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MICROALGAL BIOFILM FOR POST-TREATMENT OF WASTEWATER AND BIORESOURCE RECOVERY

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CONFIRMATION BY PANEL OF EXAMINERS

I certify that a Panel of Examiners has met on 16 December 2025 to conduct the final examination of Nik Airin Syazlin Binti Azhan in her Master of Science thesis entitled "Microalgal Biofilm for Post-Treatment of Wastewater and Bioresource Recovery" in accordance with Universiti Teknologi MARA Act 1976 (Akta 173). The Panel of Examiner recommends that the student be awarded the relevant degree. The Panel of Examiners was as follows:

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ABSTRACT

Microalgae, especially with biofilm-forming abilities, are known to be an efficient alternative to treat wastewater. On the other hand, their biomass is reported to contain valuable bioactive compounds making them useful in industries such as food, pharmaceuticals, and cosmetics. However, there is lack of study on the potential of locally isolated microalgae in wastewater treatment or their role in biotechnological applications. This study was aimed to investigate the potential of local biofilm-forming microalgae and their consortia in pollutant removal performance and bioresource recovery. Local microalgae were isolated and grown in synthetic wastewater to determine their ability in reducing nitrogen and phosphorus pollutants while their biomass and lipid production were also determined. Following that, microalgae with promising abilities were selected to form consortia and their potentials were re-evaluated. The biofilm morphology, Total Nitrogen (TN) and Total Phosphorus (TP) removal, biomass production, and lipid profiles were assessed using FESEM, standard colorimetric analyses, gravimetric measurements, and GC–MS, respectively. A total of 24 microalgae were successfully isolated and demonstrated biofilm formation, which was then confirmed by FESEM analysis for selected isolates. After cultivation in synthetic wastewater for seven days, MA18 identified as *Nodosilinea nodulosa,* and MA7 identified as *Leptolyngbya sp.,* were found to be the highest pollutant remover with 43.5% and 95.0% for TN and TP, respectively, while the highest biomass production was observed in MA23, which is molecularly identified as *Leptolyngbya sp.,* at 90.0% as compared to the initial growth. GC–MS analysis of lipid extraction revealed that C16-C18 were the predominant fatty acids produced by the isolates, indicating their potential for biofuel production, with MA23 producing the broadest fatty acids profile. A total of ten consortia from the combination of microalgae displaying potential abilities was then constructed. Consortium 7 (MA18–MA5–MA22) displayed the highest TN removal rate at 75.2%, while Consortium 1 (MA18–MA7–MA23) demonstrated exceptional TP removal at 99.4%. The highest biomass yield was recorded for Consortium 10 (MA18–MA4–MA22), representing a 50.0% increase from the initial culture. For lipid analysis Consortium 1 (MA18–MA7–MA23) displayed the most diverse fatty acid profile, dominated by saturated fatty acids of C14-C24, with monounsaturated fatty acids. The consortia outperformed individual isolates in TN and TP removal, whereas higher biomass production was observed in individual isolates. These findings demonstrate the potential of locally isolated microalgae in pollutant removal, biomass productivity and biofuel-relevant lipid profiles, indicating promising candidates for biotechnological applications.

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LIST OF SYMBOLS

Symbols

C_0	Initial concentration
C	Final concentration
˚C	Celcius
g	gram
ml	milliliter
µl	microliter
uM	micromolar
h	hour

LIST OF ABBREVIATIONS

Abbreviations

BOD	Biochemical Oxygen Demand
COD	Chemical Oxygen Demand
EDCs	Endocrine Disrupting Compounds
EPS	Extracellular Polymeric Substance
FAMEs	Fatty Acid Methyl Esters
FESEM	Field Emission Scanning Electron Microscope
PBR	Photobioreactor
PCR	Polymerase Chain Reaction
PPCPs	Polypropylene Copolymers
TN	Total Nitrogen
TP	Total Phosphorus

LIST OF NOMENCLATURE

Nomenclatures

C	Calcium
Ca	Carbon
Mg	Magnesium
Mn	Manganese
Mo	Molybdenum
N	Nitrogen
P	Phosphorus
Zn	Zinc

CHAPTER 1
INTRODUCTION

1.1 Research Background

Wastewater can contain many pollutants such as phosphorus and nitrogen, and heavy metals such as lead and zinc (Yakamercan et al., 2022; Gururani et al., 2022). There are three primary sources of wastewater, which are municipal, industrial and agricultural. Municipal wastewater typically comprises domestic, commercial, and institutional effluents, along with infiltration and inflow, including those generated from healthcare facilities such as hospitals and laboratories, which may introduce pathogens, pharmaceutical residues, and disinfectants, in addition to organic matter, microplastics, and nutrient loads of nitrogen and phosphorus. In contrast, industrial wastewater is released by a variety of sectors, including food processing, mining, and chemical manufacturing, which contain chemicals like heavy metals, dyes, and phenols, among others. Agricultural wastewater, on the other hand, is defined as the discharge from animal farms and agricultural areas with high quantities of nitrogen, phosphorus, and organic carbon and contains contaminants such as pesticides, manure, pathogens, and antibiotics (Amaro et al., 2023).

Each year, approximately 380 billion m^3 of municipal wastewater is generated worldwide (Bortolini et al., 2022; Spínola et al., 2024). The amount of wastewater produced, however, keeps increasing due to the growing population, water supply expansion, improved living conditions, and economic expansion (Al-Jabri et al., 2021; Plöhn et al., 2021). By 2050, it is estimated that the world's water consumption for towns, industry, and agriculture will increase by 20–30%, which would definitely contribute to the increasing production of wastewater (Plöhn et al., 2021).The continuous increase in wastewater production is of great concern due to its high load of contaminants and pollutants, leading to potential impact on the environment and endangering public health in general. Hence, these wastewaters need to be treated appropriately before they can be released safely to the environment and reused.

Different treatment approaches are required according to the distinct characteristics of wastewater. Currently, conventional methods include physical, chemical and biological treatments. Physical treatment includes filtration, screening,

sedimentation and membrane filtration. In contrast, chemical treatment involves chlorination, coagulation, ultraviolet light and ozonation. Physical and chemical methods demand high operational and maintenance costs, increasing the overall budget and process complexity (Abdelfattah et al., 2023; Gururani et al., 2022).

On the other hand, biological approaches involve the use of microorganisms, such as bacteria, fungi, yeast and microalgae (Abdelfattah et al., 2023). The employment of biological agents is a cost-effective, eco-friendly alternative to the physical and chemical methods of environmental restoration in wastewater treatment (Saini et al., 2023). One of the biological agents with potential in wastewater treatment is microalgae (Arita et al*.,* 2015; Gururani et al*.,* 2021). Microalgae have been reported to exhibit a remarkable ability to remove pollutants in wastewater, with removal rates of up to 80-100% (Gururani et al., 2022). Their effectiveness is primarily attributed to their capacity for nutrient assimilation, rapid growth, and photosynthetic oxygen production, which supports both nitrogen and phosphorus removal while reducing the need for external aeration (Lee & Lei, 2022; Yaakob et al., 2021). A wide range of microalgae was documented in wastewater treatment, including *Chlorella variabilis,* which was able to remove total nitrogen and total phosphorus by 93.8–96.1% and 97.1–99.9%, respectively (Tran et al., 2021a) while *Scenedesmus obliquus* demonstrated a higher removal efficiency, at 99% (Ling et al., 2019). In addition, microalgal-based wastewater treatment systems are often regarded as environmentally sustainable due to their low energy requirements (Al-Jabri et al., 2021; Srimongkol et al., 2022), and potential for biomass valorisation (Al-Jabri et al., 2021).

Microalgal biomass can be harvested and used for a variety of purposes, as they contain valuable bioactive compounds like proteins, fatty acids, pigments, and vitamins (Chen et al., 2022), making them useful in food, pharmaceuticals, and cosmetics (Al-Jabri et al., 2021; Machado et al., 2022). Examples are *Chlorella* which is widely commercialized for nutraceuticals due to its high protein, carotenoids, and vitamin content, while *Spirulina* is used in medicine for its antioxidant properties and as a source of phycocyanin, a natural food colorant (Bortolini et al., 2022; Spínola et al., 2024).

Microalgae can also be utilised for biofuel production (Al-Jabri et al., 2021; Delrue et al., 2016), with some strains reaching up to 70% oil content (Alishah Aratboni et al., 2019). Compared to traditional crops, microalgae can efficiently absorb CO_2 (Machado et al., 2022; Srimongkol et al., 2022), which helps them to grow faster and can adapt easily to the environment (Srimongkol et al., 2022), while providing an

economical source for biofuel production (Nanda et al., 2021). Microalgae species like *Nannochloropsis* sp., *Chlorella* sp., *Scenedesmus* sp., *Pavlova lutheri*, and *Isochrysis* sp. are frequently researched for biodiesel production. *Botryococcus braunii* and *Chlorella vulgaris* are notable for their high lipid content, with *B. braunii* reaching up to 44.97% and *C. vulgaris* containing about 28.07%, making them promising candidates for biofuel production (Chhandama et al., 2021).

For bioremediation applications, microalgal biofilms grown offer several advantages as compared to their planktonic floating cells. Microalgae biofilms are more robust in withstanding fluctuating environmental conditions (Zhang et al., 2020). They were also reported to be more efficient in treating wastewater as compared to their planktonic counterparts (Ugya et al., 2021). In addition, due to their nature of growing on surfaces, biofilm-based microalgae are easier to collect with less energy and space requirements (Hu et al., 2021).

Hence, the current study aims to isolate local biofilm-forming microalgae and determine their ability in reducing nitrogen and phosphorus pollutants in wastewater, while producing lipids and biomass that would probably be beneficial for biotechnology applications. In addition, the prospective of multiple consortia of different types of microalgae in wastewater treatment will also be explored to investigate if they can perform better as a collective as compared to single microalgae cells. Data from this study will help to discover the potential of locally isolated microalgae in wastewater treatment and subsequently encouraging a circular economy by promoting a sustainable and ecologically friendly future prospective.

1.2 Problem Statement

Conventional wastewater treatment methods are widely used to mitigate wastewater discharge and manage pollutants. However, these methods suffer from significant limitations, such as inconsistent efficiency, high operational costs, and the generation of secondary pollution due to successive treatment stages. These drawbacks highlight the urgent need for innovative wastewater treatment solutions that simultaneously produce valuable bioresources.

Microalgae-based wastewater treatment systems have emerged as a promising alternative. Microalgae align with biorefinery and circular economy principles, offering advantages such as low carbon emissions, high resource recovery rates, production of

non-toxic byproducts and reduced energy consumption. Despite these potentials, the application of locally isolated microalgal strains for wastewater treatment remains underexplored, particularly their ability to form biofilms and produce value-added components.

1.3 Significance of Study

This research may aid in developing an eco-friendly alternative that uses microalgae for efficient treatment methods to reduce nitrogen, and phosphorus pollutants in wastewater to a minimal level, while simultaneously determining the ability to produce fatty acids as potential candidates for biofuel and produce biomass to be used for other applications. This work supports sustainable water management, the National Policy on Biological Diversity 2022-2030 (NPBD), and the Sustainable Development Goals (SDGs) for a clean environment.

1.4 Research Objectives

The objectives of this research are:

a) To isolate and characterize locally isolated microalgae with abilities to form biofilm
b) To determine the ability of the locally isolated microalgae biofilm in reducing Total Nitrogen (TN) and Total Phosphorus (TP) in wastewater
c) To investigate the production of biomass and lipids from the local microalgal biofilm
d) To determine the reduction of TN and TP, and the production of biomass and lipid using different microalgae biofilm consortia

1.5 Scope and Limitation of Study

This study focuses on the capability of microalgae biofilm in treating post-treated wastewater and producing valuable bioresources. However, this study concentrates solely on samples obtained from a single ecological niche and uses only municipal wastewater, to develop a more sustainable treatment method for this type of wastewater. Another limitation is that the research may have been conducted under

controlled lab conditions, which may not fully represent the complexities of natural environments. Factors like light intensity, temperature, and nutrient availability in the lab might differ significantly from those in the actual ecosystems.

CHAPTER 2
LITERATURE REVIEW

2.1 Microalgae

Microalgae are unicellular photosynthetic microorganisms capable of generating chemical energy from solar energy. Through photosynthesis, they convert sunlight, carbon dioxide and nutrients into glucose, oxygen and valuable biomass. They are major contributors to oxygen production (Udayan et al., 2021). They can be found in diverse aquatic environments, including freshwater and marine habitats, as well as on soil surfaces (Kassim et al., 2022; Brasil et al., 2017). Based on their pigmentation, habitat and cellular structure, they can be classified into prokaryotic cyanobacteria and eukaryotic microalgae (Gururani et al., 2022; Renuka et al., 2013).

2.1.1 Classification of Microalgae

Prokaryotic microalgae are blue-green algae (*Cyanophyceae*), also known as cyanobacteria. They are commonly found in fresh, brackish and salt water, as well as in soils and even in extreme environments (Bozan et al., 2024). They lack membrane-bound organelles and share structural similarities with Gram-negative bacteria. Their internal thylakoid membranes, responsible for photosynthesis, are loosely arranged within the cytoplasm. The cell wall comprises multiple layers of peptidoglycan and lipopolysaccharides, forming a relatively loose envelope that simple enzymatic systems can easily digest. This makes cyanobacteria, such as *Spirulina*, suitable for use as a single-cell protein in food supplements (Nagarajan et al., 2020). Although cyanobacteria are taxonomically classified as prokaryotic bacteria, they are often referred to as prokaryotic microalgae in wastewater treatment and environmental biotechnology literature (Arias et al., 2020; Touliabah et al., 2022). This terminology reflects their functional similarity to eukaryotic microalgae, particularly in terms of oxygenic photosynthesis, nutrient assimilation, biomass production, and biofilm formation (Pinevich & Averina, 2024). Consequently, many applied studies adopt a functional rather than phylogenetic classification when evaluating microalgae-based

wastewater treatment systems. Hence, the "microalgae" label in wastewater and biotechnology literature is mainly functional or ecological, not taxonomic (Żymańczyk-Duda et al., 2022).

In contrast, eukaryotic microalgae include diverse groups such as green algae (*Chlorophyceae*), brown algae (*Phaeophyceae*), red algae (*Rhodophyceae*) and diatoms (*Bacillariophceae*) (Emparan et al., 2019) as shown in Figure 2.1. These microalgae possess a defined nucleus, chloroplasts, and other membrane-bound organelles. In eukaryotic microalgae, thylakoid membranes are enclosed within chloroplasts and are highly organized. Their rigid cell walls are primarily composed of fibrillar polysaccharides such as cellulose and hemicellulose (Chen et al., 2022), distinguishing them structurally from their prokaryotic counterparts. Additionally, their higher surface-to-volume ratio enhances nutrient uptake and growth efficiency (Yin et al., 2020). Table 2.1 presents the main classes and representative species of each group.

Table 2.1
Main Classes of Microalgae

Classes	**Examples**
Prokaryotic blue-green (*Cyanophyceae*)	Nostoc, Microcystis, Oscillatoria, etc
Eukaryotic green (*Chlorophyceae*)	Botryococcus, Chlamydomonas, Chlorella, Scenedesmus, etc
Eukaryotic brown (*Phaeophyceae)*	Mallamonas, Ochromonas, Uroglena, Synura etc
Eukaryotic red (*Rhodophyceae)*	Porphyridium
Eukaryotic diatoms (*Bacillariophyceae*)	Asterionella, Cyclotella, Fragilaria, etc

Classes and examples of microalgae. Source: Emparan et al. (2019)

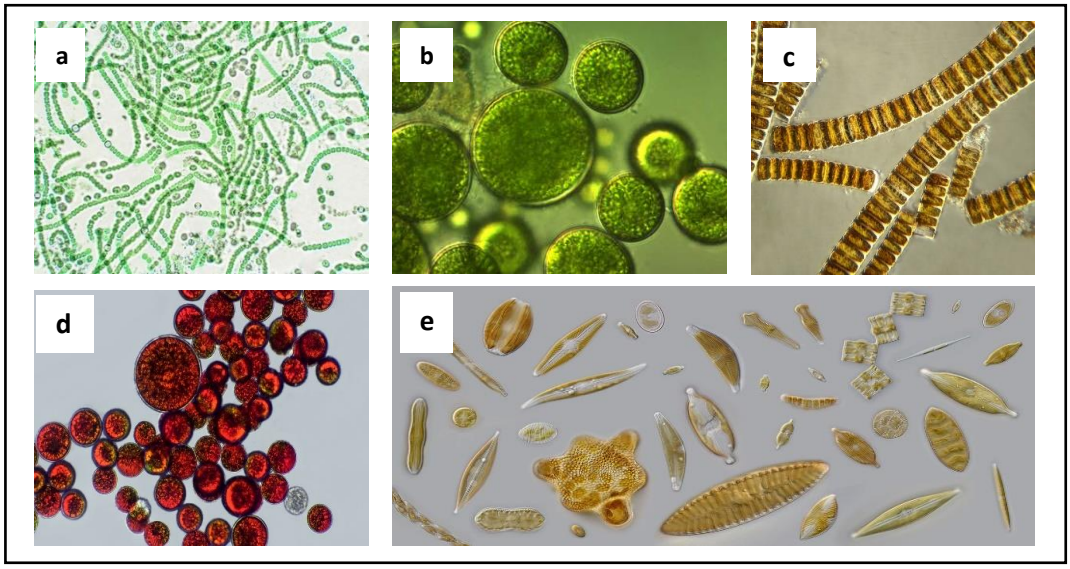


Figure 2.1 Different Classes of Microalgae

Note. The figure shows (a) prokaryotic blue-green microalgae (cyanophyceae) and (b-e) eukaryotic microalgae (green, brown, red microalgae and diatoms). The figures obtained from Mapstone et al. (2022).

Microalgae are highly adaptable to various environments due to their versatile metabolic pathways that enable microalgae to assimilate nutrients and survive. They exhibit diverse metabolic modes including autotrophy, heterotrophy, and mixotrophy (Chhandama et al., 2021).

2.1.2 Metabolism of Microalgae

Microalgae can thrive and adapt under diverse environmental conditions by exhibiting a range of metabolic processes, which include photoautotrophic, heterotrophic, and mixotrophic metabolisms (Chhandama et al., 2021). These different metabolic strategies influence their growth rate, biomass yield, and nutrient uptake efficiency.

Photoautotrophic microalgae convert inorganic carbon (CO_2) into organic compounds, such as glucose, using sunlight as an energy source through photosynthesis (Emparan et al., 2019). In contrast, heterotrophic microalgae rely entirely on external organic carbon sources for energy and growth, typically in the absence of light. Some species also exhibit photoheterotrophy, wherein light is utilised as the energy source

while organic compounds serve as the carbon source (Abdelfattah et al., 2023).

Mixotrophic microalgae can switch between photoautotrophic and heterotrophic modes depending on environmental conditions, allowing them to use CO_2 and organic carbon sources simultaneously or alternately. This metabolic flexibility enhances their adaptability and resilience (Abdelfattah et al., 2023; Markou & Georgakakis, 2011).

While heterotrophic growth depends mainly on the availability of organic carbon, photoautotrophic, photoheterotrophic, and mixotrophic growth are influenced by both light intensity and carbon source concentration (Abdelfattah et al., 2023; Markou & Georgakakis, 2011; Nagarajan et al., 2022). Although most microalgae are fundamentally autotrophic, many species have evolved the ability to metabolize organic substrates under favorable conditions, as illustrated in Figure 2.2.

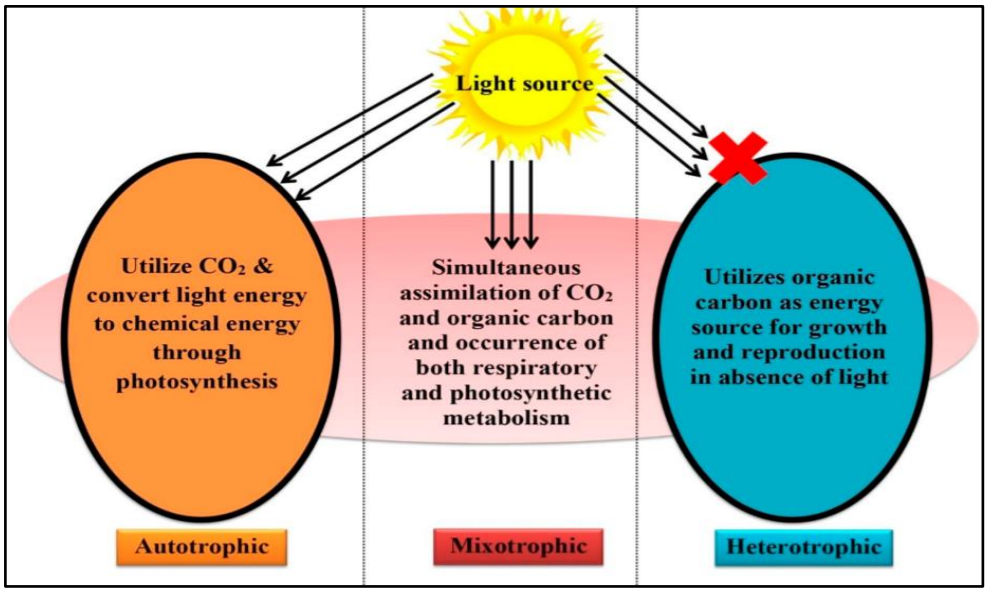


Figure 2.2 Different Types of Microalgae Metabolisms

Note. Schematic representation of microalgal metabolic modes: autotrophic (light-dependent CO_2 assimilation via photosynthesis), heterotrophic (organic carbon utilization in the absence of light) and mixotrophic (combined use of CO_2 and organic carbon in the presence of light). Source: Adapted from Gururani et al. (2022).

The type of metabolic mode employed affects not only growth rate but also nutrient assimilation and biochemical composition. This enables them to modulate internal structures, secrete extracellular substances, compete with other microorganisms, and adapt to changing environmental conditions (Emparan et al., 2019).

2.2 Wastewater

Wastewater is a byproduct of various human and industrial activities, containing a complex mix of organic and inorganic substances, pathogens, nutrients, and emerging pollutants (Al Hamedi et al., 2023). It originates from municipal, industrial and agricultural sources, each contributing unique contaminant profiles (Plöhn et al., 2021). With increasing global urbanization, population growth, and industrial activities, the volume and complexity of wastewater have risen, posing significant environmental and public health concerns (Pratap et al., 2023). Effective management and treatment of wastewater are essential to protect water resources, reduce pollution, and enable water reuse (Al-Jabri et al., 2021). Understanding the different types and sources of wastewater is fundamental in developing targeted and sustainable treatment approaches. Generally, there are three types of wastewaters, which are municipal, industrial and agricultural wastewater.

