitrite removal in overall. These findings suggest that LED light is more effective for maintaining stable conditions for microalgal wastewater treatment and nitrite reduction.

Figure 4.29 showed the performance of the nitrate removal by *C. vulgaris* when in different light source, which is the LED grow light and sunlight. Both LED grow light and sunlight condition showed a similar trend of nitrate removal, where the nitrate was being removed initially and then followed by slight fluctuate in concentration. The LED grow light condition achieved the highest nitrate removal of 47% at Day 2 while sunlight condition achieved highest nitrate removal of 43% at Day 1.

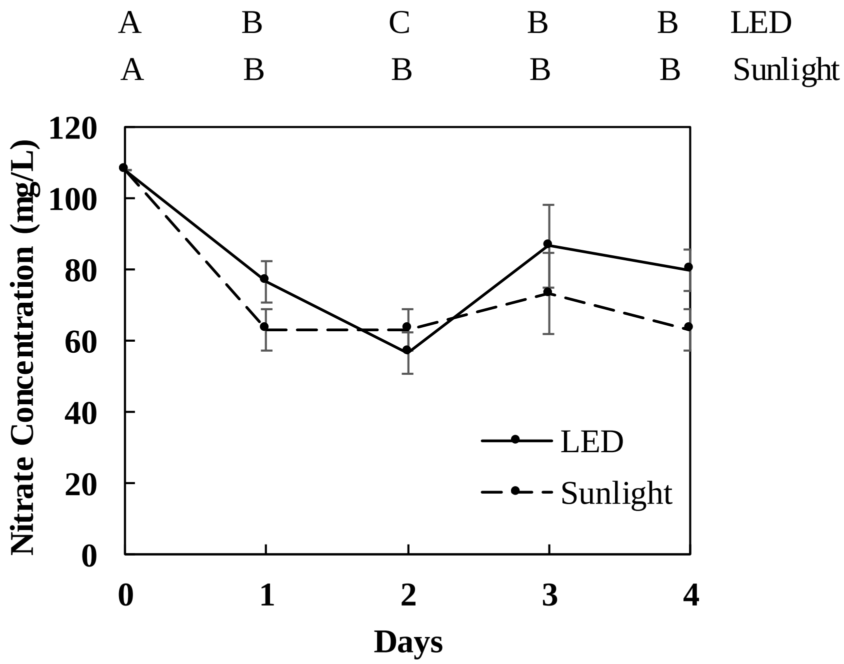

Figure 4. 29: Nitrate Concentration in Wastewater under LED Light and Sunlight. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

The result showed that the different light source did not cause any significant different on the nitrate removal. Only the phenomenon of nitrate release into the wastewater at Day 3 and 4 is more significant when under LED grow light compared to that of the sunlight. This is mainly caused by the higher biomass concentration in wastewater under LED grow light exhibited higher self-shading effect and caused cell death and lysis to release the nitrate.

Figure 4.30 showed the performance of the orthophosphate removal by *C. vulgaris* when in different light source, which is the LED grow light and sunlight. Both light sources did not promote any orthophosphate assimilation but promoted orthophosphate release into the poultry wastewater. The differences between the orthophosphate releases under both light sources were not significant. Result proved that the LED grow light and sunlight do not cause any significant impact on the orthophosphate removal.