2.2.1 Municipal Wastewater

Municipal wastewater, or sewage, is generated in urban areas from domestic activities such as cooking, washing and bathing. It includes wastewater from households, commercial buildings, healthcare and other public facilities connected to the sewage system (Srimongkol et al., 2022; Thebo et al., 2017). This type of wastewater contains a wide range of contaminants, including organic materials, pollutants, mainly nitrogen and phosphorus, pathogens, suspended particles and heavy metals such as chromium, cadmium, cobalt, iron, and arsenic (Abdelfattah et al., 2023; Kong et al., 2021). Among these, carbon, nitrogen, and phosphorus are the primary pollutants. The amounts of nitrogen and phosphorus in municipal wastewater typically range from 10 to 100 mg L^{-1} (Bhatti et al., 2021). In addition to conventional pollutants, municipal wastewater also contains micropollutants such as persistent organic pollutants (POPs), pharmaceuticals, hormones, surfactants and plasticizers (Plöhn et al., 2021).

2.2.2 Industrial Wastewater

Industrial wastewater is a byproduct of industrial processes such as manufacturing, processing, cleaning, cooling, and rinsing. The composition of industrial wastewater varies greatly depending on the type of industry. For instance, chemical plants, textile factories, and food processing facilities each produce different types and amounts of pollutants (Srimongkol et al., 2022). In general, industrial wastewater tends to have high concentrations of heavy metals, synthetic chemicals, organic contaminants and suspended solids that can be toxic to the environment and human health. The main source of POPs globally is industrial wastewater, which are difficult to break down and harmful to the environment (Plöhn et al., 2021).

2.2.3 Agricultural Wastewater

Agricultural wastewater refers to the wastewater produced from agricultural activities and practices. According to the United Nations Environment Programme (UNEP) (2013), there are several sources of agricultural wastewater, which include water runoff from fields, wastewater from animal farms, and cleaning water from equipment or storage areas. The characteristics of agricultural wastewater depend on factors such as the type of crops grown, livestock reared, farming practices, and the use of fertilizers and chemicals (Serejo et al., 2019). Contaminants, including nutrients, pesticides, herbicides, sediments, pathogens, and organic matter, can be obtained in this particular wastewater (Amaro et al., 2023). Agricultural wastewater can also contain a variety of other components, such as antibiotics or any other pharmaceutical given to animals. It is a major contributor to eutrophication and groundwater contamination due to the nutrients from the manure, which typically comes with the wastewater and is used as fertilizer, that are not taken up by crops, will enter the water body (Plöhn et al., 2021).

2.2.4 Wastewater Treatment

Wastewater is treated in wastewater treatment facilities before being discharged into the water systems. The treatment processes are divided to three steps physical (primary), biological (secondary) and chemical (tertiary) as shown in Figure 2.3 (Wang

et al., 2022). Primary treatment involves physical approaches of screening and removing solids using sedimentation to separate particles. In contrast, the secondary treatment primarily relies on aerobic biological processes, such as the activated sludge system, in which aerobic microorganisms degrade biodegradable organic matter under oxygen-rich conditions (Lima et al., 2020). Tertiary treatment applies physical, chemical or biological advancements through membrane systems, disinfection or nature-based systems to remove remaining contaminants, including dissolved solids, nutrients (especially nitrogen and phosphorus), and pathogens (Pratap et al., 2023; Renuka et al., 2013; Wang et al., 2022).

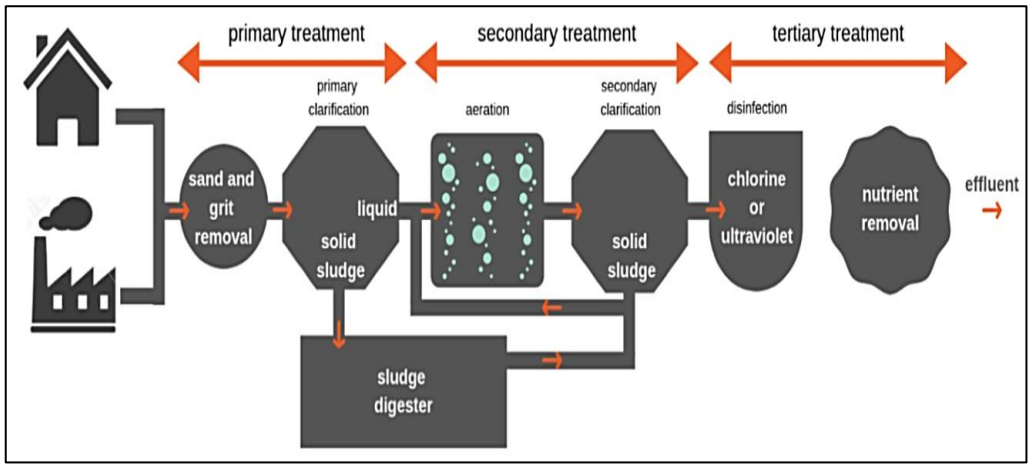


Figure 2.3 Schematic Process Flow Diagram of a Typical Wastewater Treatment Plant

Note. The image outlines a wastewater treatment process, including primary treatment (sand and grit removal, primary clarification), secondary treatment (aeration, secondary clarification) and tertiary treatment (disinfection with chlorine or ultraviolet, nutrient removal), producing treated effluent. Sludge is processed in a digester. Adapted from Lima et al. (2020).

Depending on the types of wastewaters, each requires a specific treatment due to the differences in contaminants contained in the wastewaters. Municipal wastewater treatment aims to eliminate or reduce the concentration of contaminants in receiving water bodies to levels that meet regulatory standards. The treated wastewater, also known as effluent, is considered safe for release into rivers, lakes, or oceans. It can also be treated further for reuse applications like irrigation, industrial operations, or groundwater recharge (Amaro et al., 2023). Industrial wastewater often requires specialized treatment to meet regulatory standards and prevent environmental contamination due to its complex and hazardous nature. Agricultural wastewater

treatment, particularly focusing on degrading herbicides, insecticides and other organic contaminants, implements biological processes, adsorption processes and advanced oxidation processes (AOPs) to reduce nutrients and organic matter (Yin et al., 2020).

2.2.5 Pollutants in Wastewater

Wastewater contains a wide range of pollutants that pose risks to both the environment and human health. Organic pollutants mainly come from human waste, food scraps, fats, proteins, sugars, and soaps. These substances may be dissolved in the water or present as suspended solids (du Plessis, 2022). In addition to organic waste, wastewater often contains inorganic pollutants such as heavy metals like lead, copper, zinc and salts. These can come from industrial activities, stormwater runoff, or damaged pipes. Unlike organic matter, inorganic substances are more stable and harder to break down (Orfanos & Manariotis, 2019). Pathogens such as bacteria, viruses, and parasites are also commonly present in wastewater. They can be harmful if they contaminate water supplies or food sources like shellfish (Kilbane, 2022; Wang et al., 2022).

Nutrient pollution, mainly nitrogen and phosphorus, is another major concern. In excess, these nutrients can lead to eutrophication, which harms aquatic ecosystems by triggering algae blooms, reducing oxygen levels, and threatening aquatic life (Saini et al., 2023).

2.2.5.1 Total Nitrogen (TN)

Total Nitrogen (TN) is a critical indicator of nutrient pollution in wastewater, referring to the combined concentration of all nitrogenous compounds in a water sample, including organic nitrogen, ammonia (NH_4^+), nitrate (NO_3^-), and nitrite (NO_2^-). These compounds can originate from agricultural runoff, industrial discharge, sewage, and aquaculture waste such as undigested feed and feces. Elevated levels of nitrogen, particularly nitrates and nitrites, pose significant environmental and health risks. While nitrates are toxic at concentrations exceeding 45 mg/L, nitrites are even more harmful (as low as 0.1–0.5 mg/L, and toxic effects can occur even below 1 mg/L, depending on species and exposure duration), to aquatic life due to their instability and reactivity (Al-Jabri et al., 2021). High TN concentrations, typically exceeding 20–30 mg/L in

wastewater, can lead to eutrophication, a process of nutrient enrichment that promotes the growth of harmful algal blooms and excessive aquatic plant growth. This can deplete oxygen levels, disrupt ecosystems, and lead to the long-term degradation of water bodies, transforming lakes and rivers into marshy or dry land areas (Butler, 2024).

In Malaysia, the Water Services Industry (Prohibited Effluent) Regulations 2021 stipulate a maximum allowable limit of 50 mg/L for total nitrogen in effluents discharged into public sewage systems. This regulatory benchmark is crucial for assessing the effectiveness of nitrogen removal strategies, such as nitrification and denitrification, in wastewater treatment processes.

2.2.5.2 Total Phosphorus (TP)

Total phosphorus (TP) is a measure of all phosphorus present in a water sample, whether dissolved or particulate. It is a critical parameter in water quality monitoring, especially in wastewater treatment, as phosphorus is a major contributor to eutrophication. Phosphorus is present in a variety of chemical forms, primarily as inorganic phosphorus, which includes orthophosphates (PO_4^{3-}) and polyphosphates, as well as organic forms. It comes from various sources, including soil, rocks, wastewater treatment plants, fertilized lawns, cropland runoff, and failing septic systems.

The U.S. Environmental Protection Agency (EPA) set a recommended limit of 0.05 mg/L for total phosphorus in streams that enter lakes and 0.1 mg/L for total phosphorus in flowing waters (Tuser, 2022). In comparison, Malaysia's Water Services Industry (Prohibited Effluent) Regulations 2021 set the permissible limit for total phosphorus at 10 mg/L in effluents discharged into public sewage systems, regulated by the National Water Services Commission (SPAN). This higher threshold reflects a different regulatory approach that considers local treatment infrastructure and environmental factors (Aoki, 2021).

Since conventional treatment processes may not always achieve optimal removal of these contaminants, alternative and sustainable approaches such as microalgal systems have gained increasing interest due to their efficiency in nutrient removal while simultaneously generating valuable biomass.

2.3 Microalgae in Wastewater Treatment

Commercial use of algal cultures stretches about 75 years, beginning with the application in wastewater treatment and production of strains such as *Chlorella* and *Dunaliella.* In the 1950s, Oswald and Golueke first suggested the possibility of microalgal bioremediation of wastewater (Gonçalves et al., 2017; Tran et al., 2021b). Due to their photosynthetic capabilities of converting solar energy into valuable biomass by assimilating the nutrients they reserve, microalgae are an excellent choice for treating wastewater (Amaro et al., 2023; Kilbane, 2022). Various microalgae like *Chlorella*, *Scenedesmus*, *Spirulina*, and *Chlamydomonas* have shown strong potential for cleaning wastewater by removing pollutants, heavy metals, and pathogens. *Scenedesmus, Chlorella, Euglena, Oscillatoria, Chlamydomonas*, and *Ankistrodesmus* thrive in wastewater and also tolerate its toxic compounds well (Abdelfattah et al., 2023).

Microalgae-based systems have been applied in the tertiary treatment process. In this treatment process, organic ions such as ammonia, nitrate and phosphate are removed, which can be achieved either biologically or chemically. Compared to biological treatment, chemical treatment is too costly and may require additional treatment steps to control the secondary pollution produced. Nevertheless, the overall cost of a wastewater system rises significantly with each additional treatment stage. Hence, the employment of microalgae in tertiary treatment serves as an attractive solution, as microalgae possess the ability to use inorganic nitrogen and phosphorus for growth (Abdelfattah et al., 2023; Abdel-Raouf et al., 2012).

2.3.1 Conventional Wastewater Treatment vs Microalgae-Based Wastewater Treatment

Conventional wastewater treatment has long served as the primary method for managing municipal and industrial effluents, utilizing physical, chemical, and biological processes to remove contaminants. However, these systems primarily focus on pollutant removal and often require significant energy and chemical inputs (Abdelfattah et al., 2023; Gururani et al., 2022).

In contrast, microalgae-based wastewater treatment offers a sustainable and resource-efficient alternative by integrating pollutant removal with nutrient recovery and the production of valuable biomass. Microalgae can efficiently remove residual nitrogen and phosphorus in wastewater effluent, while the cost of growing microalgae can be reduced by utilising nutrients in wastewater (Abdelfattah et al., 2023). This highlights the shift from traditional removal-focused systems toward more circular and environmentally friendly approaches. Table 2.2 highlights the pollutant removal efficiencies of conventional versus microalgae-based treatment systems based on findings from recent studies.

Table 2.2
Comparisons Between Conventional and Microalgal-Based Wastewater Treatment in Removing COD, TN, TP, Pathogens and Micropollutants

Parameter of Removal	**Conventional Treatment**	**Microalgae-Based Treatment**	**References**
Reduction of COD (%)	75–90%	80–95%	(Arashiro et al., 2020; Zhang et al., 2023)
Reduction of TN (%)	40–60%	70–95%	(Arashiro et al., 2020; Wang et al., 2023)
Reduction of TP (%)	50–70%	85–100%	(Arashiro et al., 2020; Wang et al., 2023)
Reduction of Pathogens	Moderate (requires disinfection)	Moderate to high (algal antimicrobial effects)	(Cheah & Chan, 2022; Wang et al., 2020)
Reduction of Micropollutants	Low (<20%)	Moderate to high (40-60%)	(Bhatti et al., 2021)

COD: Chemical Oxygen Demand; TN: Total Nitrogen; TP: Total Phosphorus. Removal efficiencies may vary with system configuration, retention time, and operational parameters. Data compiled from Arashiro et al. (2020), Zhang et al. (2023), Wang et al. (2023), Wang et al. (2020), Cheah & Chan (2022) and Bhatti et al. (2021).

Wastewater represents a significant opportunity for microalgae since it has the potential to serve as a medium for cultivation. This not only cuts the cost of chemical nutrients needed but also ensures the availability of phosphorus, in particular, is sufficient to support microalgae growth. The economic and environmental drawbacks, such as high production cost, nutrient availability and water scarcity can also be

improved (Kilbane, 2022).

Additionally, microalgae could also adapt to various living conditions and are highly resilient to environmental stress. They are capable of physiological adaptations that alter their nutrient uptake and biomass composition. Due to this, they can thrive in nutrient-rich, extremely salinised water and arid environments, which makes them an attractive candidate for treating municipal, industrial and agricultural wastewater (Al-Jabri et al., 2021).

In addition to removing pollutants, microalgae can develop exponentially in 3.5 hours, doubling their biomass in 24 hours, and thrive in any environment provided they have access to enough nutrients. The produced biomass can be harvested and reused for biofuel production, animal feed and fertiliser (Moreno Osorio et al., 2021; Yap et al., 2021).

Besides that, they can efficiently utilise the free and abundant sunlight available for their growth through photosynthesis while also capturing CO_2, which helps reduce the global atmospheric CO_2 concentration and mitigate greenhouse gas emissions (Al-Jabri et al., 2021; De Francisci et al., 2018). They also do not produce any toxic residues, unlike conventional treatments that may generate carcinogenic compounds from untreated toxic by-products (Amaro et al., 2023).

Furthermore, microalgae do not compete with crops for arable land (Miranda et al*.,* 2017) and require lower nutrient requirements than terrestrial crops (Boelee et al*.,* 2011; Moreno Osorio et al*.,* 2018). They may be grown on land unsuitable for food production (Berner et al*.,* 2015).

Table 2.3 summarizes the differences between using conventional and microalgal-based wastewater treatment. While conventional treatment remains the standard for municipal and industrial wastewater, microalgal-based systems are promising, particularly for nutrient-rich wastewater, offering an efficient and environmentally friendly alternative for future wastewater treatment (Fito & Alemu, 2019).

Table 2.3
Performance Comparisons Between Conventional and Microalgae-Based Wastewater Treatment

Characteristics	**Conventional Wastewater**	**Microalgae-Based**

	Treatment	Wastewater Treatment
Main Process	Physical, chemical, and biological treatments	Through photosynthesis (Bioadsorption, Bioaccumulation and Biodegradation)
Nutrient Removal	Uses membranes, chemicals or bacteria	Naturally uptake nutrients for growth
Energy Requirement	High (aeration, filtration and chemical treatment)	Low (sunlight for photosynthesis)
Carbon Sequestration	Produces CO_2	Absorbs CO_2
End Products	Treated water, secondary pollutant	Biomass (biofuels, fertilizers or animal feeds)
Sludge Production	High	Minimal
Environmental Impact	Can contribute to secondary pollutants (excess sludge and chemical residues)	Eco-friendly and sustainable, with minimal chemical use
Wastewater Suitability	Municipal, industrial and agricultural wastewater	Municipal, industrial and agricultural wastewater

Evaluation based on general performance trends and may differ based on site-specific conditions and system designs. Comparative summary based on reported literature: Abdelfattah et al. (2023), Berner et al. (2015) and Gururani et al. (2022).

While suspended microalgae systems have traditionally been used for wastewater treatment, they often face challenges such as low biomass retention, difficulty harvesting, and sensitivity to environmental fluctuations (Tang et al., 2020; Udayan et al., 2021). Microalgal biofilm-based systems offer a better alternative, with higher biomass density, easier harvesting, and greater resilience, making them a promising alternative for efficient and scalable wastewater treatment (Kilbane, 2022).

2.4 Microalgal Biofilm

In nature, microorganisms grow in two structures: either freely suspended cells or attached to a surface. Microalgal biofilm is a surface-attached microbial community in which microalgae constitute the dominant and functional group, forming a cohesive matrix embedded within extracellular polymeric substances (EPS). It is structured communities of photosynthetic microorganisms like cyanobacteria, diatoms, and green

algae, often found in natural habitats such as estuaries and sandy shores. Unlike conventional bacterial biofilms, microalgal biofilms are primarily driven by photosynthetic organisms, which play the central role in biomass accumulation, nutrient assimilation, and oxygen production. In such systems, microalgae act as the structural and metabolic backbone of the biofilm, while associated bacteria function synergistically, benefiting from algal-derived oxygen and organic exudates. The EPS production helps these organisms to adhere, store nutrients, and protect against environmental stress (Saini et al., 2023). Non-photosynthetic bacteria are also present and contribute to biofilm formation (Hu et al., 2021; Zhu et al., 2023).

Biofilms form on various surfaces, such as natural or artificial, living or non-living, and can grow in air, at liquid interfaces, or underwater, such as in wastewater systems or water pipes (Philipp et al., 2024; Saini et al., 2023; Zhang et al., 2017). Compared to free-floating cells, biofilms offer higher biomass yield, better nutrient uptake, and greater resistance to harsh conditions, making them ideal for wastewater treatment and bioresource recovery (Zhu et al., 2023). The formation of biofilm involves several stages, which are initial attachment, irreversible attachment, maturation, and detachment.

Each stage involves complex interactions among microalgal cells, their secreted polymers, and the surrounding environment, ultimately resulting in a structured community with enhanced resilience and ecological function, as illustrated in Figure 2.4 (Mantzorou & Ververidis, 2018; Saini et al., 2023). The transition of free-living planktonic cells to sessile, stationary cells that adhere to the surface marks the beginning of biofilm growth (Moreno Osorio et al*.,* 2021; Ennaceri et al*.,* 2023).

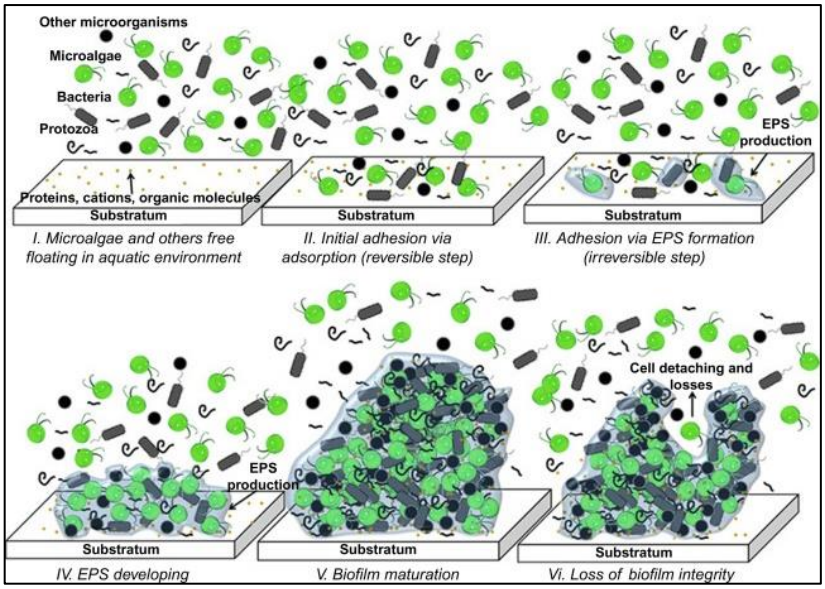


Figure 2.4 Stages of Microalgal Biofilm Formation

Note. Stages in microalgal biofilm development: (I) free-floating microorganisms, (II) initial reversible adhesion via adsorption, (III) irreversible attachment through EPS formation, (IV) EPS development, (V) biofilm maturation and (VI) biofilm deterioration and cell detachment. Adapted from Abu Hasan et al. (2021).

2.4.1 Initial Attachment

In the first stage of attachment, microalgae initially attach to a substrate through physical and chemical interactions, including van der Waals forces, electrostatic interactions and hydrophobic effects. This attachment is weak and reversible. The attachment process is significantly influenced by surface roughness, hydrophobicity and charge. Figure 2.5 illustrates this process, where microalgae settle within the grooves of the substratum, highlighting how surface topography can enhance or hinder cell adhesion.

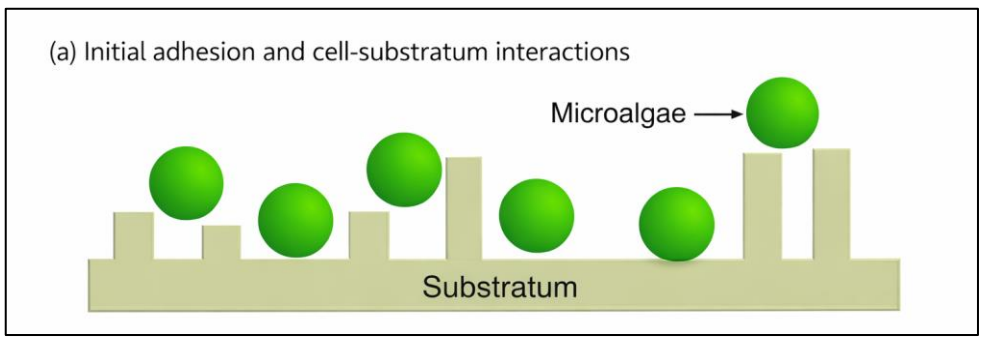


Figure 2.5 Initial Attachment of Microalgae Cells

Note. Schematic representation of initial adhesion and cell–substratum interactions during the early stage of biofilm formation. Microalgal cells adhere to the surface through physical interactions such as van der Waals forces and electrostatic attraction. Adapted from Rosmahadi et al. (2021).

Hydrophilic and negatively charged surfaces promote higher cell attachment (Ennaceri et al., 2023). Additionally, these interactions are also influenced by environmental factors such as pH, ionic strength, and temperature (Ennaceri et al., 2023; Moreno Osorio et al., 2021). Microalgal cells can move to attach to surfaces voluntarily (for motile cells) through the use of flagella or gravitationally as a result of hydrodynamic pressure (Hu et al., 2021).

The adsorption of microalgae to surfaces is significantly aided by extracellular polymeric substances (EPS), particularly transparent exopolymeric particles (TEPs). TEPs are acidic, sticky, transparent, and abundant in nutrients, resulting from the aggregation of polysaccharides secreted by phytoplankton. TEPs assist in preparing surfaces for cell adhesion and aggregation, which creates a stable structure for the growth of biofilms. TEPs also contribute to stabilizing biofilm layers and enhancing nutrient entrapment, increasing the chance of successful colonization (Ennaceri et al., 2023).

2.4.2 Irreversible Attachment

In this stage, microalgae cells inside the biofilm constantly release sticky EPS to promote cell immobilization (Cheah & Chan, 2021) by irreversibly adhering to the surface. The secretion of polysaccharides, proteins, lipids and nucleic acids is also increased (Ennaceri et al., 2023) to act as biological adhesives that anchor and establish the biofilm. The formation of the EPS matrix is essential for the stabilization and long-term development of the biofilm (Ennaceri et al., 2023; Sharma, 2022).

About 90% of the dry weight of the biofilm is made up of the EPS matrix, which surrounds the biofilm community and acts as a diffusive barrier that shields it from environmental stressors such as phagocytes, toxic chemicals, shear forces and nutrient fluctuations (Ennaceri et al., 2023; Moreno Osorio et al., 2021). EPS also serves as a carbon storage site and a food source for the microcolonies within the biofilm (Cheah & Chan, 2022; Ennaceri et al., 2023).

In this stage, the cells adapt based on cell-surface interactions. They can change their surface charge and hydrophobicity or develop surface structures like pili, curli, fimbriae, and flagella to strengthen attachment. They may also regulate EPS production to form stable colonies and enable cell-cell contact. This change makes it easier for other microorganisms to attach and grow, leading cells to shift permanently from a planktonic to a sessile state, forming a more complex biofilm (Moreno Osorio et al., 2021).

2.4.3 Maturation

In this stage, the cells focus on the growth and structural organization of the biofilm. As microalgae proliferate, the production of EPS also increases, which helps the development of biofilm on the surface and increases its thickness, improving the biofilm stability (Toyofuku et al*.,* 2016). The biofilm's population grows as a result of cell division among the cells. The overabundance of EPS attracts a variety of bacteria through chemotaxis and facilitates their incorporation along with other particulate matter. This causes the biofilm to become more structurally complex and heterogeneous (Ennaceri et al., 2023).

During this stage, the cells release soluble acidic polysaccharides (SAPs) into the surrounding environment. These polysaccharides help to bind cells together and to the surface. They enhance biofilm stability by creating a protective layer that shields microalgae from stressors like UV radiation or nutrient fluctuations. They also assist in retaining nutrients within the biofilm matrix by adsorbing and entrapping nitrogen and phosphorus compounds. This nutrient retention sustains microbial growth, enhances biomass accumulation, and supports the development of a stable and functionally active microalgal biofilm, as the biofilm matures (Rosmahadi et al., 2021), as shown in Figure 2.6.

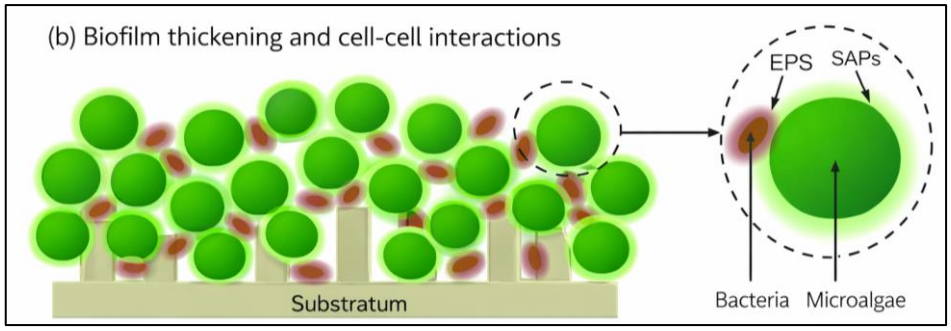


Figure 2.6 EPS and SAPs Developing During the Biofilm Maturation Stage

Note. The image shows a biofilm with microalgal and bacterial cells on a substratum, thickened by EPS and SAPs that enhance adhesion and stability. Adapted from Rosmahadi et al. (2021).

Environmental factors, such as the availability of nutrients and light, play a major role in shaping the biofilm (Ennaceri et al., 2023). The microbes in the biofilm absorb nutrients primarily for the synthesis of EPS rather than for biomass increase. In the absence or lack of nutrients, they must leave the EPS matrix by producing enzymes that can degrade the EPS matrix. The biofilm's growth and cellular and polymeric biomass are inextricably related. Hence, when the EPS is degraded, this includes the loss of biofilm biomass, which leads to biofilm detachment (Moreno Osorio et al., 2021).

2.4.4 Detachment

Once the biofilm has reached full maturity, several environmental factors can cause the biofilm to detach. For instance, the biofilm tends to disperse and transform into free-living cells under stressful conditions such as nutritional deficiency (Wijeyekoon et al., 2004; Moreno Osorio et al., 2021), the presence of toxic metabolites, and the secretion of enzymes that can dissolve the EPS matrix and release the cells. The production of surfactants, cell lysis and sloughing off, the impact of shear forces, and erosion are also associated with biofilm detachment. Detachment serves multiple ecological purposes, such as dispersal to new niches, population control, and stress avoidance (Ennaceri et al., 2023). Detachment is important for harvesting biomass in biofilm systems and knowing the causes can help improve controlled harvesting and keep systems efficient for wastewater treatment or biofuel production.

2.5 Comparison Between Microalgal Biofilm Systems and Suspended Microalgal Systems

Traditionally, planktonic or suspended microalgal systems, where microalgae grow freely and mix in the water column, have been widely employed in large-scale cultivation and wastewater treatment due to their simplicity and ease of monitoring. These systems have shown effectiveness in nutrient uptake and are commonly used in open ponds and photobioreactors (Tran et al., 2021a). However, in recent years, biofilm-based microalgal systems have gained increasing attention as a more sustainable and efficient alternative. Unlike suspended systems, biofilm cultivation involves the attachment of microalgae to a solid surface, allowing for higher biomass density, reduced harvesting costs and improved tolerance to environmental fluctuations (Bozan et al., 2024; Ennaceri et al., 2023).

Structurally, biofilm has significantly different characteristics from those of its planktonic counterparts, including a 3D structure with tightly packed cells, EPS and voids. Biofilms exhibit significant temporal and spatial heterogeneity. In other words, planktonic cells are mobile and dispersed, whereas biofilm cells are stationary and attached to a surface (Moreno Osorio et al., 2021). Compared to planktonic forms, biofilms are more complex and organised because they demonstrate changes over time and at various depths, including in oxygen levels, nutrients, and cell activity (Gao et al., 2024).

In suspended microalgal systems, the cells remove nutrients by direct uptake from the medium, as compared to microalgal biofilms, which primarily use adsorption and assimilation to remove nutrients from wastewater. Pollutants are gradually drawn closer to the cells after being absorbed into the microalgae's extracellular polymer (EPS). The microalgae eventually transform some of the contaminants they ingest and use into microalgal biomass. These are directly assimilated into proteins, nucleic acids, and carbohydrates, effectively becoming microalgal biomass while reducing nutrient loads in wastewater (Lin-Lan et al., 2018).

In addition, suspended microalgal cells are dispersed throughout the culturing liquid, and the total solid content is less than 1%. Due to the low biomass concentration, complex harvesting processes are required. Centrifugation, chemical flocculation and

filtration are some of the commonly used methods (Hu et al., 2021) to collect microalgal biomass from the liquid medium, which can be labour- and energy-intensive (Lin-Lan et al., 2018).

Other than that, suspended microalgal systems are more sensitive to environmental changes, such as shifts in light, toxins, or nutrient levels, which can affect their performance and growth. Biofilm-based systems are more robust and stable because the biofilm matrix protects the microalgae from environmental stressors and fluctuations in nutrients (Kilbane, 2022).

In contrast, microalgal biofilm provides biomass harvesting at a substantially lower cost. During the harvesting phase, the biomass can be readily isolated from the liquid medium because the microalgal cells are immobilised within an algal biofilm matrix. Several studies have demonstrated that the existence of biofilms not only facilitates the flow of nutrients and energy but also concentrates and immobilises microalgae cells, making harvesting much easier (Huang et al., 2023). Harvesting can be done with a basic scraping approach, which reduces energy consumption. Additionally, this system reduces water and space needs, as well as the possibility for increased biomass productivity (Ennaceri et al., 2023; Huang et al., 2023).

Hence, the usage of traditional suspended cultures is found to be considerably more inefficient compared to microalgal biofilm systems. Table 2.4 compares the microalgal biofilm and suspended microalgal systems.

Table 2.4
Comparisons Between Microalgal Biofilm Systems and Suspended Microalgal Systems

Feature	**Microalgal Biofilm**	**Suspended Microalgal**
Structure	Attached to surfaces, forming dense biofilm layers	Free-floating (planktonic) cells in liquid medium
Biomass Harvesting	Easier, requires less energy	Requires centrifugation or filtration, energy-intensive
Growth Rate	Often slower but more stable	Faster initial growth but less stable over time
Nutrient Uptake	Higher efficiency due to prolonged contact with	Lower nutrient retention;

	wastewater	relies on mixing
Space Requirement	Compact, can grow vertically or on carriers	Requires large volume of water for cultivation
Light Penetration	Limited to surface layer, may need optimization	Better light distribution in shallow, well-mixed systems
Contamination Resistance	More resistant due to EPS matrix and biofilm barrier	More prone to contamination and predator attacks
Operational Stability	More resilient to environmental changes	Sensitive to fluctuations in pH, temperature, or light
Cost & Maintenance	Lower harvesting cost, simple operation	Higher maintenance and operation cost

Adapted and summarized from various sources including Cheah & Chan (2021), Moreno Osorio et al. (2021), Ozkan & Berberoglu (2013), and Emparan et al. (2019).

2.5.1 Integration of Wastewater Treatment Technologies Associated with Microalgal Biofilms

In recent years, microalgal biofilm-based systems have received increasing attention as a reliable alternative to suspended cultures with practical advantages in wastewater treatment and biomass production. Biofilm reactors enable microalgae to grow attached to surfaces, yielding higher biomass density, easier harvesting, and improved operational stability. Various reactor designs have been developed to optimize these benefits, each with its own mechanisms for nutrient delivery, light exposure, and biomass collection (Hu et al., 2021; Moreno Osorio et al., 2021). Some of the biofilm reactors currently in use include Rotating Algal Biofilm Reactors (RABRs), Algal Turf Scrubbers (ATS) and fixed-bed systems.

Rotating algae biofilm reactors (RABRs) are innovative systems designed to grow algae as biofilms on rotating surfaces, which can be disks or drums, partially submerged in wastewater, as shown in Figure 2.7. This rotating motion alternates the exposure of the microalgae to air and wastewater for light exposure and nutrient absorption, optimizing their growth conditions (Hoh et al., 2016; Nguyen et al., 2023). These reactors are primarily used for efficient wastewater treatment and the production

of valuable algal biomass, which can be further processed into biofuels and other bioproducts (Hodges et al., 2017; Nguyen et al., 2023).

Compared to traditional suspended algae systems, the rotating design improves light exposure and gas exchange, supporting higher growth rates, improving productivity and energy efficiency (Hodges et al., 2017; Nguyen et al., 2023). RABRs have a high efficiency in the removal of nitrogen, phosphorus, organic pollutants, and even complex contaminants like dyes and polyacrylamide from various types of wastewaters, including municipal, industrial, and aquaculture sources. Besides, the biofilm format naturally concentrates the algal biomass, making it easier and less costly to harvest compared to suspended cultures (Nguyen et al., 2023). Productivity can reach up to 31 g/m²/day at pilot scale (Jones et al., 2023). The harvested algae can be used for biofuel production, nutrient recovery, and as feedstock for other bioproducts (Jones et al., 2023).

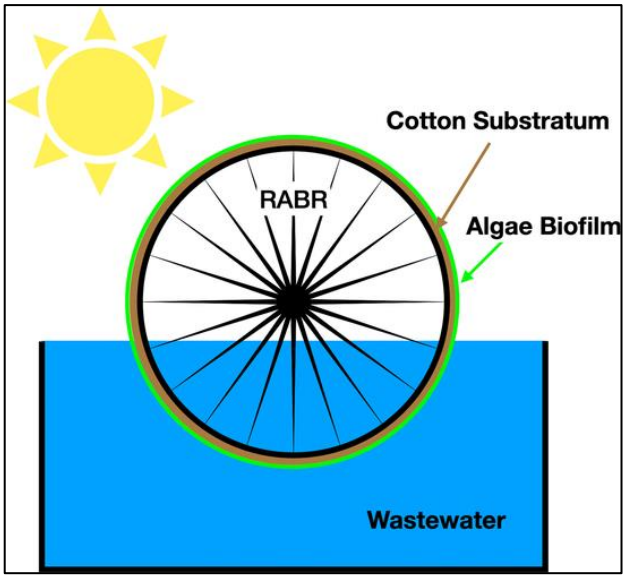


Figure 2.7 Schematic Illustration of the Rotating Algal Biofilm Reactors

Note. RABR: Rotating algal biofilm reactor. The image illustrates a rotating algal biofilm reactor with a cotton substratum supporting an algae biofilm, exposed to sunlight and submerged in wastewater. The design facilitates nutrient uptake and biomass growth, with sunlight enhancing photosynthesis and EPS production for biofilm stability. Adapted from Jones et al. (2023).

In contrast, Algal Turf Scrubbers (ATS) systems use shallow, inclined flow-ways, like artificial streams, where microalgae naturally grow as a turf on mesh or rough surfaces. Wastewater continuously flows over this surface as the algae absorb the

nutrients as they grow (Kangas et al., 2017; Leong et al., 2021). ATS systems are designed to mimic natural algal communities and are used for water treatment, pollution prevention, and resource recovery. This system efficiently removes nitrogen, phosphorus, and other contaminants from various water sources while producing valuable algal biomass, with advantages such as high photosynthetic efficiencies and adaptability to harsh environments (Gan et al., 2022; Leong et al., 2021). ATS uses low energy, yields very high biomass, mimics natural stream ecosystems and is great for large-scale applications (Leong et al., 2021). Figure 2.8 illustrates the model of ATS with algae.

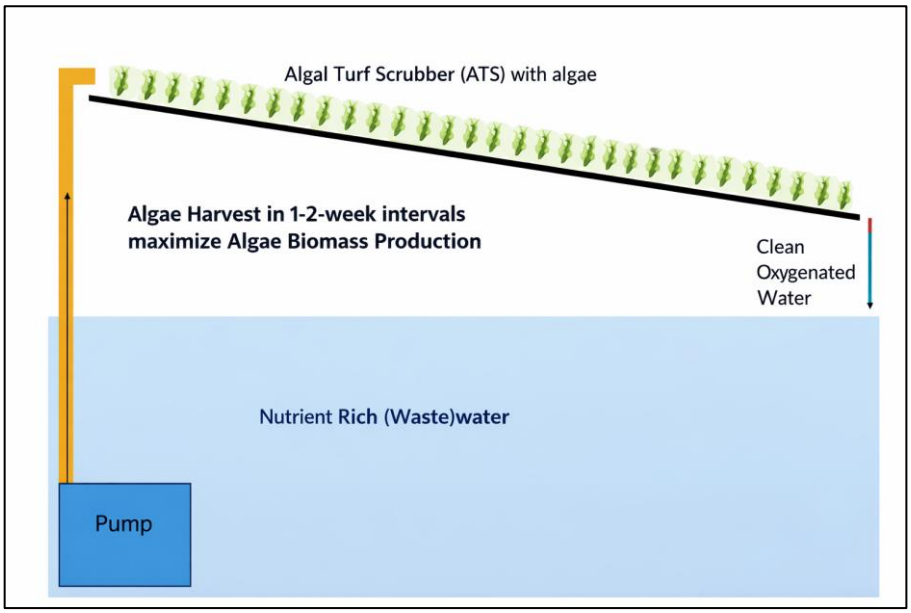


Figure 2.8 Model Design of Algal Turf Scrubber

Note. The image depicts an Algal Turf Scrubber (ATS) with algae, harvested every 1-2 weeks to maximize biomass production. Nutrient-rich wastewater is circulated via a pump, supporting algal growth, while producing clean, oxygenated water. Adapted from Siville & Boeing (2020).

On the other hand, fixed-bed or attached-growth systems are reactors with packed microalgal biofilm grown on fixed substrates that stay stationary while the wastewater flows as shown in Figure 2.9. This system is widely used in wastewater treatment and bioremediation, as it enables efficient degradation of organic pollutants and nutrients in wastewater (Abyar et al., 2024; Waqas et al., 2020). Common designs include fixed-bed bioreactors, moving bed biofilm reactors (MBBR), integrated fixed-film activated sludge (IFAS), and submerged fixed-bed biofilm reactors (SFBBR). This

system is simple, low cost and generate moderate to high biomass (Waqas et al., 2020). Recent research has explored their efficiency, environmental impact, and applications in various contexts, including municipal, industrial, and aquaculture wastewater, as well as microalgae cultivation for biodiesel (Irani et al., 2020; Waqas et al., 2020).

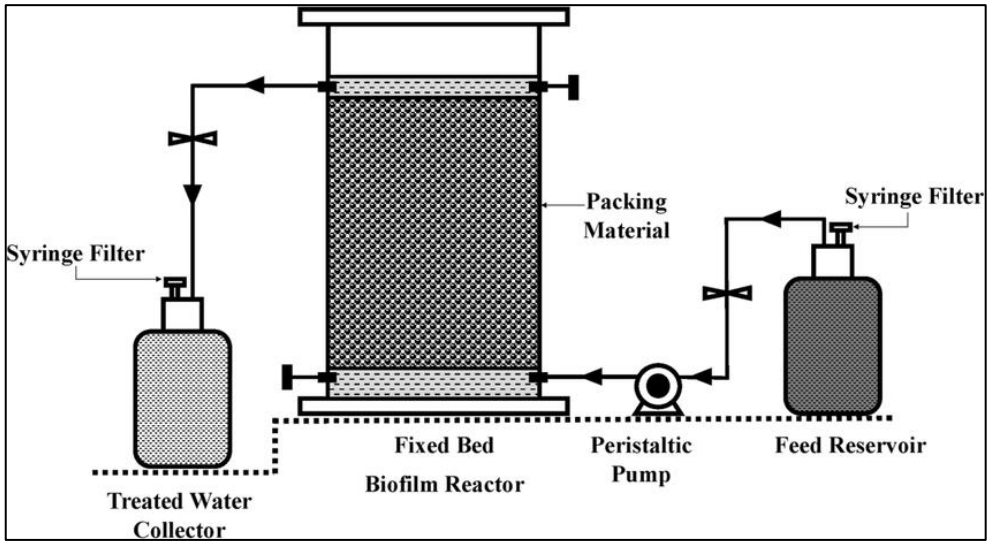


Figure 2.9 Schematic of Fixed-Bed Biofilm Reactor

Note. The image shows a fixed-bed biofilm reactor with packing material supporting biofilm growth, circulated by a peristaltic pump from a feed reservoir. Treated water is collected using a syringe filter, enhancing water purification. Adapted from Madhushika et al. (2022).

While these reactors generally fall as microalgal biofilm technologies, when treating real wastewater, they often function as hybrid algae–bacteria systems. In these configurations, microalgae absorb inorganic nutrients and produce oxygen via photosynthesis, while heterotrophic bacteria embedded in the biofilm matrix break down organic materials. This biological synergy shows that current microalgal biofilm reactors may be effectively integrated with bacterial treatment processes without the need for additional treatment units, improving nutrient and organic pollutant removal while lowering aeration needs (Xu et al., 2024).

2.6 Mechanisms in Removing Nitrogen and Phosphorus from Wastewater by Microalgae

Although numerous microalgal strains have the capacity to remove contaminants from wastewater, a chosen number appears to be utilized more frequently

than others. This is likely due to their quick growth rate, low production costs, and strong endurance to harsh, possibly stressful environmental conditions (Plöhn et al., 2021). For instance, cyanobacteria are known for their ability to rapidly absorb nutrients like nitrate and phosphate (Nawaz et al., 2024).

Nitrogen and phosphorus are the two main nutrients microalgae require for growth, along with a few other micronutrients (Sharma, 2022). Wastewater has significant levels of nitrogen, phosphate, and organic carbon in general, but also a wide range of additional pollutants such as pharmaceuticals and heavy metals due to a mixing of extraneous water and rainy water (Plöhn et al., 2021). It is well-recognized and well-documented that microalgae can remove pollutants such as mineralized nitrogen (ammonium, NH_4^+ or nitrate, NO_3^-) and phosphorus (phosphate, PO_4^{3-}) (Salbitani & Carfagna, 2021; Schaedig et al., 2023a). Although excessive nitrogen and phosphorus concentrations cause eutrophication in natural water bodies, these nutrients can be effectively exploited in controlled microalgae-based systems, where selected freshwater microalgae rapidly assimilate them into biomass, making the process a viable bioremediation strategy (Collotta et al., 2018; Mohsenpour et al., 2021; Kong et al., 2021).

Microalgae use different mechanisms to remove pollutants from wastewater, which are biosorption, bioaccumulation and biodegradation, as illustrated in Figure 2.10 (Al-Jabri et al., 2021). Biosorption is a passive process that displaces material from a liquid phase to bind to a solid surface. This mechanism includes physical, chemical and metabolic-independent processes supported by different mechanisms such as ion exchange, absorption, adsorption, precipitation, surface complexation and electrostatic interaction (Abdelfattah et al., 2023).

Bioaccumulation, on the other hand, is an active metabolic process that uses various substrates in the cell lumen. This process requires energy and is comparatively slower than biosorption. The cells absorb the substances and accumulate or metabolize them. Inorganic and organic pollutants (such as sulfates, nitrates, phosphates, heavy metals and pesticides) can be transferred into the cells, and this process is a crucial pathway to eliminate pollutants (Abdelfattah et al., 2023).