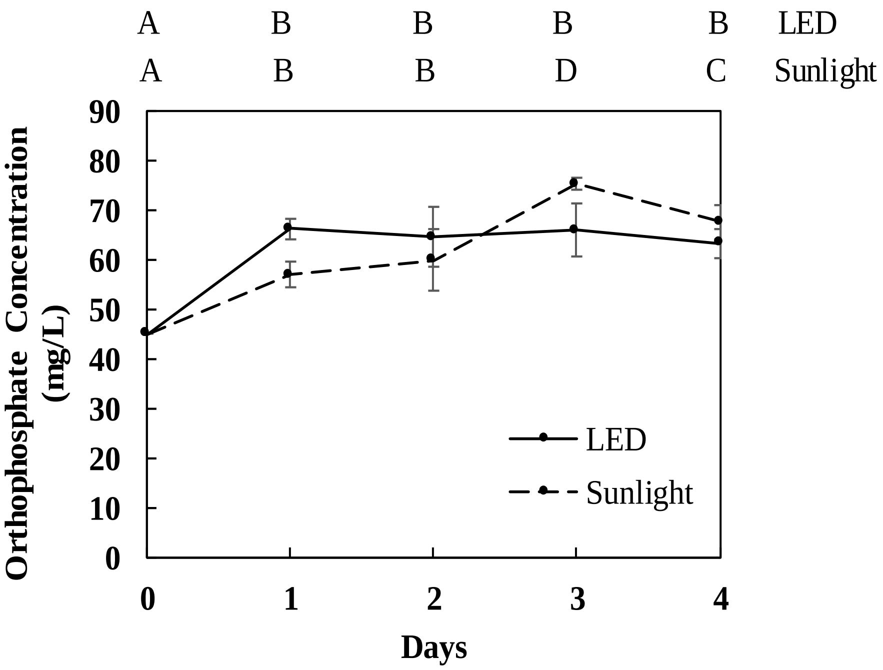

Figure 4. 30: Orthophosphate Concentration in Wastewater under LED Light and Sunlight. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

In summary, the stable and consistent LED grow light is preferable than the sunlight due to the LED grow light tend to promote higher microalgae growth and higher removal of COD, ammoniacal nitrogen, and nitrite than the sunlight. However, the using of LED grow light might cause higher costing compared to that of sunlight. Due to the development in technology, the sunlight can be used as solar energy supply for LED grow light in order to overcome the drawback of sunlight.

4.4.3 Harvesting method

The microalgae harvesting method after the wastewater treatment is one of the key factors that will affect the operating cost of the microalgae treatment process and the performance of wastewater treatment. Figure 4.31 showed the comparative effectiveness of the flocculation method by using chitosan and centrifugation method in removing various contaminants from poultry wastewater. The centrifugation method was found more effective in biomass harvesting compared to the flocculant method by chitosan, which achieved cell separation efficiency at 81% and 92% for flocculation method and centrifugation method respectively. Both method was found tend to further remove the remaining COD and nutrients from the treated wastewater together with biomass harvesting, especially the COD removal that achieved 82 – 85% in efficiency. Moreover, the flocculation method by chitosan was found outperformed the centrifugation method on the removal of remaining nutrients such as nitrite, ammoniacal nitrogen, and orthophosphate from the treated poultry wastewater.

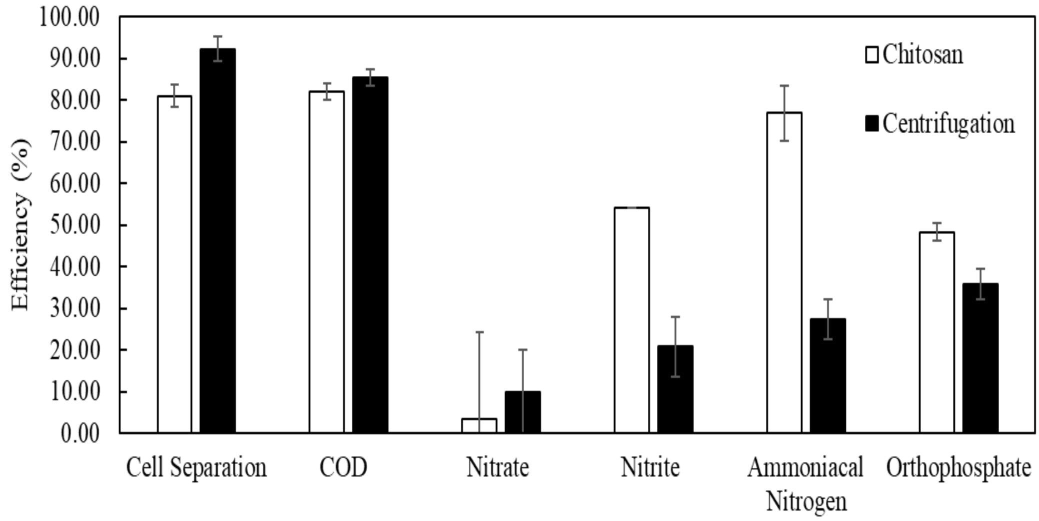

Figure 4. 31: Cell Separation, COD, Nitrate, Nitrite, Ammoniacal Nitrogen and Orthophosphate Removal Efficiency in Wastewater

The *C. vulgaris* used in this study appears in micron sized suspended solid form. The cell separation and COD removal in this study showed a high efficiency reflects that the two separation processes are effective to target suspended solids and organic particles. The centrifugation may be a better option for systems with significant organic loads, as it removes larger suspended particles more efficiently. Centrifugation method is recognized as an effective method for microalgae harvesting due to its efficiency can be optimised by adjusting the rotation speed and the duration of rotation.