In contrast, biodegradation is one of the most effective processes to remove pollutants from effluents. The complex compounds are degraded into simple and safe

chemical building blocks. This process involves the breakdown of target pollutants through the complete mineralisation of parent molecules to CO_2 and water or through biotransformation, which consists of a series of enzymatic reactions that produce different metabolic intermediates (Abdelfattah et al., 2023). Due to the slow breakdown of persistent organic compounds and sensitivity to environmental conditions, hybrid treatment systems that integrate biofilm-based reactors, microbial consortia, and complementary physicochemical processes are increasingly adopted (Armanu et al., 2025). In particular, microalgal–bacterial biofilm systems enhance process stability, broaden degradation pathways, and reduce dependence on strict operational conditions, resulting in improved overall treatment efficiency (Liu et al., 2022; Wu et al., 2025).

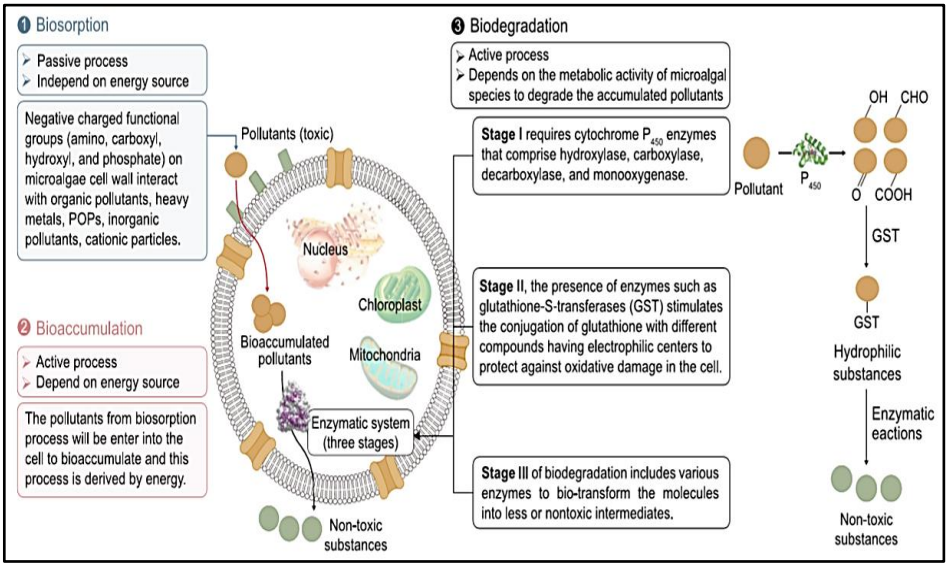


Figure 2.10 Bioremediation Mechanisms of Pollutants Through Microalgal Metabolism

Note. The image illustrates microalgal pollutant removal via biosorption (passive, energy-independent), bioaccumulation (active, energy-dependent), and biodegradation (active, enzyme-mediated). Processes involve EPS, enzymatic systems and GST to transform toxic pollutants into non-toxic substances. Adapted from Abdelfattah et al. (2023).

Although the removal of organics by microalgal sorption was rather modest, the cell walls of microalgae may include a number of polymer groups that could provide potential sorption sites for organic pollutants (Al-Jabri et al., 2021). At the initial stage of growth, the absorptive capacity of algae for these pollutants is low, however, as their growth reaches a peak, the absorptive capacity will synchronously increase (C. Wang et al., 2022).

Some microalgae could utilize different organics in either mixotrophic or heterotrophic mode, even though the majority of them are photosynthetic. This would allow the mixing of organic-rich streams (such as food processing wastewater, biodiesel plant glycerol, etc.) into other wastewater for combined treatment (Yaakob et al., 2021).

2.6.1 Microalgal Removal of Nitrogen

Nitrogen makes up 14% of the dry weight of microalgae and is involved in metabolism that supports the synthesis of proteins and nucleic acids (Maltsev et al., 2023). Nitrate, nitrite, nitric acid, ammonium, ammonia, molecular nitrogen, nitrous oxide, nitric oxide, and nitrogen dioxide are the most prevalent inorganic nitrogen forms (Glass & Rousk, 2024).

In microalgae-based wastewater treatment systems, nitrogen is removed through a range of complementary mechanisms, such as direct incorporation into biomass (Shen et al., 2023), indirect contributions from nitrifying bacteria (Fallahi et al., 2021), and ammonia stripping induced by pH elevation (Shen et al., 2023). These mechanisms often work simultaneously to effectively reduce total nitrogen (TN), particularly in microalgal biofilm and hybrid algae-bacteria systems.

The primary mechanism of nitrogen removal is the assimilation of dissolved inorganic nitrogen into microalgal biomass. The removal of nitrogens from wastewater is done by assimilating both inorganic forms (NH_4^+, NO_2^-, NO_3^-) and organic nitrogen sources such as urea and amino acids, which are subsequently incorporated into essential cellular components such as amino acids, proteins, nucleic acids, and chlorophyll during growth (Sägesser et al., 2023; Salbitani & Carfagna, 2021). Among these, ammonium (NH_4^+) is the most energy-efficient and preferred nitrogen source, as it can be directly incorporated into amino acids via the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle. In contrast, nitrate (NO_3^-) and nitrite (NO_2^-) must first be reduced to NH_4^+ through energy-demanding steps involving nitrate and nitrite reductases (Amaro et al., 2023), as shown in Figure 2.11. This process represents a permanent removal pathway, as nitrogen is retained within the biomass and can be removed from the system through harvesting (Abdelfattah et al., 2023).

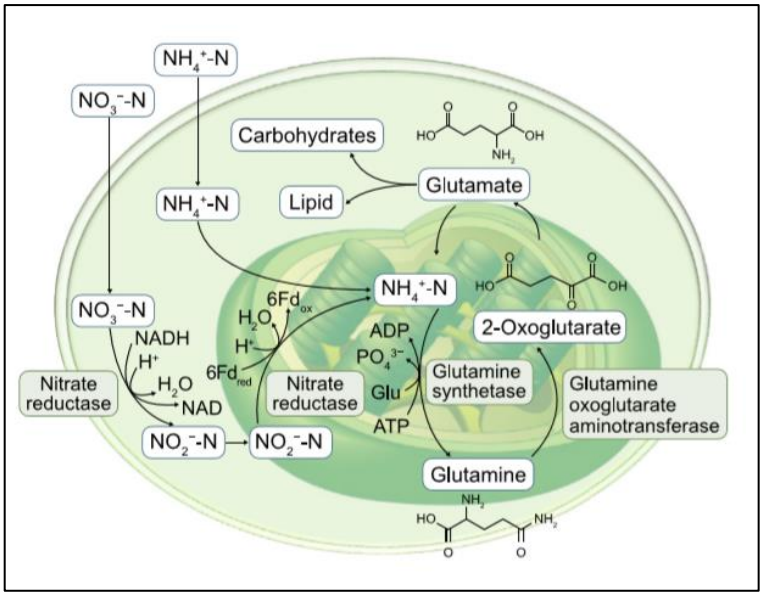


Figure 2.11 Nitrogen Removal Mechanisms by Microalgal Cells in Wastewater

Note. The image depicts nitrogen metabolism in microalgae, showing nitrate reduction to nitrite and ammonium (NH_4^+) via nitrate and nitrite reductase, with glutamate and glutamine synthesis involving 2-oxoglutarate, glutamine synthetase and glutamine oxoglutarate aminotransferase. Carbohydrates and lipids, supported by ATP and NAD(P)H, contribute to the process. Adapted from Abdelfattah et al. (2023).

In contrast, in hybrid microalgal–bacterial systems, nitrogen removal is further enhanced by the indirect contribution of nitrifying bacteria. Ammonia-oxidizing bacteria convert ammonium into nitrite, which is subsequently oxidized to nitrate by nitrite-oxidizing bacteria under aerobic conditions (Li et al., 2023). Although microalgae do not directly perform nitrification, their photosynthetic activity supplies dissolved oxygen that supports bacterial nitrification. The nitrate produced can then be assimilated by microalgae, linking bacterial nitrogen transformation with algal nutrient uptake and improving overall nitrogen removal efficiency (Li et al., 2023; Li et al., 2024; Li et al., 2023).

On the other hand, microalgal photosynthesis can also elevate the pH of the wastewater due to the uptake of carbon dioxide and bicarbonate (Latagan et al., 2024). Under alkaline conditions, the equilibrium between ammonium and ammonia shifts toward the formation of un-ionized ammonia (NH_3), which can volatilize and be stripped from the wastewater (Zangeneh et al., 2021). While ammonia stripping is generally considered a secondary removal mechanism, it may contribute significantly to nitrogen reduction in systems exhibiting high photosynthetic activity and elevated pH (Chen et al., 2021).

The capacity of microalgae to remove nitrogen is closely linked to their nitrogen metabolism and taxonomic classification. Prokaryotic microalgae, such as cyanobacteria, have the ability to fix atmospheric molecular nitrogen (N_2-N), turning it into ammonia-nitrogen (NH_3- N) through nitrogenase activity, particularly under nitrogen-limited conditions (Chen et al., 2022). The fixed nitrogen can either be assimilated into amino acids, proteins and cellular biomass (Fallahi et al., 2021). However, in wastewater treatment systems where inorganic nitrogen is typically abundant, nitrogen removal predominantly occurs through assimilation rather than nitrogen fixation (Zhang et al., 2021). In contrast, eukaryotic microalgae possess the ability to ingest dissolved inorganic nitrogen forms or fixed nitrogen, such as NH_4-N, NO_3-N, and nitrite-nitrogen (NO_2-N) (Carletti et al., 2024). Through active transport across the plasma membrane, these nitrogen sources get into the microalgae cells (Salbitani & Carfagna, 2021) . Among these forms, nitrate is frequently detected in aquatic environments due to its high thermodynamic stability (Kermorvant et al., 2021).

Different microalgae species have specific preferences for nitrogen sources. For instance, *Arthrospira platensis* and *Arthrospira sp.* prefer urea over nitrates, while, *C. vulgaris* favors ammonium. Similarly, *Chlamydomonas*, *Botryococcus braunii* and *Dunaliella tertiolecta* thrive on nitrate (Maltsev et al., 2023).

Overall, the combined action of microalgal assimilation, hybrid bacterial nitrification, and physicochemical ammonia stripping promotes the effectiveness of microalgae-based systems for nitrogen removal in wastewater treatment.

2.6.2 Microalgal Removal of Phosphorus

Phosphorus mediates energy transmission and the production of nucleic acids, phospholipids and nucleotides. Similar to nitrogen, phosphorus plays a crucial role in the growth of microalgae. Phosphorus is available as soluble phosphate and makes up less than 1% of the total biomass of microalgae (Maltsev et al., 2023; Yaakob et al., 2021).

In microalgal-based wastewater treatment systems, removal of phosphorus occurs through three primary mechanisms, such as biological uptake (assimilation), intracellular storage, and phosphorus precipitation (Amaro et al., 2023). Microalgae can

uptake phosphorus mainly in the form of polyphosphate or orthophosphate for cellular growth and metabolism (Yaakob et al., 2021). Through active transport across the plasma membrane, this nutrient penetrates microalgal cells as H_2PO_4 and HPO_4^{2-}. Phosphates (PO_4-P) are incorporated into organic compounds through phosphorylation at the substrate level, oxidative phosphorylation and photophosphorylation. Adenosine diphosphate (ADP) and energy input are used to create ATP in these processes (Gonçalves et al., 2017). Under light conditions, they can also be converted into polyphosphates by polyphosphate kinase, supporting DNA and protein synthesis (Amaro et al., 2023). During active growth, microalgae take up dissolved phosphate from the surrounding medium and incorporate it directly into cellular biomass, thereby reducing the soluble phosphorus concentration in wastewater. This assimilation-driven uptake is often very effective under nutrient-rich conditions and contributes significantly to total phosphorus (TP) removal (Amaro et al., 2023; Bossa et al., 2024; Mao et al., 2021).

Beyond immediate metabolic requirements, microalgae possess the ability to store excess phosphorus intracellularly through a process known as luxury uptake (Amaro et al., 2023). In this mechanism, phosphate is accumulated and stored as polyphosphate granules within the cells when phosphorus availability exceeds growth demand. This stored phosphorus can later be mobilized under phosphorus-limited conditions to sustain cellular metabolism. Intracellular storage enhances overall phosphorus removal efficiency, as phosphorus remains immobilized within the harvested microalgal biomass even after growth has slowed (Amaro et al., 2023; Schaedig et al., 2023b).

In addition, phosphorus removal may also occur through chemical precipitation, indirectly induced by microalgal activity. During photosynthesis, microalgae consume dissolved CO_2, leading to an increase in pH in the surrounding medium (Amaro et al., 2023; Molitor et al., 2024). Phosphorus precipitation may happen at pH levels higher than 8.0 and high oxygen concentrations. Elevated pH conditions promote the precipitation of phosphate with multivalent cations such as calcium (Ca^{2+}), magnesium (Mg^{2+}), and iron (Fe^{3+}), forming insoluble phosphate minerals. These precipitates can settle or become associated with biofilms and biomass, further reducing dissolved phosphorus concentrations in wastewater (Amaro et al., 2023).

As shown in Figure 2.12, excess phosphate is converted into polyphosphate granules and stored in the vacuole, supporting long-term phosphorus immobilization through biomass harvesting.

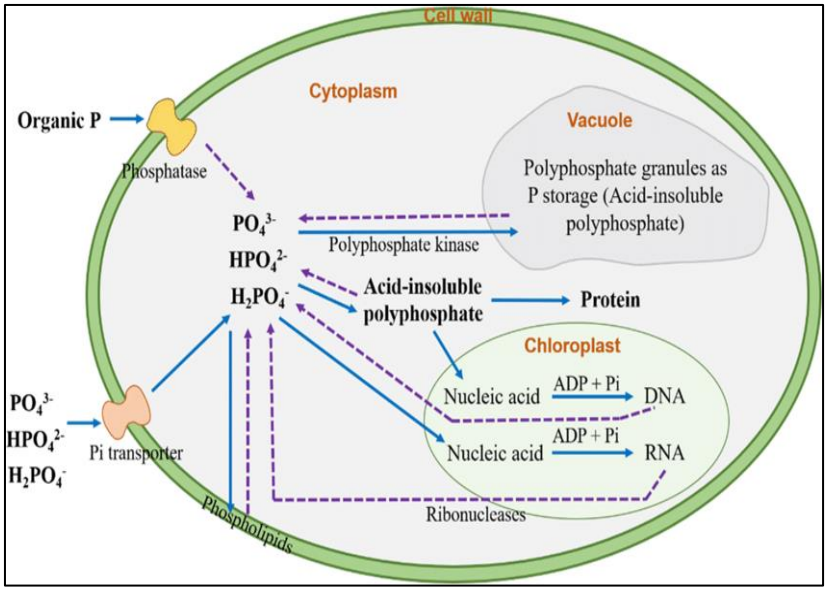


Figure 2.12 The Phosphorus Absorption and Transformation Pathway in Microalgae

Note. The image illustrates phosphorus metabolism in microalgae, showing uptake of organic P and PO_4^{3-} via phosphatases and Pi transporters, storage as polyphosphate granules in vacuoles and utilization in chloroplasts for nucleic acid synthesis (DNA, RNA) and phospholipids, regulated by polyphosphate kinase and ribonucleases. Derived from Liu & Hong (2021).

These biological processes translate into measurable reductions in pollutant concentrations at the system level. Accordingly, Table 2.5 summarizes the reported pollutant removal efficiencies of microalgae across different types of wastewater, reflecting the combined effects of assimilation, intracellular storage, and physiochemically-induced removal mechanisms under varying operational conditions.

Table 2.5
Microalgae-based Wastewater Treatment for Pollutants Removal

Microalgae	**Wastewater Type**	**Location**	**Removal Efficiency (%)**		**Reference**
			N	**P**	
Halochlorella rubescens	Secondary municipal	Germany	83.2%	73.2%	(Shi et al., 2014)
Scenedesmus obliquus	Piggery	Brazil	83.74%	54.69%	(Prandini et al., 2016)
Chlorella vulgaris	Brewery	South Korea	83.74%	54.69%	(Choi, 2016)
Scenedesmus spp.	Anaerobic membrane bioreactor effluent	Spain	90-99%	90-99%	(González-Camejo et al., 2018)
Chlorella sorokiniana and *Scenedesmus obliquus*	Industrial and municipal and stormwater	Denmark	4.2%	82.7%	(De Francisci et al., 2018)
Scenedesmus obliquus	Stimulated municipal	China	99.2%	91.2%	(Qu et al., 2019)
Chlorella sorokiniana-activated sludge consortium	Synthetic municipal	China	98%	96%	(Fan et al., 2020)
Microalgae-activated sludge co-culture	Synthetic	Vietnam	86%	98%	(Nguyen et al., 2020)
Chlorella vulgaris with activated sludge	Synthetic municipal	Philippines	81%	39%	(Corpuz et al., 2021)
Microalgae biofilm	Municipal	China	98.6%	95.5%	(Li et al., 2022)

N, Nitrogen; **P,** Phosphorus. The table represents removal efficiency of pollutants of several species of microalgae in several locations. Data were compiled from the cited studies.

2.7 Bioresource Recovery

Bioresource recovery promotes environmental circularity by utilizing biomass from microalgae to produce lipids, which are then converted into biofuel, creating a sustainable cycle that reduces waste and enhances renewable energy production. Bioresource recovery brings a breath of fresh air to environmental circularity by harnessing the power of microalgae biomass to produce lipids, which are then transformed into biofuel, creating a sustainable cycle that reduces waste and boosts renewable energy production. Additionally, the biomass can be transformed into valuable byproducts like high-protein animal feed, nutraceuticals and bioplastics, which can be marketed to diverse industries, thereby boosting economic viability. Similarly, biofuel can be refined into marketable products such as biodiesel blends and biojet fuel, catering to transportation and aviation sectors, while supporting a greener economy (Karim et al., 2019).

2.7.1 Environmental Circularity

Microalgae offer a smart and sustainable approach to tackling pollution while converting waste into a valuable resource. They can naturally clean up wastewater by removing things like carbon dioxide, heavy metals, and excess nutrients. What's even better is that they thrive in wastewater, which means we can grow them without needing clean water or added nutrients, making the process cheaper and more eco-friendly (Morais Junior et al., 2020; Naik et al., 2024; Udayan et al., 2021).

As they grow, microalgae form biofilms that absorb nutrients from different types of wastewaters, though their growth can vary depending on the nutrient balance (Hu et al., 2021). For instance, biofilms grown in landfill leachate with a nitrogen-to-phosphorus ratio of 16:1 produced the highest biomass (Quan et al., 2020).

The algae harvested are rich in valuable materials, including lipids, proteins, and pigments. These can be used to make biofuels, fertilizers, and even products for industry or agriculture (Wang et al., 2024). In this way, microalgal systems support environmental circularity, cleaning up waste while creating resources that can be reused, reducing our environmental footprint and supporting a greener economy as shown in Figure 2.13 (Yap et al., 2021).

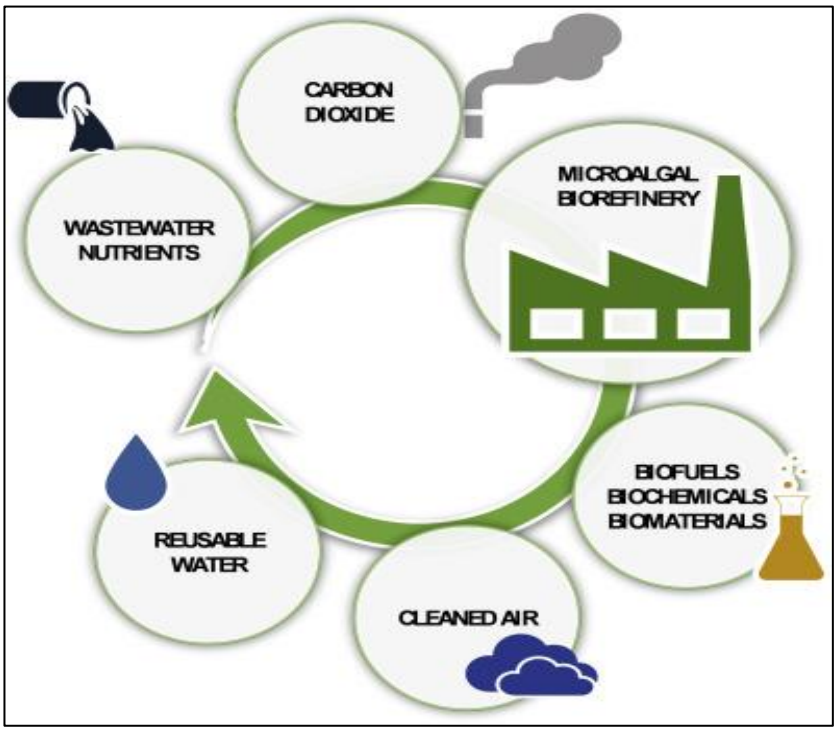


Figure 2.13 Role of Microalgae in Circular Economy

Note. The image shows a microalgal biorefinery cycle, utilizing wastewater nutrients and carbon dioxide to produce biofuels, biochemicals and biomaterials, while generating reusable water and cleaned air. Adapted from Calicioglu & Demirer (2022).

2.7.2 Biomass

Microalgae are a potential source of food, biofuel, animal feed, fertiliser, cosmetics, and pharmaceuticals as shown in Figure 2.14. The quality of the biomass composition of microalgae determines the quality of these bioproducts. Numerous methods, including cell counting, volume measurement, optical density, and weight can quantify microalgae biomass. Microalgae growth rates are generally directly associated with microalgae biomass and its quality. In a short period, a greater growth rate would yield more microalgal biomass, as shown in Table 2.6 (Yaakob et al., 2021).

Compared to the traditional suspended technique, microalgal biofilm harvesting offers much lower costs. According to this method, the biomass may be readily isolated from the liquid medium during harvesting because the microalgal cells are immobilized

inside an algal biofilm matrix (Gross et al*.,* 2015). Harvesting may be done using a straightforward mechanical removal via a scraping approach. This system also has potentially greater biomass production and requires less water and space. Factors like species, biofilm transporters, nutrient intake, and other growth features influence the increased microalgal biomass productivity (Ennaceri et al*.,* 2023).

Table 2.6
Microalgal Biomass Production in Wastewaters

Microalgae	**Wastewater**	**Biomass Production**	**Reference**
Scenedesmus sp.	Municipal	1.1 g L^{-1}	(Arias et al., 2018)
Chlorella sorokiniana	Municipal	1.0 g L^{-1}	(Leite et al., 2019)
Scenedesmis obliquus	Municipal	0.22 g L^{-1}	(Ling et al., 2019)
Scenedesmus sp.	Municipal	1.81 g L^{-1}	(Tripathi et al., 2019)
Scenedesmus abundans	Domestic	3.55 g L^{-1}	(SundarRajan et al., 2020)
Chlorella variabilis	Domestic	1.72 g L^{-1}	(Tran et al., 2021a)

The table presents biomass production rates of various microalgae species in municipal and domestic wastewaters, ranging from 0.22 to 3.55 g L^{-1} d^{-1}, with references to studies between 2015 and 2020. Data were compiled from the cited studies.

Microalgae are rich in a variety of bioactive compounds, such as protein (essential amino acids and bioactive peptides), polyunsaturated fatty acids (EPA and DHA), pigments (chlorophylls, carotenoids and phycobiliproteins), polysaccharides, vitamins, polyphenols and phytosterols. Compared to conventional meat, *Spirulina* can produce protein rich in essential amino acids, and its content can exceed 60% of dry weight, which is better than conventional meat (Soni, Sudhakar, & Rana, 2017). It is confirmed as “One of the best protein sources” by the Food and Drug Administration (FDA) (Chen et al., 2022).

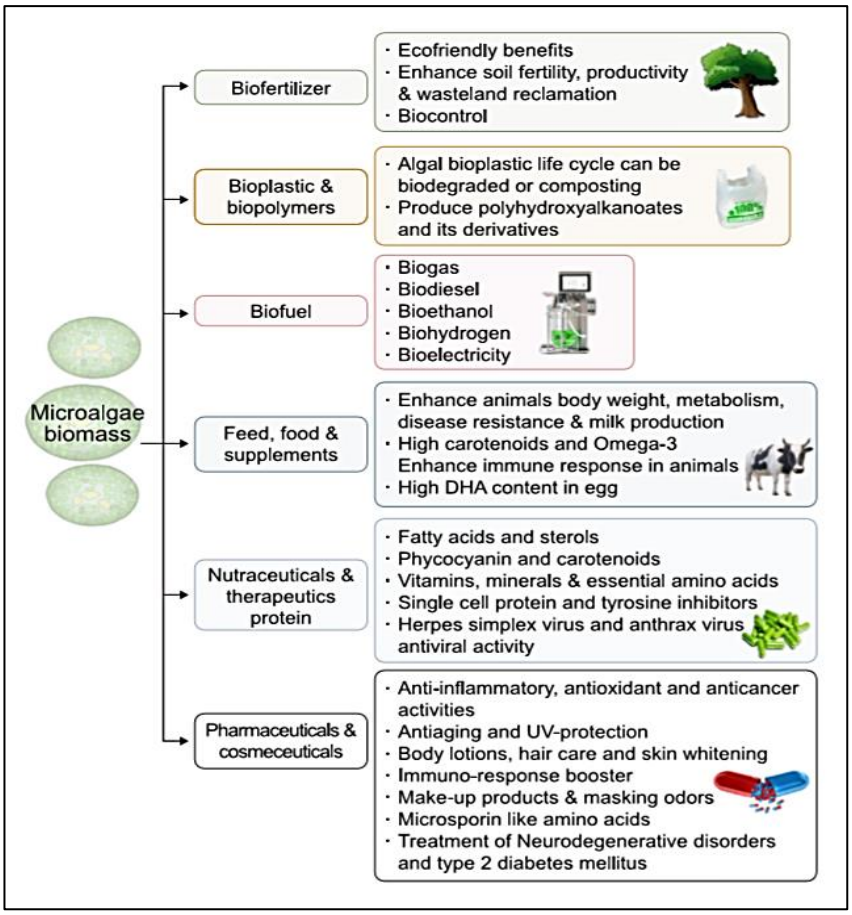


Figure 2.14 Production of Biofuel, Bio-Fertilizer and Value-Added Products of Microalgal Biomass After Wastewater Treatment

Note. The image outlines products from microalgae biomass, including biofertilizer, bioplastic, biopolymers, biofuel, feed/food supplements, nutraceuticals and pharmaceuticals/cosmeceuticals. This highlights ecological benefits like waste reclamation and health benefits like immune enhancement of microalgae biomass. Adapted from Abdelfattah et al. (2023).

2.7.3 Lipid and Biofuel

Lipids are accumulated by microalgae for cell division, growth, and storage of energy during times of stress. Microalgae oils have been produced for third-generation biofuels over the past ten years. However, their complex lipid composition, which includes saturated, monounsaturated, polyunsaturated, branched fatty acids, and fatty acid amides, allows for a wide variety of possible uses. Polyunsaturated fatty acids including omega-3, omega-6, docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) are abundant in microalgae lipid profiles. In aquatic environments, microalgae are the main producers of EPA and DHA, which facilitates higher species' subsequent

bioaccumulation of these compounds (Sierra et al., 2021).

Due to the inability to produce polyunsaturated fatty acids (PUFAs) by the human body, there is a need to obtain them through food consumption. Microalgae are high in polyunsaturated fatty acids (PUFAs), which have been demonstrated to protect against cardiovascular disease (Caporgno & Mathys, 2018; Ampofo & Abbey, 2022), obesity, diabetes (Caporgno & Mathys, 2018), and arthritis, as well as to support eye and brain health. Some of the well-known PUFAs microalgal producers are *Crypthecodinium, Schizochytrium*, and *Ulkenia sp*., while additional genera, including *Phaeodactylum, Monodus, Nannochloropsis*, and *Porphyridium* have also demonstrated significant quantities of DHA and EPA (Ampofo & Abbey, 2022).

Interestingly, the lipid (triglyceride), which is widely edible, produced by microalgae can be extracted and converted to biodiesel through the transesterification process, as depicted in Figure 2.15. Biodiesel is a substitute for diesel and is defined as alkyl ester of long fatty acid chain obtained from vegetable oil, animal fats, and compounds containing TAGs, through transesterification reaction in presence of acid or base catalyst (Karpagam et al., 2021).

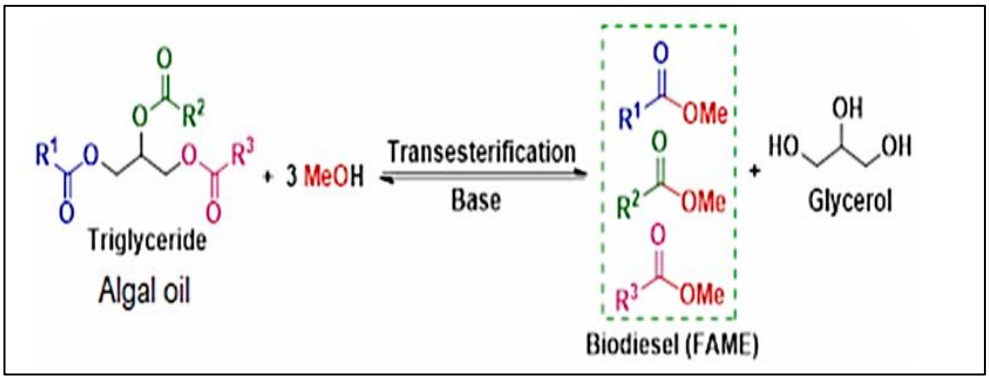


Figure 2.15 Synthesis of Biodiesel from Microalgae Using Transesterification Reaction

Note. The image depicts the transesterification process of converting algal oil triglycerides with methanol (MeOH) and a base catalyst into biodiesel (FAME) and glycerol, which is a key step in biofuel production. Adapted from Chhandama et al. (2021).

Common microalgae explored for biodiesel production are cyanophyte, chlorophyceae (green algae), and diatomaceous groups. Species like *Chlorella, Dunaliella sp*., and *Scenedesmus sp*. were observed to have high lipid contents of 20–30%, 17–67%, and 11–55%, respectively. Previous studies observed lipid content of

55% of *Chlorella protothecoide*s under heterotrophic condition, whereas *Chlorella sorokiniana* showed 55% lipid content when glucose as substrate. After the lipid is extracted from microalgae, it is trans- esterified in the presence of an acid or base catalyst (Islam et al*.,* 2013; Nascimento et al*.,* 2013; Sydney et al*.,* 2011).

Due to their rapid development and high lipid content, microalgae are also distinguished by enhanced lipid output. In addition, compared to conventional crops, the production of microalgae requires far smaller growth areas. As a result, with a 30% (w/w) oil content in algal biomass (Chisti, 2007; Tu et al*.,* 2015), the rivalry for arable land with other food crops is greatly decreased in the case of algae farmed for energy, which is up to 49 or 132 times less when compared to rapeseed or soybean crops (Mata et al. 2010). Table 2.7 illustrates the total lipid content and free-fatty acid profile of several microalgal species that have been previously studied.

Table 2.7
The Total Lipid Content and Free-Fatty Acid Profile of Several Microalgal Species

Microalgae species	**Total lipid content (% wt)**	**Fatty acid profile**
Botryococcus braunii	44.97	C14, C16, C17:1, C18, C18:1, C18:2, C18:3
Chlorella vulgaris	28.07	C14, C15:1, C16, C16:1, C17, C17:1, C18, C18:1, C18:2, C18:3, C20
Chlamydomonas sp.	15.07	C6, C8, C10, C12, C14, C16, C17, C18, C18:1, C18:2, C18:3
Desmodesmus brasiliensis	17.99	C6, C14, C16, C17:1, C18, C18:1, C18:2, C18:3
Scenedesmus obliquus	16.73	C4, C6, C8, C14, C16, C18, C18:1, C18:2, C18:3
Botryococcus terribilis	49.00	C12, C16, C18, C18:1, C18:2. C18:3
Coelastrum microporum	20.55	C4, C6, C10, C14, C16, C16:1, C17:1, C18, C18:1, C18:2, C18:3
Kirchneriella lunaris	17.30	C6, C14, C16, C17:1, C18, C18:1, C18:2, C18:3
Chlamydocapsa bacillus	13.52	C6, C8, C10, C11, C12, C14, C16, C16:1, C17, C17:1, C18, C18:1, C18:2, C18:3
Pseudokirchneriella subcapitata	28.43	C4, C14, C16, C17:1, C18, C18:1, C18:2, C18:3
Chlorella emersonii	18.6	C16, C18, C18:1, C18:2, C18:3,

		C20:1
Dunaliella sp.	22.00	C16, C16:1, C18, C18:1, C18:2, C18:3, C20:1

The table presents total lipid content (wt%) and fatty acid profiles of several microalgal species, with fatty acid compositions including C14 to C20:1, as reported by Chhandama et al. (2021).

2.8 Microalgae Consortia

Microalgae have been proven to be superior in its capability to remove pollutants from various wastewater. However, sustaining monocultures of microalgae during the processes of wastewater treatment is found to be difficult. In comparison to monocultures, mixed consortia consisting of many microalgal species and/or bacteria are considered to be advantageous in wastewater treatment, bioresource optimization and more stable, especially during large-scale cultivation (Gururani et al., 2022; Naseema Rasheed et al., 2023). The mixed consortia consisting of photosynthetic microalgae and heterotrophic bacteria (microalgal–bacterial consortia), or photosynthetic microorganisms (microalgal consortia), and microalgal–fungi or yeast may develop naturally or by artificial engineering (Zhu et al., 2023). Artificial consortia can boost the abundance of a particular species with high growth rates and nutrient removal efficiencies in comparison to engineered consortia. However, the chosen species' adaptability to the environment and compatibility with the wastewater should be considered (Gururani et al., 2022). Figure 2.16 shows the employment of microalgae consortia in treating wastewater and biorefinery.

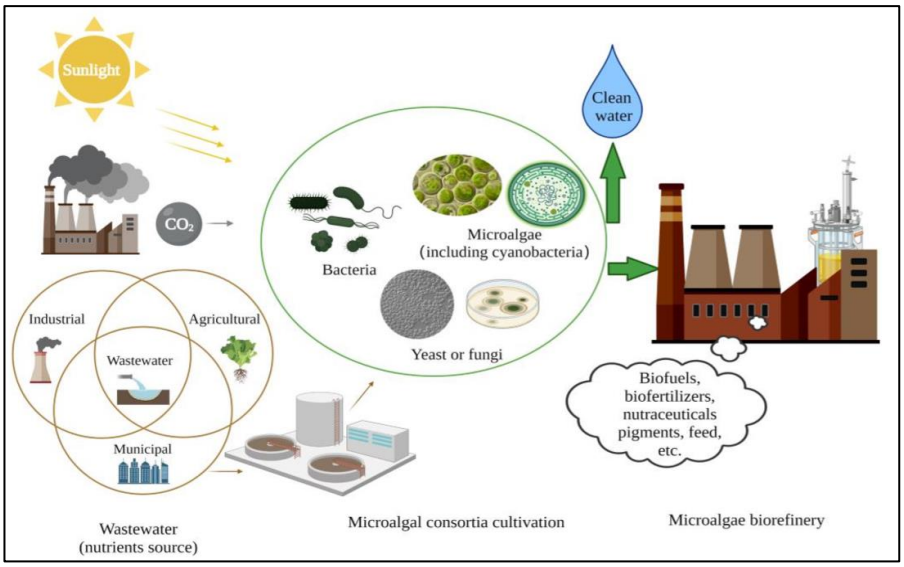


Figure 2.16 Microalgae Consortia in Wastewater Treatment and Biorefinery

Note. The image depicts microalgal consortia cultivation using wastewater (industrial, agricultural, municipal) as a nutrient source, with sunlight and CO_2 enhancing growth of microalgae, bacteria, yeast, and fungi. The process yields clean water and supports a microalgal biorefinery producing biofuels, biofertilizers, nutraceuticals, pigments, feed and more. Based on Zhu et al. (2023).

2.8.1 Microalgal-Microalgal Consortia

The interactions between microalgal consortia offer various benefits in the wastewater remediation techniques (Gururani et al., 2022; Iasimone et al., 2021; Zhu et al., 2023). As distinct microalgae have varied nutritional requirements, they can, firstly, increase the overall nutrient intake. Second, due to the induction of allelochemicals, they may be resistant to pollutants and invading predators. Thirdly, combining filamentous and unicellular microalgal species can enhance settleability, which is advantageous for algae harvesting. Additionally, by using microalgal consortia, the treatment process may be ensured sustainable and stable since the growth of other species within the consortia can compensate for the loss of one species of microalgae (Gururani et al., 2022).

Co-cultivation supports pollutant removal and renewable energy generation, with mutualistic interactions aiding nutrient uptake and resilience in wastewater environments (Naseema Rasheed et al., 2023). Notably, biomass yields of 0.98 $mg \cdot L^{-1} \cdot d^{-1}$ were obtained in domestic wastewater, and up to 96.2% TN and 89.6% TOC removal was reported in slaughterhouse wastewater (Taskan et al., 2016). Co-

cultivation of microalgae also enhances large-scale production by improving stability, nutrient use efficiency, and boosting biomass and lipid yields, while reducing biodiesel production costs (Naseema Rasheed et al., 2023). For instance, *Chlorella* sp. and *Monoraphidium* sp. co-culture achieved higher biomass (62 $mg \cdot L^{-1} \cdot d^{-1}$), lipid content (47.72%), and lipid productivity (29.52 $mg \cdot L^{-1} \cdot d^{-1}$) than monocultures (Devi et al., 2012).

In addition, a variety of microalgae species, including *C. vulgaris, C. sorokiniana, Tetradesmus sp., Ascomycota sp., C. saccharophila, Chlamydomonas sp., Chlamydomonas sp., Scenedesmus sp., Neochloris oleoabundans,* and *Coelastrum microporum*, have been used to treat wastewater from a variety of sources, including dairy, meat processing, tannery, and activated sludge (Gururani et al., 2022; Moreno-García et al., 2021). Furthermore, the viability of the decontamination process is ensured by the use of microalgae consortia in wastewater treatment. This is due to the possibility that some other microbes included in a consortium could compensate for the loss of one microorganism (Gururani et al., 2022).

2.8.2 Microalgal-Bacterial Consortia

Until recently, bacteria in microalgal cultures were considered as contamination. However, research has shown that microalgae and bacteria can form beneficial relationships, making their consortia highly promising for various applications. This synergy has gained significant attention, especially in wastewater treatment, where their interactions enhance pollutant removal and biomass production. Microalgae convert solar energy into chemical energy, producing oxygen (O_2) and organic matter through photosynthesis. In turn, bacteria utilize the oxygen for respiration, breaking down organic compounds and releasing CO_2, which microalgae need for growth. Additionally, bacteria provide growth-promoting factors and essential vitamins such as B12, B1, and B7, further enhancing microalgal growth and productivity. This mutual exchange creates a balanced and efficient ecosystem beneficial for various applications, including wastewater treatment and bioresource recovery (Amaro et al., 2023; Hu et al., 2021). Figure 2.17 shows how microalgal-bacteria consortia systems work simultaneously to treat wastewater while producing biomass.

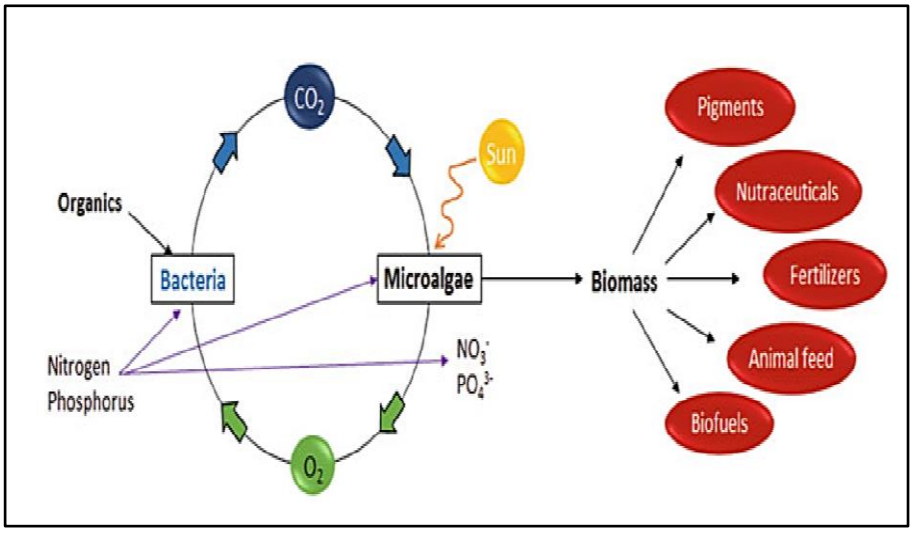


Figure 2.17 Microalgal-Bacterial Consortia Systems for Resource Recycling

Note. The image illustrates a microalgae cycle where bacteria utilize organics, nitrogen and phosphorus, supporting microalgal growth with CO_2 and sunlight to produce biomass. This biomass yields pigments, nutraceuticals, fertilizers, animal feed and biofuels, while releasing O_2. Adapted from Gonçalves et al. (2017).

It has been observed that bacteria from the genus *Halomonas* that live in close proximity to microalgae supplied the source of vitamin cobalamin for the microalga *Amphidinium operculatum*. The presence of cobalamin-producing bacteria shielded the green alga *Chlamydomonas reinhardtii* against heat stress. Additionally, several bacterial genera have been shown to produce antibiotics that protect microalgae against cell lysis (parasitism, management of algal blooms) or other microbes (mutualism/commensalism) (Zhu et al., 2023). Moreover, some bacteria (such as *Escherichia coli*, *Pseudomonas* sp. and *Bacillus* sp.) can provide inorganic phosphate for their host microalgae (Ahmad et al., 2022).

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Materials

The materials used in this study are as listed below.

3.1.1 Antibiotics

The antibiotics used in this study were ampicillin (10 mg/ml), kanamycin (5 mg/ml), cycloheximide (5 mg/ml), nystatin (100 μg/ml), and imipenem (100 μg/ml). These antibiotics were purchased from Sigma-Aldrich, Germany.

3.1.2 Buffers

All buffers and solutions used in this study were prepared using distilled water and sterilised by autoclaving at 15 psi for 15 minutes at 121°C unless stated otherwise.

1X Tris-Borate-EDTA (TBE) buffer, pH 8.0

TBE buffer at 10X concentration was purchased from Sigma Aldrich, Germany. For gel electrophoresis, the buffer was diluted by adding 100 ml of the 10X TBE buffer in 900 ml of distilled water.

1X Tris-EDTA (TE) buffer, pH 8.0

A volume of 10 ml of 10X TE buffer was prepared by adding 10 ml of 1M Tris-HCl, 2 ml of 0.5M EDTA and distilled water until the volume reached 88 ml. The pH of the buffer was adjusted until the desired pH of 8.0 was obtained. Distilled water was added to the solution to a final volume of 100 ml before autoclaving.

To reconstitute primers, the 10X TE buffer was diluted by adding 10 ml of the prepared buffer into 90 ml distilled water to obtain 100 ml of 1X TE buffer.

3.1.3 Chemicals and Reagents

Chloroform, 37% (v/v) formaldehyde, methanol and n-hexane used in this study were from Merck, Germany.

Sodium acetate trihydrate ($NaAc{\cdot}3H_2O$), Ammonium chloride (NH_4Cl), Dipotassium phosphate ($K_2HPO_4 \cdot 3H_2O$), Potassium dihydrogen phosphate (KH_2PO_4), Calcium Chloride ($CaCl_2$), Magnesium Sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$), Ethylenediaminetetraacetic acid (EDTA) and Sodium bicarbonate (NaHCO3) used were all purchased from Merck, Germany.

F.A.M.E Mix Internal Standard C8 – C24

F.A.M.E mix standard was prepared by dissolving 50 mg of the standard (Merck, Germany) in 50 ml n-hexane.

Sodium Methoxide

A volume of 50 ml of sodium methoxide was prepared by dissolving 1.15 g of sodium hydroxide (NaOH) pellets in 50 ml of methanol.

DNA Ladder

The DNA ladders used in this study were 1 kb and 100 base pair (bp) (Solis BioDyne, Estonia). The DNA ladders were stored at -20˚C upon purchase.

Ethidium Bromide Stain

The ethidium bromide stain used in this study was purchased from Bio-Rad, Singapore and stored at room temperature upon purchase.

3.1.4 Kits

The kits used to measure the TN and TP in this study were Total Nitrogen LR Test 'N Tube Reagent Set (CAT No. 2672245) (Hach, USA), Total Phosphorus LR Test 'N Tube Reagent Set (CAT No. 2742645) (Hach, USA). Spin Plant Mini Kit (Invisorb, Germany) was used for genomic DNA extraction for microalgal isolates.

3.1.5 Media

Blue Green-11 medium (BG-11) and synthetic wastewater were the culture media used in this study. These media were autoclaved at 15 psi for 15 minutes at 121°C.