The flocculation method by chitosan also tend to promote high cell separation efficiency and COD removal same with the study by Zheng et al. (2023), where the flocculation by chitosan tends to achieve up to 100% of cell separation and enhanced the COD removal from 54% to 83%. In this study, the centrifugation showed a better result than the chitosan as the excess chitosan that remained in the treated wastewater might contribute to the increment of COD (Ariyani et al., 2021).

On the other hand, chitosan was particularly successful in removing nitrogenous pollutants, such as ammoniacal nitrogen and nitrite, and orthophosphate from the treated poultry wastewater more efficiently than the centrifugation method (Tan et al., 2019). Chitosan contains –OH and $–NH_2$ functional groups that capable of forming hydrogen bond with ammoniacal nitrogen and also it's positively charged amino group tends to adsorb the negatively charged nitrite and orthophosphate from the wastewater effectively (Toh et al., 2014; Loo et al., 2021) Centrifugation, although effective in separating solid matter, showed limited success with nutrients removal, indicating that physical separation alone cannot effectively address all nitrogen forms, especially dissolved ones.

In conclusion, the flocculation method was found more preferable for large scale industrial application for microalgae biomass harvesting. Flocculation by chitosan tends to further polish the treated wastewater and improve the water quality effectively. The microalgae method for poultry wastewater treatment is proven feasible for the real industrial application.

4.5 Study on *Chlorella vulgaris* Biomass on Optimal Parameters

One of the novelties by employing the microalgae method for wastewater treatment is the nutrients resources that leak into the wastewater can be recovered and then generated the biomass for by-product production in order to meet zero-waste mission. *C. vulgaris* is well known with its high lipid yield that have the potential to become the biofuel. The following study focuses on the investigation for the potential of biomass that generated from the poultry wastewater.

4.5.1 Biomass Production

The results in Figure 4.32 clearly indicated the potential of poultry wastewater as a growth medium for microalgae, with significant biomass increase observed over the 21-day period. The microalgae were pre-acclimated to the same wastewater medium, which played a key role in the absence of a lag phase. As the microalgae were already adapted to the wastewater, they were able to directly begin their growth process without the typical initial delay. The wastewater, with an initial concentration of COD (1060 mg/L), nitrate (120 mg/L), nitrite (800 mg/L), ammoniacal nitrogen (49 mg/L), and orthophosphate (90 mg/L) was used in this study.

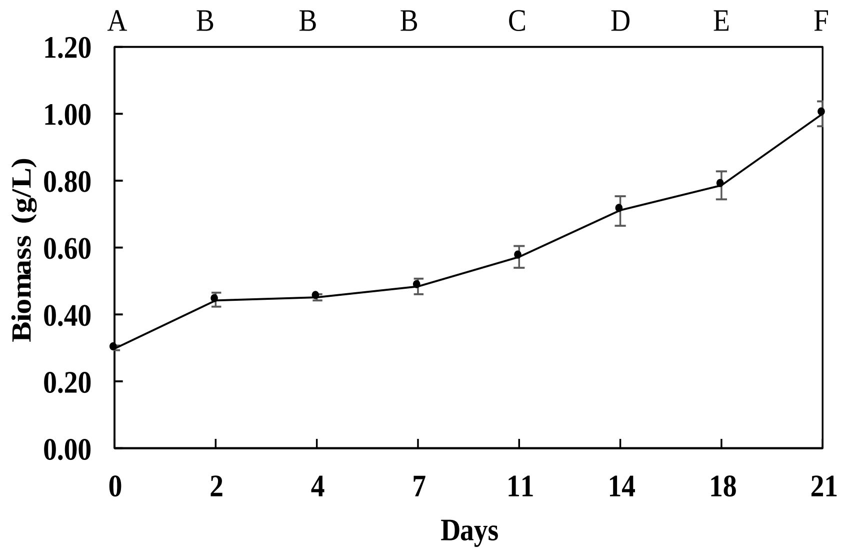

Figure 4. 32: Biomass Concentration of *C. vulgaris* in Wastewater under Optimised Condition. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$.