1X BG-11 Medium

BG-11 medium of 1X concentration was prepared by diluting 20 ml of the 50X concentrated stock (*Phyto* Technology Labs, USA) in 980 ml of distilled water, according to the manufacturer's instructions.

Synthetic Wastewater

Synthetic wastewater was prepared as shown in Table 3.1. Each of the components was dissolved one after another in 800 ml of distilled water. The pH of the solution was then adjusted to pH 7.0 and the final volume topped up to 1000 ml using sterile distilled water before autoclaving.

Table 3.1
Compositions of Synthetic Wastewater

Components	**g/l**
Sodium acetate trihydrate ($NaAc \cdot 3H_2O$)	2.44
Ammonium chloride (NH_4Cl)	0.38
Dipotassium phosphate ($K_2HPO_4 \cdot 3H_2O$)	0.15
Potassium dihydrogen phosphate (KH_2PO_4)	0.05
Calcium Chloride ($CaCl_2$)	0.05
Magnesium Sulphate heptahydrate ($MgSO_4 \cdot 7H2O$)	0.04
Ethylenediaminetetraacetic acid (EDTA)	0.04
Sodium bicarbonate ($NaHCO_3$)	**0.08**

The synthetic wastewater was prepared based on Xu *et al.,* 2015.

3.1.6 Microalgae Samples and Maintenance

The isolated microalgae were maintained at 25 ± 2 °C in 50 ml of BG-11 medium under continuous blue-red LED lighting with a light intensity of 1000–2000 lux with a 12:12 h light-dark cycle (Miranda et al., 2017).

3.1.7 Primers

The primers used for the amplification of *cya* is as shown in Table 3.2. Each primer was reconstituted at 100 µM by using an appropriate amount of 1X TE, pH 8.0 buffer according to the manufacturer's instructions. The primers were stored at -20°C. To prepare a working solution of 100 µl of 10 µM primers, 10 µl of the 100 µM were diluted in 90 µl of 1X TE buffer and kept at -20°C.

Table 3.2
cya Gene Primer

Primer	Sequence	Size (bp)	Reference
cya	F: 5'-CGGACGGGTGAGTAACGCGTGA-3' R: 5'-GACTACTGGGGTATCTAATCCCATT-3'	675	Nu¨bel et al., 1997

The nucleotide sequence is returned in 5' to 3' direction.

3.1.8 Solutions

0.1 M Hydrochloric Acid (HCl) Solution

The HCl used in this study was purchased from Sigma Aldrich, Germany. To prepare 0.1 M HCl, 10 ml of HCl was mixed into 990 ml of distilled water.

1.0 M Chitosan Solution

The chitosan powder used in this study was purchased from Sigma Aldrich, Germany. For biomass harvesting, 100 mg of chitosan powder was mixed with 10 ml 0.1 M HCl solution until completely dissolved. The mixture was then diluted in 100 ml of distilled water to obtain a 1.0 mg/ml solution.

Bligh and Dyer Lipid Extraction Solution

For lipid extraction, a ratio of 1:2:0.8 (chloroform: methanol: distilled water) was added individually into the microalgal samples. One part of chloroform was first added, followed by two parts of methanol, before vortexing, and the mixture was left to incubate overnight. Following that, distilled water was added accordingly, and the mixture was vortexed and centrifuged to allow phase separation (Bligh & Dyer, 1959).

3.2 Methodology

3.2.1 Overall Flow of Methodology

An overview of the experimental design and workflow adopted in this study is presented in Figure 3.1.

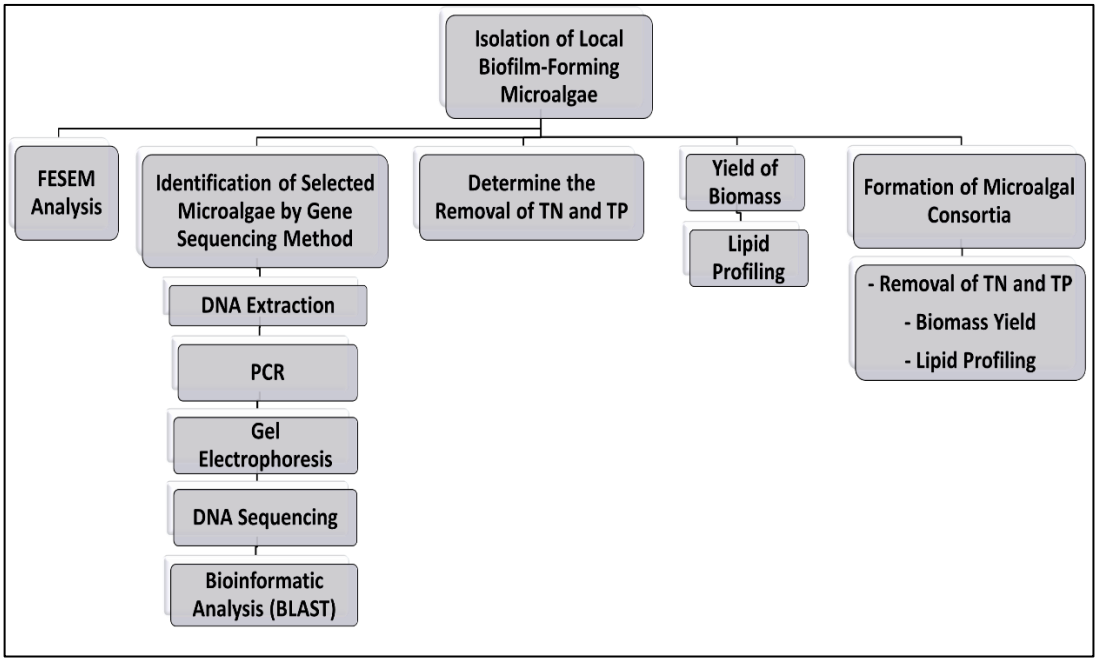


Figure 3.1 The Overview of Methodology

3.2.2 Collection and Maintenance of Microalgae

The microalgae samples were collected from the influent area of a maturation pond, which received treated wastewater at Sungai Buloh, Selangor, Malaysia

(3°14'42.0"N 101°28'22.8" E). Each sample was carefully scraped from rock surfaces using a sterile tweezer, transferred into sterile plastic containers, and transported to the laboratory immediately under aseptic conditions.

The collected microalgae samples were washed with sterile distilled water to remove any attached dirt or debris. The samples were then cultured in sterile plastic containers containing 50 ml of BG-11 medium supplemented with antibiotic cocktails prepared as detailed in Section 3.1.1 (Miranda et al., 2017). The microalgae were grown under conditions stated in Section 3.1.6. Each of the microalgae samples was subcultured at least 5 times to achieve homogeneous cultures.

Following that, the microalgae samples were then cultured with sterilized 22 x 22 mm glass coverslips placed at the bottom of the sterile bottle to serve as a substrate for biofilm attachment and immobilization purposes (Sunoj et al., 2021). Only microalgae with biofilm-forming ability were kept for further study, while the remaining were discarded.

3.2.3 Preliminary Characterization and Identification of the Microalgae

For preliminary characterization, the microalgae samples were grown in BG-11 medium together with sterile glass coverslips until they reached a maximum growth of 28 days (Moreno Osorio et al., 2021).

A light microscope (OLYMPUS CH20, Japan) was used to determine the cell size, shape, and morphology of each planktonic microalgae for preliminary identification at the genus level. Similarly, the structural biofilm of the microalgae samples was observed and recorded.

3.2.4 Structural Analysis of Biofilm using Field Emission Electron Scanning Microscopy (FESEM)

Selected microalgae samples were observed for biofilm formation under FESEM. To compare the structural variations of the microalgal biofilm, the samples were taken on day 0 and at the end of the 28-day incubation period, where the biofilm had matured (Zhu et al., 2019). The samples were meticulously processed for the FESEM examination. To eliminate the water content, each sample was subjected to a dehydration

process. The harvested samples were centrifuged for 15 minutes at 3000 rpm, washed with distilled water, fixed with 37% (v/v) formaldehyde and dehydrated with 25% ethanol. The dehydration process gradually continued using 50%, 75% and 100% EtOH. The samples were spread onto the glass slide and left to air-dry. The samples were then coated with gold using a sputter coater (Bal-Tec SCD005, USA). The high-resolution observation and documentaries of the biofilm structure of each of the selected isolates and consortia were analyzed.

3.2.5 Molecular Identification

Selected microalgae isolates were subjected to molecular identification through genomic extraction. The total genomic DNA was isolated using the Invisorb Spin Plant Mini Kit (Germany) according to the manufacturer's instructions. The extracted DNA was maintained at -20°C before further use.

3.2.6 PCR Amplification of *cya* Gene

For identification purposes, PCR amplification using the *cya* primer was performed on the selected microalgae isolates based on their earlier performances. The *cya* gene was selected as this study focused on cyanobacteria-dominated microalgal biofilms. Cyanobacteria-specific primers were used to confirm the presence of cyanobacteria within the biofilm system. As this study focused on the functional performance of cyanobacteria-dominated biofilms rather than detailed species identification, universal microalgal primers were not employed. The targeted use of the *cya* gene was therefore appropriate and sufficient for the scope of this study.

The PCR reaction mix was prepared using a total volume of 50 µl. The mix was prepared using 25 µl of MyTaq RedMix PCR mixture (Bioline, United Kingdom), 2.5 µl of 10 µM of each primer, 5 µl of 150 ng of DNA template and 15 µl of deionized water.

The amplification of target genes was performed using the MasterCycler Thermocycler (Eppendorf, Germany). Optimization was applied as needed to the amplification conditions. Table 3.3 shows the PCR conditions for the *cya* gene.

Table 3.3
Amplification Conditions for the *cya* Gene (Nu¨bel et al., 1997)

Conditions	Temperature (°C)	Time	Cycle
Initial Denaturation	94	5 min	1
Denaturation	94	30 sec*	
Annealing	60*	1 min	30
Extension	72	1 min*	
Final Extension	72	10 min	1
Hold	4	∞	

*Optimized PCR conditions

3.2.7 Agarose Gel Electrophoresis

The agarose gel was prepared by adding the appropriate amount of the agarose powder into 1X TBE buffer to make a 1.5% agarose gel. The mixture was heated in the microwave to dissolve the agarose completely and allowed to cool down to about 50˚C to 60˚C. Then, to give a final concentration of 1 ug/ml, the 1X concentration of Ethidium Bromide (Vivantis Technologies, Malaysia) was added. The gel was cast following the appropriate instructions after being mixed carefully.

After the spin-down of all PCR products, they were carefully loaded into the hardened agarose gel well. The first well of the gel was loaded with 5 µl of DNA ladder. Prior to that, 1X TBE buffer was poured into the chamber until the agar was submerged. Electrophoresis was then carried out under appropriate voltage and time. The separated bands were visualized using the gel visualization and documentation system, ChemiDoc Imaging System (Bio-Rad, USA). The band size was determined using the DNA ladder.

3.2.8 The *cya* Gene Sequences Identification by BLAST

The samples were sent to a commercial company (Bio3 Scientific, Malaysia) for sequencing. DNA sequencing was performed using the forward primer. Each sample was processed as paired-end reads with lengths of 675 base pairs.

The resulting sequence data of the *cya* and 18S rRNA were then submitted to the Genbank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using the BLAST algorithm. The match with the highest percentage of similarity (with a minimum similarity of 99%

and 99% coverage) was considered the correct identification.

3.2.9 Pollutant Removal Efficiency – Total Nitrogen (TN) and Total Phosphorus (TP) Analysis

The efficiency of the microalgae isolates to reduce pollutants in synthetic wastewater was investigated. Each microalgae sample with an initial weight of 0.15 ± 0.01 g was grown in similar conditions as in Section 3.1.6. A bottle of synthetic water without the microalgae was included as a negative control. At the end of the seven-days incubation period (Miranda et al., 2017), the synthetic wastewater was microfiltered using a nylon membrane filter (0.45 μm) to remove microalgal biomass and floating debris.

The total nitrogen (TN) and total phosphorus (TP) concentrations were measured using standard wastewater analysis techniques using Hach reagents. The percentage of nitrogen or phosphorus removal was calculated using the following formula as shown at (3.1) (Renuka et al., 2013):

$$((C_0 - C)/ C_0) \times 100) \quad (3.1)$$

whereby C_0 is the initial concentration of nutrients in the wastewater, while C is the concentration of nutrients left in the wastewater after treatment.

All the experiments were conducted in three independent replicates.

3.2.10 Biomass Production

To evaluate the biomass production, each of the microalgae samples was prepared to a standard wet weight of 1.00 ± 0.01 g. They were cultivated in 50 ml of synthetic wastewater together with glass coverslips for biofilm attachment, as mentioned earlier. At the end of seven days incubation period, the biofilm cells were scraped off and removed from the coverslips. The collected cells were then pooled and harvested by the coagulation-flocculation process using chitosan solution and washed with distilled water (Divakaran & Sivasankara Pillai, 2003). Following that, the microalgal biomass was collected through dewatering by filtration using Whatman GF/C glass fiber filter paper

(CAT No. 1822-047, pore size 1.2 μm), centrifuged at 6000 rpm, and washed twice with sterile distilled water. The centrifugation and washing steps were repeated twice. The biomass was then collected and dried until a constant weight was obtained (Qin et al., 2015).

The procedure was repeated to determine the biomass production of the microalgae grown for 14, 21 and 28 days (Zhu et al., 2019). All the experiments were conducted in three independent replicates.

3.2.11 Extraction and Analysis of Fatty Acid Content

The fatty acid content of the microalgae was determined according to the protocols by Bligh & Dyer, (1959), Kialashaki et al., (2019) and Qin et al., (2016) with some modifications. The samples were first grown in 150 ml BG-11 medium in conical flasks with sterile cover slips for 28 days (Zhu et al., 2019). Following that, 1.0 g of the microalgae biomass was collected by centrifugation at 9000 rpm for 10 minutes and washed three times with distilled water to remove the salt content (Cheng et al., 2013).

The total lipid was then determined using a modified Bligh and Dyer's method. The biomass was lysated using a pestle and mortar in liquid nitrogen (-196 ˚C) for 5 minutes. The Bligh and Dyer solution was added and mixed vigorously by vortexing. The mixture was then centrifuged at 9000 rpm for 10 minutes to separate the lipid-containing chloroform from the aqueous layer and kept at 4˚C overnight. After 24 hours, the top aqueous layer was removed, and the lipid phase was transferred to pre-weighed Falcon tubes. The remaining solvent was dried at 40°C under a low-pressure nitrogen atmosphere. The remaining residue was considered as crude lipid. The extracted lipid was measured gravimetrically to obtain the total lipid weight (Cheng et al., 2013). The total lipid contents were then calculated according to the following equation, as shown at (3.2):

$$\text{Total lipid content, (L \%)} = (W_L / W_B) \times 100\ \% \qquad (3.2)$$

whereby W_L is the weight of the extracted lipids, and W_B is the dry biomass (Qin et al., 2016).

The extracted lipids were converted into fatty acid methyl esters (FAMEs) via an alkaline-catalyzed transesterification reaction for fatty acid profiling. After the solvent had evaporated completely, the total lipids were subjected to transesterification by adding 3 ml of sodium methoxide and 3 ml of hexane (Kialashaki et al., 2019). The upper phase, which was the FAMEs, was then transferred into GC vials to be analyzed qualitatively by using Gas Chromatography-Mass Spectrometry (GC-MS) with a Varian 450 gas chromatograph fitted with an HP-5ms µultra Inert GC column (30 m 0.25 mm 0.25 m, Agilent J&W). For 3.5 minutes, a split ratio of 10:1 was used with a helium carrier gas flow rate of 1.0 ml/min. The samples were kept at 135°C for 4 minutes, the oven temperature was raised to 250°C at a rate of 4°C per minute and held for 10 minutes to complete. A Varian 240 Ion Trap Mass Spectrometer detector was used to identify the FAME peaks by comparing spectral data and retention times with FAME standards (Supelco C8:0–C24:0, Sigma Aldrich, Germany). A negative control was included in the FAME analysis to ensure that all detected peaks were specific to the microalgae samples and not contaminants (Massimi & Kirkwood, 2016).

3.2.12 Microalgal Consortium

Selected microalgal strains were grown in consortia to determine their combined potential in removing pollutants and biomass production. These strains were chosen individually based on their earlier performances in biomass production and also efficiency in removing TN and TP pollutants.

Each consortium of three microalgae samples was first grown in BG-11 medium in a 1:1:1 ratio of biomass wet weight (0.15 ± 0.01 g each strain) (Renuka et al., 2013). A light microscope was used for early observation of cell distribution and morphology. The biofilm structure of the consortia was also observed and monitored to confirm long-term functionality. The consortium was allowed to adapt and acclimate for 28 days under static conditions to ensure compatibility.

Following that, the ability of each consortium to remove pollutants and produce biomass and lipid was compared. The analysis was done as described in Sections 3.2.9, 3.2.10 and 3.2.11. All experiments were tested in triplicate.

3.2.13 Statistical Analysis

Comparison of means within groups was performed using-single factor ANOVA and Tukey's Honest Significance Differences (Tukey's HSD) tests (https://www.icalcu.com/stat/anova-tukey-hsd-calculator.html). Comparison between two means was performed with Student's t-test assuming equal variance, using Microsoft Excel's built-in data analysis tool pack. A *p*-value of less than 0.05 indicates a significant difference between the means. Error bars were calculated as the standard deviation of the means.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Collection and Maintenance of Microalgae

A total of 28 microalgae strains were collected and grown in sterile BG-11 medium supplemented with antibiotics for 28 days. The results are shown in Figure 4.1.

The progressive growth of the microalgae is indicated by the increasing turbidity of the culture medium, deepening green pigmentation and the density of the green biomass production (Arrojo et al., 2022; Ramlee et al., 2021). Low growth rate was observed in isolates MA1, MA2, MA6, MA7 and MA8 due to the pale green pigmentation observed with low density of biomass production. In contrast, MA5 and MA10 displayed medium growth rate, represented by the medium biomass produced, while the remaining isolates grew really well in the medium, indicating a higher growth rate. No growth was observed in isolates MA25, MA26, MA27 and MA28, and they were therefore excluded from this study.

The observed variation in microalgal growth rates among the isolates can be attributed to several factors. Environmental conditions such as light intensity, nutrient concentration, and temperature significantly influence the metabolic activity, photosynthetic activity and biomass yield of microalgae (Cheah & Chan, 2021; Ennaceri et al., 2023). In this study, some isolates displayed a high growth rate, indicating the ability to adapt more quickly to laboratory conditions, which includes artificial light and the type of synthetic media used. This is likely due to prior exposure to similar stressors in their native environments, or they naturally have a higher tolerance to changes. This adaptability can shorten the lag phase duration, thus increasing the overall growth performance (Ramlee et al., 2021). A study by Hotos (2021) found that some microalgae thrived at different concentrations of saline water. *Amphidinium* grew well in 20 ppt saline, while *Asteromonas* thrived best at 100 ppt saline concentration. In contrast, *Dunaliella* demonstrates consistent growth across all tested salinities at 20, 40, and 60 ppt saline concentrations.

In addition, genetic variation between strains also plays a key role, as different species or even strains within the same genus may possess distinct growth capacities,

stress tolerance and nutrient uptake efficiencies. Some strains are naturally fast-growing and can achieve higher biomass and productivity under the same conditions, while others are inherently slower. These variations contribute to differences in growth behaviour even under the same culture conditions (Hirano et al., 2021). In 2021, Hirano et al. (2021) compared a fast-growing strain of *Botryococcus braunii*, known as Showa, with wild strains of *B. braunii*. Although under the same cultivation conditions, the rate of biomass and hydrocarbons productivity differ, with the wild strain (OIT-678) being 36% higher biomass productivity, 34% higher hydrocarbon productivity, and 20% higher biomass density than Showa. These differences in growth behavior are crucial when evaluating microalgae for wastewater treatment, as consistent and robust growth is often linked to more effective biofilm formation and pollutant removal.

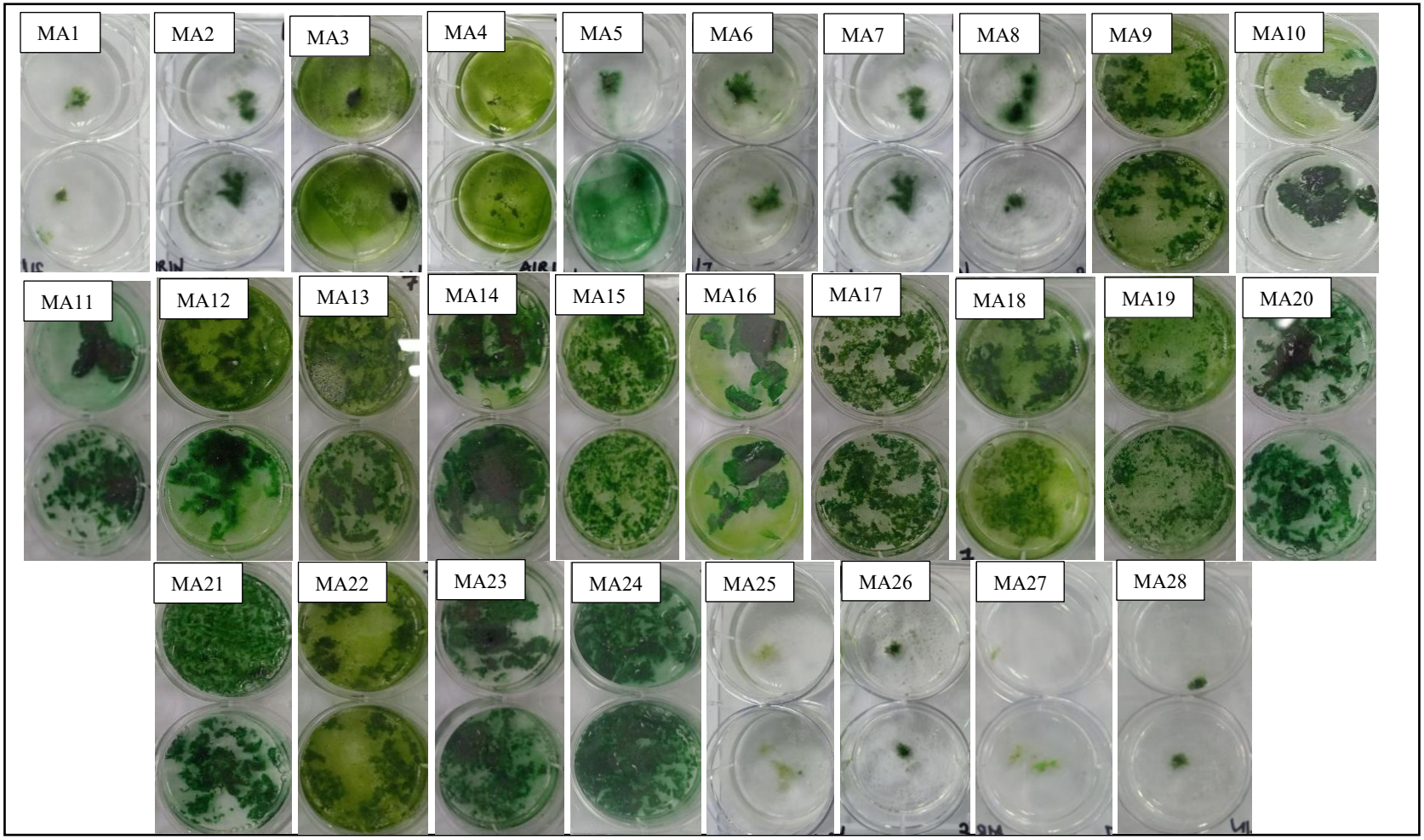


Figure 4.1 The Isolated Microalgae Samples in BG-11 Inoculated in Duplicates for 28 Days

4.2 Biofilm Formation of the Isolated Microalgae

The samples were then screened for their ability to form biofilm. They were grown in BG-11 medium together with glass coverslips, which served as a solid surface for attachment, as shown in Figure 4.2.

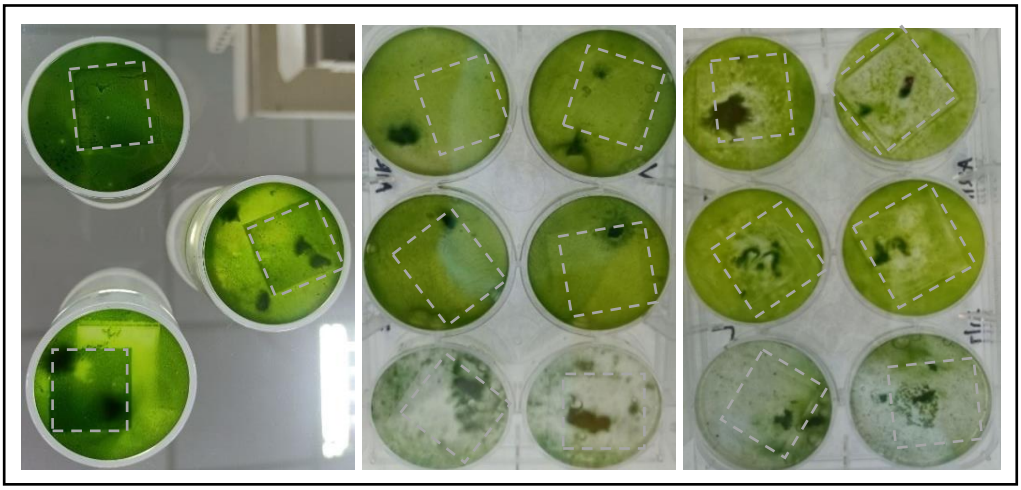
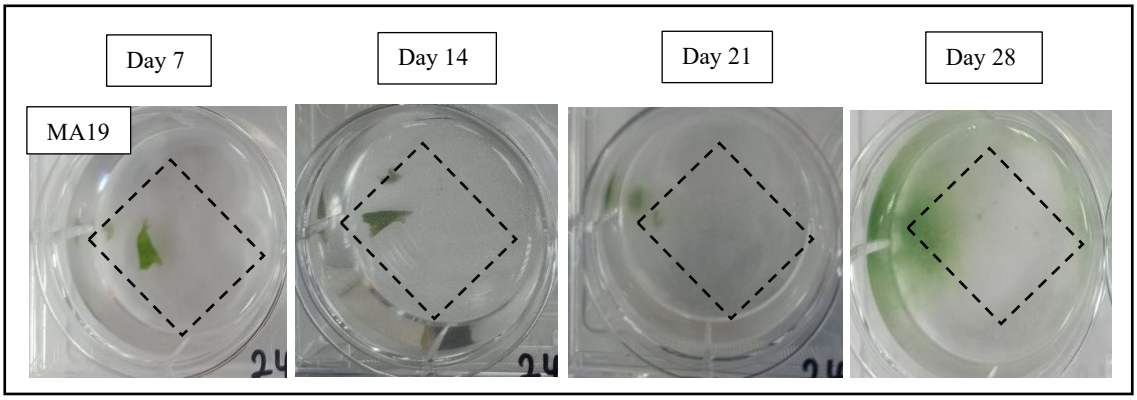


Figure 4.2 Growth of Microalgal Biofilm on Glass Coverslips in 28 Days of Incubation Period. The Dotted Square Represents the Glass Coverslips.

The growth of the biofilm was observed at intervals of 7, 14, 21 and 28 days. Figure 4.3 shows some samples of microalgae formation, while the scores of the biofilm formed by each of the isolated microalgae are summarised in Table 4.1.



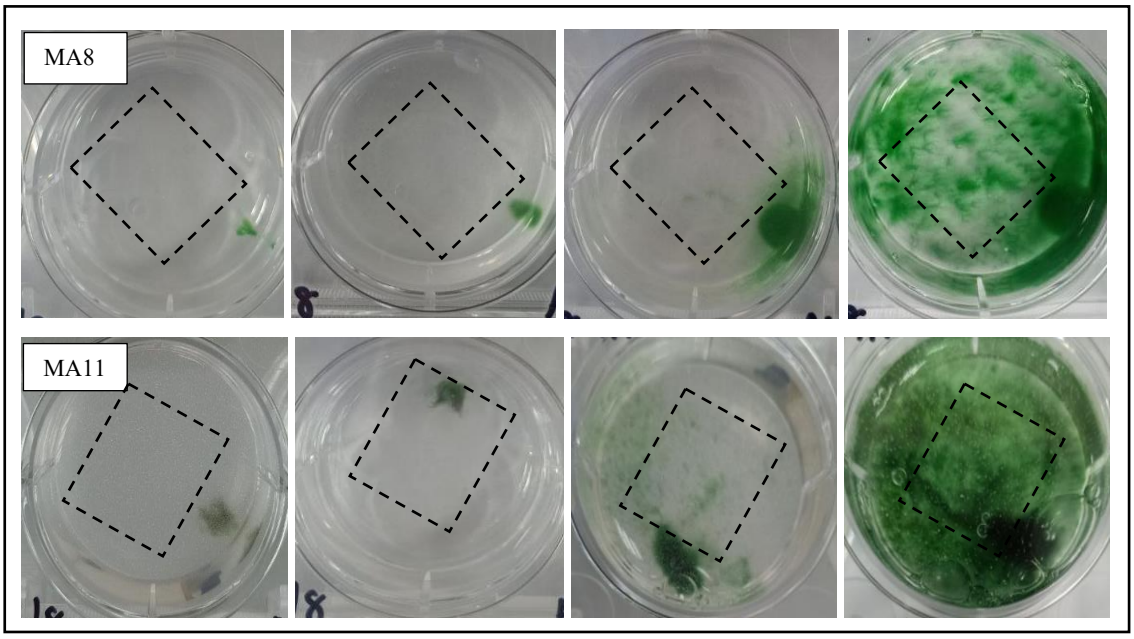


Figure 4.3 The Biofilm Formation of Samples MA19, MA8 and MA1.

MA19 represents weak microalgal biofilm formation, while MA8 and MA11 show intermediate and strong biofilm formation, respectively. The dotted square represents the glass coverslips.

Table 4.1
Microalgae Isolates' Growth and Biofilm Formation After 28 Days of Cultivation

Sample	Biofilm Formation	Sample	Biofilm Formation
MA1	+	MA13	+++
MA2	+	MA14	+++
MA3	+++	MA15	+++
MA4	+++	MA16	+++
MA5	++	MA17	+++
MA6	++	MA18	+++
MA7	+++	MA19	+++
MA8	++	MA20	+++
MA9	+++	MA21	+++
MA10	+++	MA22	+++
MA11	+++	MA23	+++
MA12	+++	MA24	+++

(+) weak biofilm former; (++) intermediate biofilm former; (+++) strong biofilm former.

The biofilm formation of the isolates can be observed by the enhanced surface attachment along the plate walls and glass coverslips. During the first seven days of cultivation, some isolates were observed to form pale greenish filaments either on the wall of the plates or on the glass coverslips. By day 14, the microalgae biofilm

attachment had become more prominent, and by day 21, it had intensified, with some isolates forming thick, visible layers along the substrate surface. By day 28, all isolates were observed to form visible biofilms on the coverslips, although the intensity and pattern of attachment varied among the strains.

Weak biofilm formation can be observed in MA1 and MA2. In contrast, MA5, MA6 and MA8 showed intermediate biofilm formation, while the remaining 19 microalgae isolates exhibited strong and robust biofilm formation. The majority of the cultures showed robust growth and clear signs of biofilm formation. These strong biofilm formers displayed dense, uniform colonization on the glass surface and visible extracellular polymeric substances (EPS).

The microalgal biofilm displayed a dense structure with a uniform surface coverage, as observed, which were represented by the formation of filaments. Structurally, the microalgal biofilm consists of filamentous cyanobacteria that form a net that traps unicellular coccal green microalgae (Sukačová & Červený, 2017). Microalgal cells secrete an EPS consisting of proteins, polysaccharides, nucleic acids, and/or phospholipids, which aid in developing biofilms (C. Wang et al., 2022). The EPS enhances adhesion to the substratum and provides a matrix for nutrient retention and microbial activity. This structural integrity may likely contribute to the biofilm's stability and effectiveness in nutrient removal. The growth of filamentous microalgae and cyanobacteria was observed until the 28th day, supposedly the last phase of species succession before the growth decline (Sukačová & Červený, 2017; Garbowski et al., 2020).

In this study, the constructed cultivation material used for the adherence of the microalgal biofilm was borosilicate glass, which was found to be a good and reliable attachment material (Heuschkel et al., 2020). This substrate was used for immobilizing the microalgal biofilm and was durable enough for scraping, a standard method to harvest algal biofilm (Rana & Prajapati, 2022). In some studies, biofilm cultivation was done using carrier materials like PVC fillers placed in plexiglass chambers. Several studies used polystyrene foam (Johnson & Wen, 2010) and pine bark (Zhang et al., 2020) as cultivation substrates, which can potentially reduce the harvesting cost, and the latter is a natural and accessible material (Sukačová & Červený, 2017; Garbowski et al., 2020).

4.3 Microscopic Analysis of Planktonic Cells and Biofilm Formation

The samples were then observed microscopically for planktonic cells and biofilm formation. Microscopic observations revealed clear morphological differences between the planktonic and biofilm growth modes across all 24 locally isolated microalgae samples (MA1–MA24). Table 4.2 shows microscopic observations of the planktonic and biofilm of the locally isolated microalgae samples.

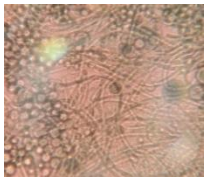
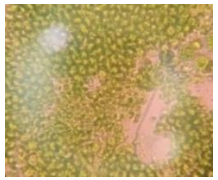
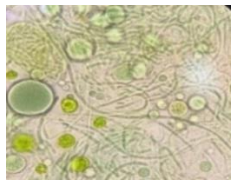
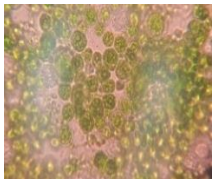
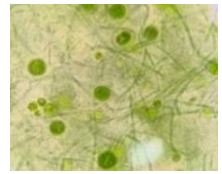
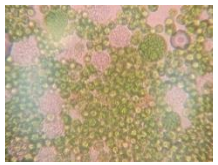
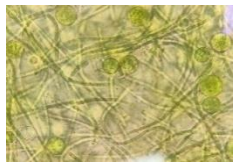
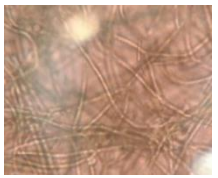
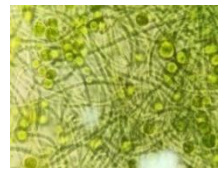
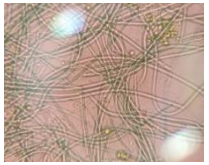
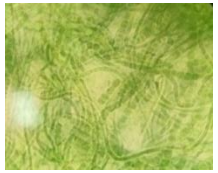
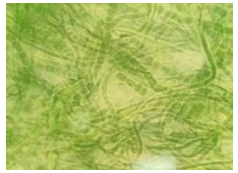
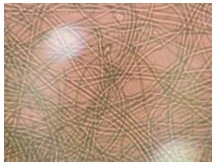
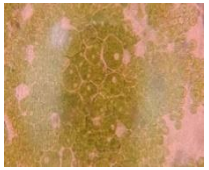
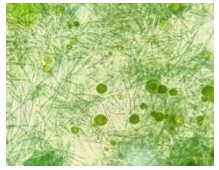
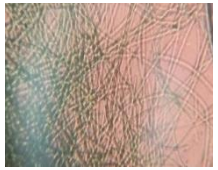
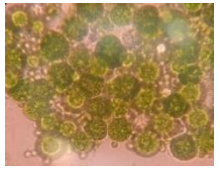
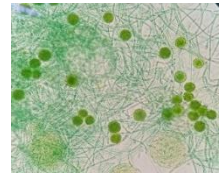
In the planktonic cultures, cells were generally observed as free-floating and dispersed, with limited aggregation and weak cell–cell interactions. Most isolates displayed individual cells or loose clusters, indicating a predominantly free-living growth state with minimal surface attachment.

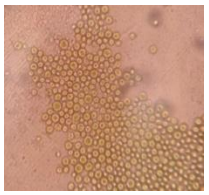
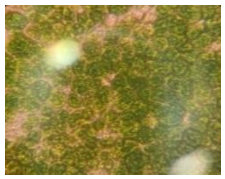
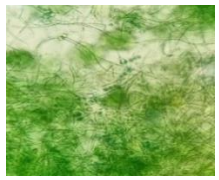
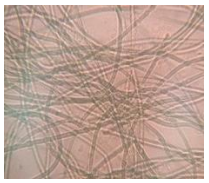
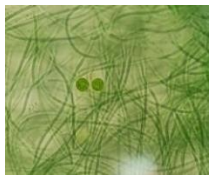
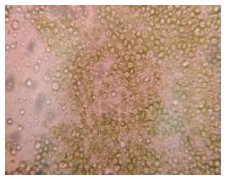
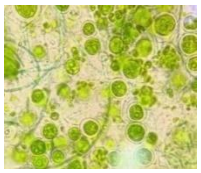
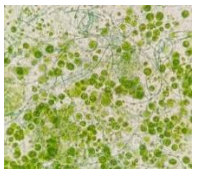
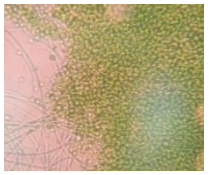
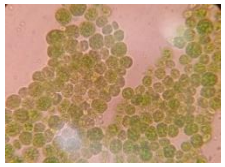
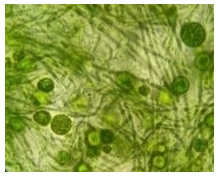
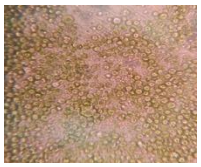
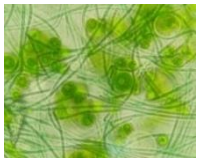
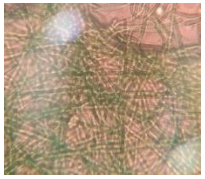
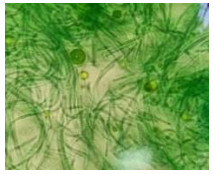
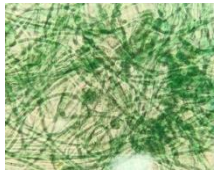
In contrast, biofilm cultures exhibited markedly higher cell density, greener pigmentation and more structured organisation compared to their planktonic forms. The biofilm forms showed extensive cellular aggregation, compact clustering and clear surface coverage, suggesting successful attachment and biofilm development. Several isolates also demonstrated filamentous or network-like structures within the biofilm, indicating the formation of complex biofilm architecture. Overall, the biofilm cultures consistently appeared denser and more organised than planktonic cultures, highlighting distinct morphological adaptations associated with surface-attached growth.

The observed morphological differences between planktonic and biofilm forms reflect the transition of microalgae from a free-living to a surface-attached lifestyle. The planktonic cells exhibited minimal aggregation and structural organisation, which is characteristic of suspended growth, where cells are not required to maintain prolonged surface attachment (Dogša et al., 2020). In this growth mode, the planktonic EPS typically involves lower polysaccharide content and less sticky, resulting in weaker intercellular interactions and less structural cohesion (Liu e al., 2024).

In contrast, the increased greenness observed in biofilm cultures suggests higher biomass accumulation and chlorophyll content, likely due to prolonged cell retention on the substrate and reduced washout. The presence of compact clusters and matrix-like structures further indicates active EPS secretion, which plays a critical role in stabilising biofilm architecture and anchoring cells to surfaces. In the biofilm form, the secretion of EPS is thicker and more adhesive, which promotes cell–cell adhesion, resulting in denser and more structured formations (Liu et al., 2024).

Table 4.2
Microscopic Observation of the Planktonic and Biofilm of the Locally Isolated Microalgae Samples

Sample	Planktonic	Biofilm	Sample	Planktonic	Biofilm	Sample	Planktonic	Biofilm
MA1			MA5			MA9		
MA2			MA6			MA10		
MA3			MA7			MA11		
MA4			MA8			MA12		

Sample	Planktonic	Biofilm	Sample	Planktonic	Biofilm	Sample	Planktonic	Biofilm
MA13			MA17			MA21		
MA14			MA18			MA22		
MA15			MA19			MA23		
MA16			MA20			MA24		

The biofilm-forming ability of the microalgae isolates corresponded closely with their observed biofilm morphology. Weak biofilm formers, such as MA1 and MA2, exhibited sparse surface attachment and thin biofilm structures, with only minor morphological differences between their planktonic and biofilm forms. These isolates showed limited cellular aggregation and weak structural organisation, suggesting lower EPS production and reduced surface adhesion capability.

Isolates categorised as intermediate biofilm formers, such as MA5, MA6 and MA8, demonstrated moderate increases in aggregation and surface coverage in the biofilm form. Although biofilm formation was evident, the resulting structures were less compact and less uniformly distributed compared to strong biofilm formers, indicating partial biofilm development.

In contrast, the rest of the isolates, which accounted for the majority of them, were classified as strong biofilm formers. The isolates displayed pronounced structural changes when transitioning from planktonic to biofilm growth. These isolates formed dense, well-organised biofilms with extensive surface coverage and thick cellular layers. In several strong biofilm formers, filamentous interconnections and network-like arrangements were also observed. This contributes to the structural integrity and stability of the biofilm. Notably, the isolates with higher aggregation in planktonic form tended to develop stronger and more complex biofilm structures, suggesting that inherent cellular morphology may influence biofilm-forming potential.

The predominance of strong biofilm-forming isolates among the locally isolated microalgae highlights their suitability for biofilm-based applications, particularly in post-wastewater treatment systems. Strong biofilm formation enhances biomass retention, resistance to hydrodynamic shear and overall system stability, which are desirable characteristics for long-term operational performance (Wang et al., 2022). Therefore, the morphological and structural characteristics observed in this study support the potential use of selected locally isolated microalgae samples in sustainable biofilm-based wastewater treatment and resource recovery processes.

4.4 Field Emission Scanning Electron Microscopy (FESEM) Analysis

Since FESEM preparation and imaging are resource-intensive, only the most representative isolate was selected. One of the isolates with strong biofilm-forming

ability, MA7, was selected for detailed structural characterisation using FESEM. Preliminary observations showed that MA7 produced the most compact and stable biofilm compared to other isolates, making it suitable as a representative model for structural analysis. The culture was grown and collected on days 7, 21 and 28 to observe different stages of biofilm development.

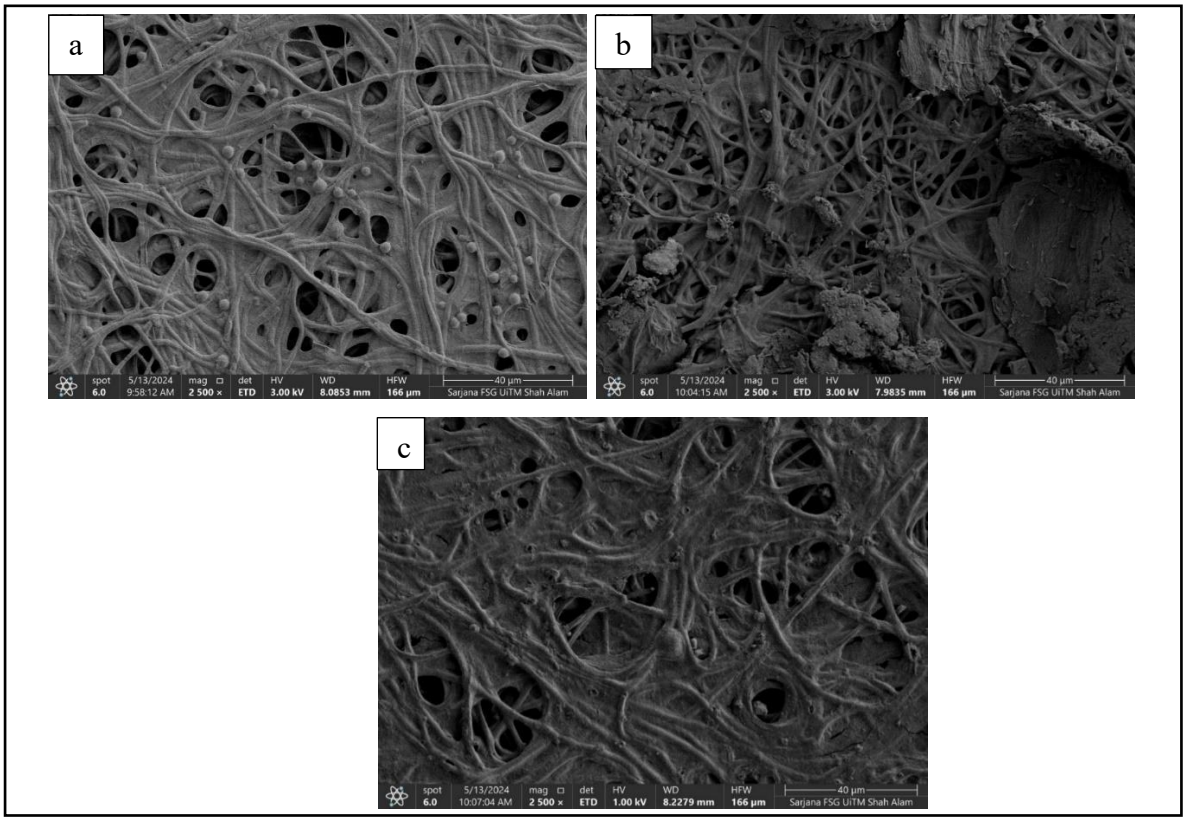


Figure 4.4 FESEM of Microalgal Biofilm Formed
(**a)** 7 days, **(b)** 21 days and **(c)** 28 days.