From Day 0 to Day 21, a steady increase in biomass was observed. Initially, the growth was relatively gradual, with biomass increasing by 48% from Day 0 to Day 2, and 50% from Day 2 to Day 4. The biomass increase accelerated between Day 4 and Day 11 (61%) and again from Day 11 to Day 18 (137%), reflecting the microalgae's increasing efficiency in utilizing the nutrients available. The highest growth rate was recorded between Day 18 and Day 21, with biomass increasing by 234%. Despite the initial imbalance in nutrient concentrations, the microalgae adapted well, showing promising potential for wastewater treatment applications with concurrent biomass production.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The use of *C. vulgaris* for poultry wastewater treatment under mixotrophic conditions demonstrated significant potential for nutrient removal and biomass production. The microalgae effectively removed 75% of COD and 98% of ammoniacal nitrogen, showing resilience even in the presence of nutrient imbalances and high nitrite levels. While phosphorus removal was limited to 22%, the strain thrived and contributed to effective pollutant removal, proving its adaptability in complex wastewater conditions. The 12:12 photoperiod was the most effective in maintaining stable nutrient removal, consistently achieving reductions of 79% in COD and 100% in ammoniacal nitrogen. In contrast, the 24:0 continuous light cycle, though promoting higher biomass growth, resulted in photoinhibition and cell lysis, leading to nutrient release back into the medium and ultimately reducing overall treatment efficiency. The 0.13 g/L inoculum size proved optimal for nutrient uptake and growth, achieving 84% COD removal, along with 50% nitrate, 50% nitrite, and 47% ammoniacal nitrogen removal. However, orthophosphate removal was the least efficient indicating nutrient imbalance in wastewater, and overcrowding in larger inocula limited nutrient diffusion, reducing overall treatment efficiency. Acclimation to poultry wastewater significantly enhanced nutrient removal, with pre-cultivated microalgae outperforming those grown in a fertilizer medium, achieving the most notable 73% COD removal. Although nitrate levels increased substantially in poultry wastewater compared to the fertilizer medium, pre-cultivated microalgae still exhibited superior removal efficiencies for other nutrients. The use of LED grow lights resulted in more stable growth and nutrient removal compared to sunlight, which exhibited fluctuating intensity. This stability contributed to 90% COD removal and complete ammonia removal, the most significant outcomes, emphasizing the critical role of consistent light conditions in optimizing treatment efficiency. Nutrient removal typically peaks between day 2 and day 4 for most nutrients, with some nutrients exhibiting enhanced removal at day 4. The utilization of a harvesting agent such as chitosan significantly improves nutrient removal efficiency, while also reducing treatment time. Chitosan flocculation achieved 76.88% ammoniacal nitrogen removal, significantly outperforming centrifugation (27.42%). However, early harvesting on Day 2 limited overall efficiency, with 3.33% nitrate removal. Despite this, chitosan flocculation outperformed centrifugation in orthophosphate removal (48.30%) and showed comparable COD removal (82.12%). In conclusion, optimizing poultry wastewater treatment through the use of microalgae presents a promising and sustainable approach for achieving zero-waste wastewater management and renewable energy generation, while simultaneously reducing treatment time.

5.2 Recommendations

To improve the quality of this study, some recommendations can be carried out:

1. Perform the study with much bigger volume of wastewater
2. Optimize further the wastewater treatment with more than 1 species of microalgae
3. Optimize further the wastewater treatment with mixed different source of wastewater treatment
4. Conduct more detailed study on harvesting by chitosan

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LIST OF PUBLICATION

Abdul Ghani, S.A., Ang, C.W., Yong, J.W., Chng, L.M., Yeap, S.P., Chan, D.J.C., Geng, J., Leong, S.S. and Toh, P.Y., 2025. Comparative analysis of cultivation methods for *Chlorella vulgaris* growth and nutrient recovery in palm oil mill effluent (POME). E3S Web of Conferences, 627, 01008. https://doi.org/10.1051/e3sconf/202562701008.