FESEM analysis revealed clear morphological changes in the microalgal biofilm over the cultivation period, as shown in Figure 4.4. At day 7 (Figure 4.4a), the biofilm structure appeared relatively loose, with thin filaments forming an initial network. Numerous voids and open spaces were observed, indicating early-stage attachment and colonization.

By day 21 (Figure 4.4b), the network became denser, with interwoven filaments creating a more compact structure. This suggests active cell growth and EPS production, which enhances biofilm cohesion and surface coverage (Ennaceri et al., 2023). The denser arrangement also indicates a transition to a mature biofilm stage, where cells are

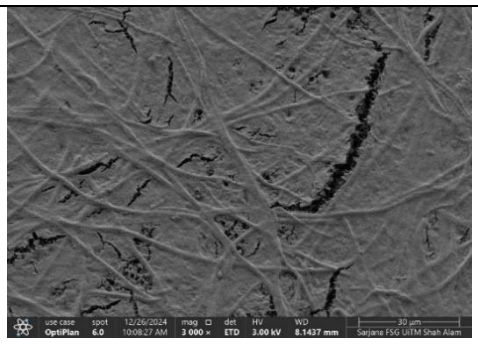
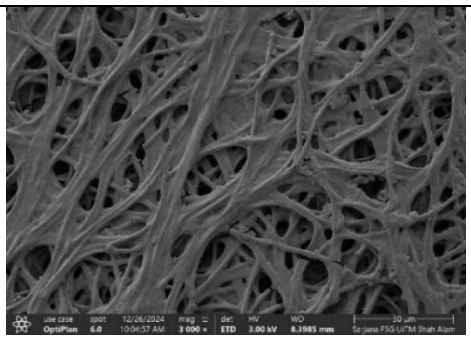
embedded in a well-developed EPS matrix (Cheah & Chan, 2021).

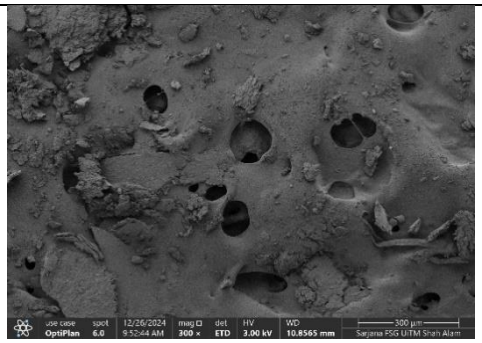
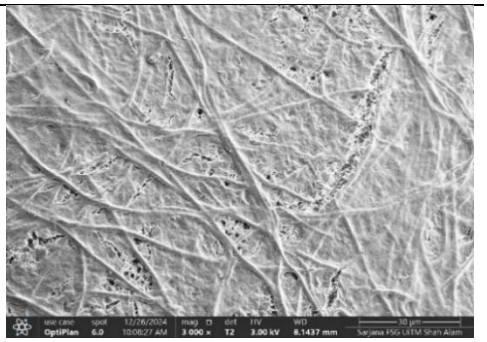
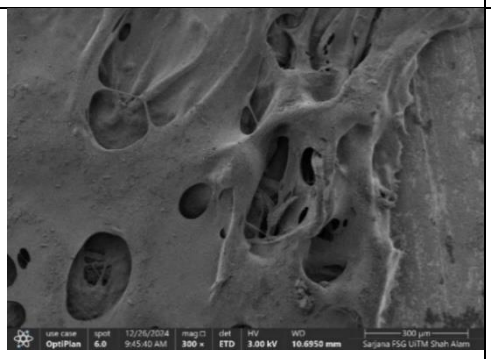
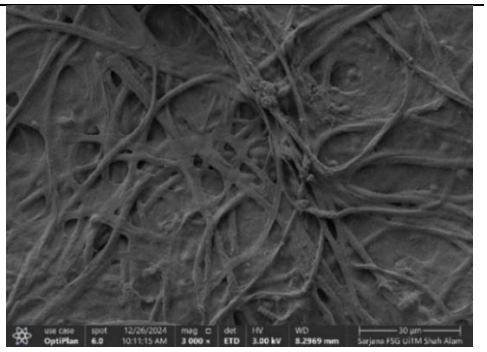
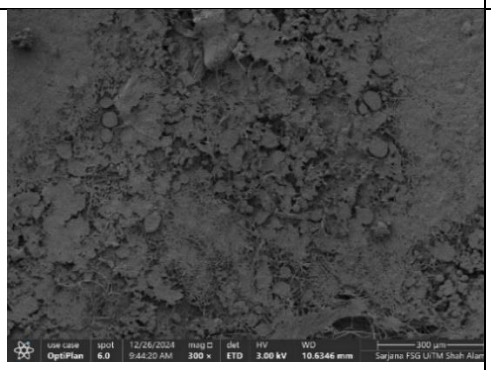
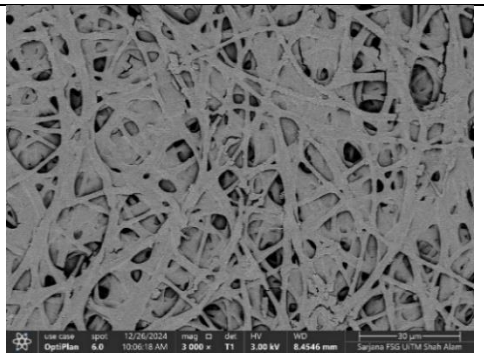
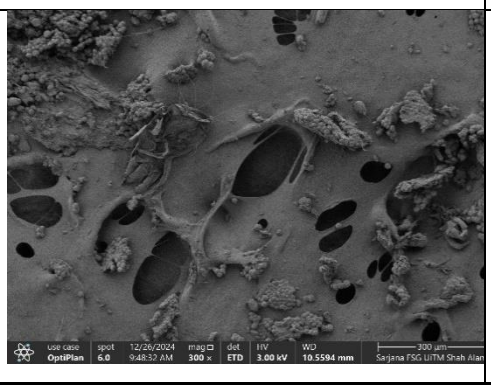
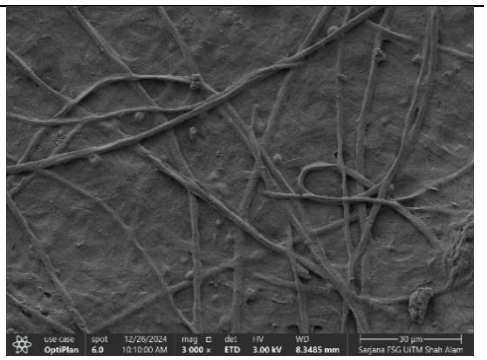
At day 28 (Figure 4.4c), the biofilm maintained a dense structure but showed signs of filament thickening and surface smoothing. The reduction in visible gaps suggests complete surface colonization. However, localized thickening could also imply nutrient or light limitations in deeper layers, potentially leading to physiological stress or reduced growth rates (Mougin et al., 2025).

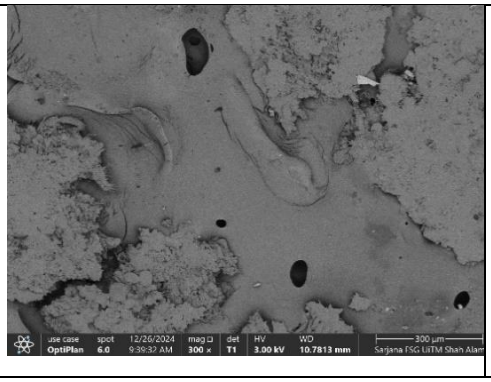
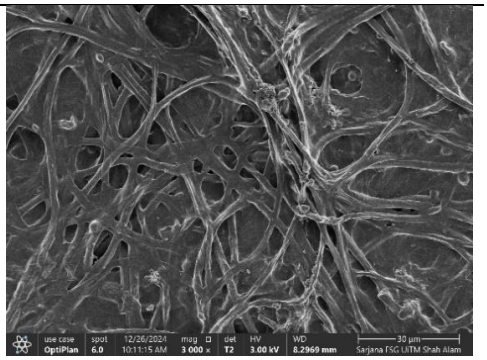
These structural changes over time reflect typical biofilm developmental stages, from initial adhesion to maturation. This relates with previous findings in cyanobacterial and microalgal systems (Mougin et al., 2025). Dense biofilms, such as those observed at 21 and 28 days, are advantageous for biofuel production due to higher biomass retention and ease of harvesting (Berner et al., 2015), although excessive thickening may limit mass transfer and require optimization of cultivation duration (Abu Hasan et al., 2021; Roostaei et al., 2018).

Selected isolates and consortia were analyzed for their biofilm structure throughout 28 days of growth. This included the top performer for TN removal (MA18), the TP removal (MA7), and the isolate with the highest biomass and FAMEs production (MA23), the best-performing consortia were those achieving the highest TN removal (MA18-5-22) and TP removal (MA18-7-23), the highest biomass yield (MA18-4-22), and the most FAMEs production (MA18-7-23).

Table 4.3
FESEM Analysis of Microalgal Biofilm After 28 Days of Growth

Isolates	0 day	28 days
MA7		

MA18		
MA23		
Consortium 1 (MA18-7-23)		
Consortium 7 (MA18-5-22)		

Consortium 10 (MA18-4-22)		

FESEM analysis was conducted to observe the biofilm structures formed by the selected isolates (MA7, MA18, and MA23) and high-performing consortia (MA18-5-22, MA18-7-23, and MA18-4-22) after 28 days of cultivation, compared to their initial state (0 day). As shown in Table 4.3, at day 0, all samples showed relatively smooth surfaces with minimal cell attachment, indicating the absence of significant biofilm development. The microalgal cells exhibit sparse attachment to the substrate. The attachment appears loose, indicating that cells have not yet formed a strong biofilm structure. EPS, which plays a crucial role in biofilm stability, is not yet prominent at this stage (Moreno Osorio et al., 2021). The attached cells appear scattered rather than forming dense clusters. At this stage, the biofilm is in its initial colonization phase, where cells are in the process of recognizing and adapting to the surface. The weak attachment suggests that biofilm formation requires further structural development through cell division, aggregation and EPS secretion (Hu et al., 2021; Moreno Osorio et al., 2021).

After 28 days, distinct morphological changes were evident in all isolates and consortia, with dense networks of EPS and filamentous structures observed, suggesting strong biofilm formation. The biofilm structure is visibly more complex, with multiple layers of microalgal cells embedded in EPS. The microalgal cells are no longer isolated but rather form dense aggregates, suggesting strong interspecies or intraspecies interactions. The biofilm extends across the substrate, covering a significantly larger area compared to Day 0. The increased EPS secretion observed in mature biofilms plays a vital role in biofilm sustainability, as it protects cells from environmental stress, facilitates nutrient exchange between microalgal cells and enhances adhesion, preventing detachment from the surface (Cheah & Chan, 2022; Ennaceri et al., 2023).

These structural transformations from scattered planktonic cells (Day 0) to a dense, interconnected biofilm (Day 28) indicate that the microalgae have successfully adapted to surface-associated growth, forming a stable biofilm system (Ennaceri et al., 2023).

Among the isolates, MA23 exhibited the most extensive and uniform biofilm coverage, consistent with its previously recorded highest biomass yield and diverse FAMEs profile. MA7 and MA18 also formed dense biofilms, with MA18 showing a more compact and layered structure, which could contribute to its high TN removal efficiency.

For the consortia, MA18-7-23 displayed the thickest and most interconnected biofilm matrix, potentially enhancing nutrient uptake and FAMEs synthesis due to synergistic interactions between strains (Gonçalves et al., 2017). MA18-4-22 showed a similarly dense arrangement but with more open channels, which may aid in nutrient diffusion and biomass production (Kumari et al., 2025; Perera et al., 2019) . Conversely, MA18-5-22 produced a comparatively less uniform biofilm, possibly explaining its lower biomass productivity despite high TN removal performance.

This suggests that biofilm structure plays a crucial role in determining the functional efficiency of both isolates and consortia, with denser, well-connected networks potentially enhancing nutrient removal and lipid accumulation. The strong correlation between biofilm density, biomass yield and FAMEs production supports previous findings that microalgal biofilms can enhance metabolic efficiency through close cell-to-cell interactions and EPS-mediated protection (Ennaceri et al., 2023; Gonçalves et al., 2017).

This analysis provides insights into the morphological and structural changes that occur during biofilm development of microalgal biofilm. The comparison highlights significant differences in cell attachment, biofilm thickness, EPS production and surface coverage, all of which contribute to the stability and functionality of the biofilm over time (Bozan et al., 2024; Ennaceri et al., 2023).

4.5 Molecular Identification of Microalgae

Some preliminary studies on the potential of isolated microalgae in removing TN, TP and producing biomass and lipid were conducted earlier. Based on these, some of the microalgae were selected and subjected to molecular identification by amplification of 16S rRNA gene. These include MA4, MA5, MA7, MA18, MA22 and MA23.

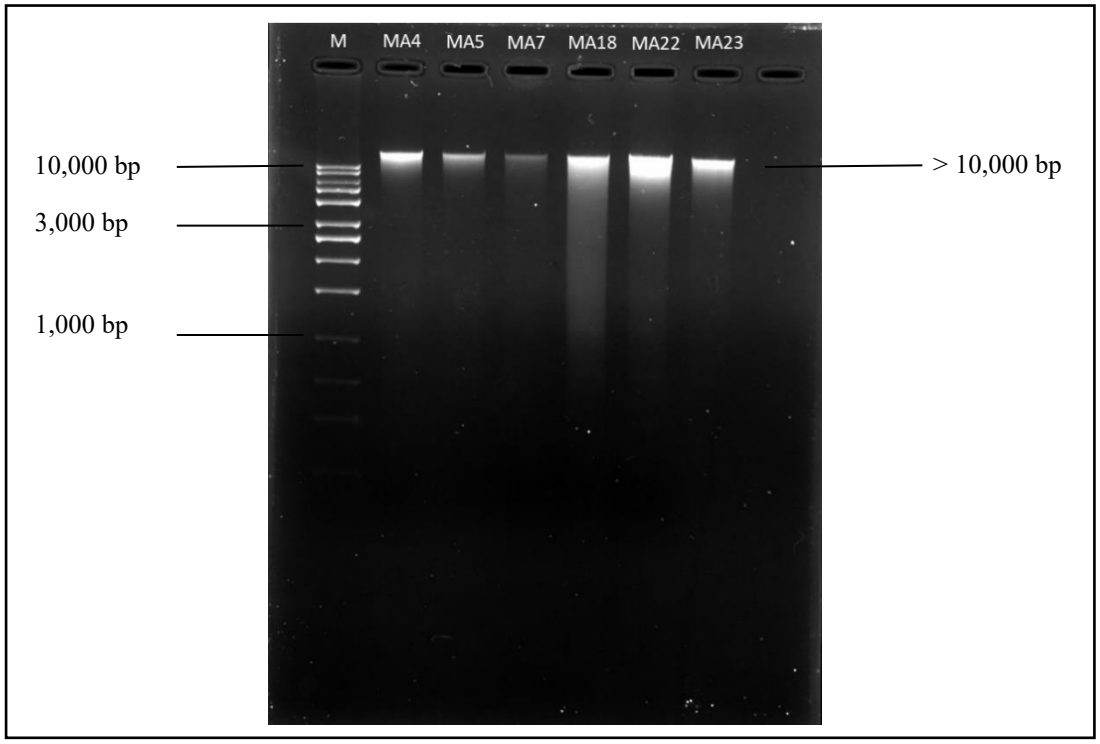


Figure 4.5 The Gel Electrophoresis of Genomic DNA of Microalgae Isolates

Lane M; 1 kb DNA ladder

Figure 4.5 shows all the genomic DNA of selected microalgae isolates successfully extracted, as confirmed by the presence of high molecular weight DNA bands on the agarose gel. Clear DNA bands were observed, indicating successful amplification and the presence of intact genomic DNA. The density and intensity of the bands reflect the relative amount of DNA present in each sample. Variations in band intensity among the isolates suggest differences in DNA yield, with fainter bands indicating lower recovered DNA concentrations. This reduced band intensity is likely associated with incomplete cell disruption and inefficient DNA release. Many microalgal and cyanobacterial isolates possess thick cell walls and extracellular

polymeric or mucilage layers, which can hinder lysis and reduce extraction efficiency (Aboul-Maaty & Oraby, 2019). Therefore, differences in extraction effectiveness likely contributed to the variation in observed band intensities.

The 16S rRNA gene sequences of the cyanobacterial isolates were amplified using cyanobacteria-specific primers (CYA106F/CYA781R). The results are shown in Figure 4.6.

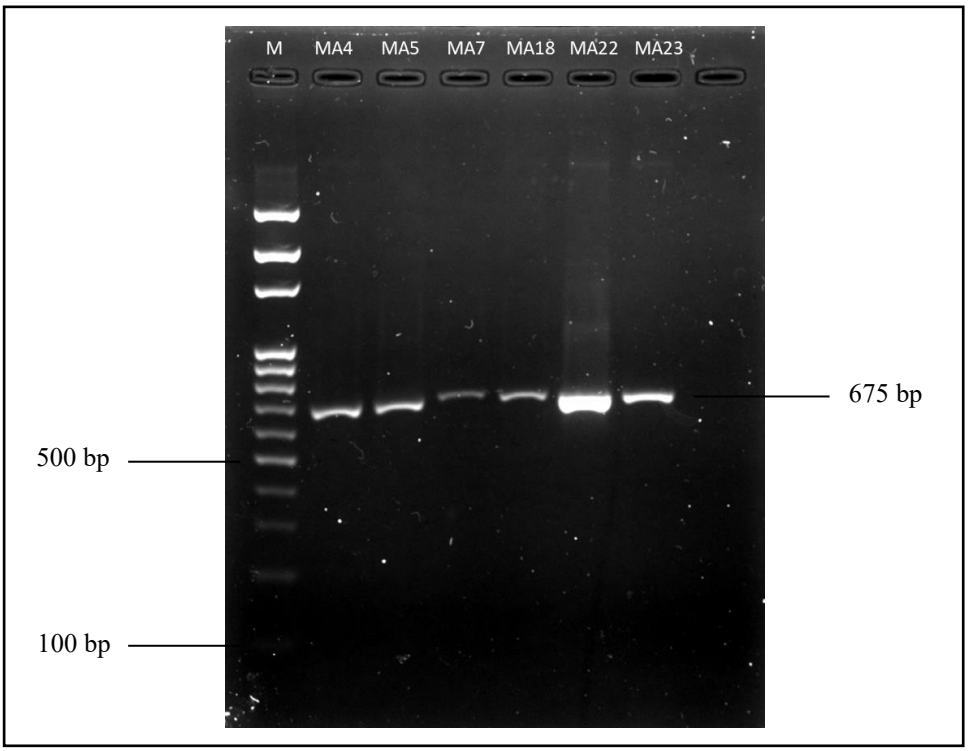


Figure 4.6 Gel Electrophoresis of PCR Products of Selected Microalgae Isolates

Lane M, 100 bp DNA ladder. The image shows an agarose gel electrophoresis result of a PCR product amplified, yielding a 675 bp band, confirming successful amplification of the target gene.

Distinct bands at approximately 675 bp, corresponding to the expected size of the amplified fragments, were observed.

The PCR products were sent to a third-party service provider for sequencing and subjected to BLAST analysis against the NCBI nucleotide database. The top match for isolates MA4, MA7 and MA23 showed 99.5%, 99.7% and 99.4% similarities to *Leptolyngbya sp.* D1C10 (GenBank accession: KJ654308.1), respectively. On the other hand, MA5 was identified as *Leptolyngbyopsis coimbrensis* with 98.2% similarity (GenBank accession: PP476847.1), while MA18 was identified to be *Nodosilinea nodulosa* UTEX 2910 with 98.2% similarity index (GenBank accession: KF307598.1),

and MA22 was 99.8% similar to *Klisinema persicum* (GenBank accession: OR426598.1). The dominant genus identified among the isolates was *Leptolyngbya sp.*, accounting for 50% of total sequences.

Following the sequencing of the 16S rRNA and BLAST analysis, the isolates were identified as *Leptolyngbya sp.* D1C10, *Leptolyngbyopsis coimbrensis*, *Nodosilinea nodulosa* and *Klisinema persicum*. These genera belong to the order Oscillatoriales, which is known for their filamentous morphology and frequent association with biofilm formation in natural and engineered environments.

Leptolyngbya sp. D1C10 is a strain within the diverse genus Leptolyngbya, a filamentous cyanobacterium, exhibiting a simple morphology typical of the Leptolyngbyaceae family, with thin trichomes ranging from 0.5 to 2 μm in width. The filaments are often straight or slightly wavy, lacking true branching or heterocysts, and may possess a thin, hyaline sheath that becomes more apparent under stress or during trichome breakage. Cells are cylindrical and divided by symmetrical binary fission. This species thrives in diverse environments, including freshwater and saline habitats, showcasing adaptability to varying conditions. This contributes to nitrogen fixation, primary productivity and the production of diverse secondary metabolites (Dutta et al., 2024). They also produce unique bioactive compounds, which are chemically diverse natural products with possible pharmaceutical applications (Dutta et al., 2024; Neupane et al., 2019). Some Leptolyngbya strains have also demonstrated high biomass and lipid productivity, making them promising candidates for biofuel and bioplastic production under optimized conditions (Kettner et al., 2022; Singh & Kumar, 2020).

Leptolyngbyopsis coimbrensis, another member of the Leptolyngbyaceae, has unbranched, thin (approximately 1-2 μm wide) and flexible, undulating structures. It is distinguished by its slightly wider cells compared to some Leptolyngbya species and may form loose mats or clusters. The sheath is typically faint or absent, and the organism is often found in terrestrial or subaerial environments, such as damp soils or stone surfaces, indicating a desiccation tolerance (Hentschke et al., 2024).

In contrast, *Nodosilinea nodulosa* is characterized by its isodiametric or slightly elongated cells, with trichomes less than 2 μm wide, and a distinctive nodular or segmented appearance due to occasional cell constrictions. The filaments lack branching and heterocysts, with a thin, sometimes absent sheath, and are commonly

found in freshwater or marine biofilms. Its morphology includes parietal thylakoids, visible under transmission electron microscopy, and it forms loose, flakelike clusters in its natural habitat (Vázquez-Martínez et al., 2018).

Klisinema persicum, a less studied species, presents thin filamentous trichomes (around 1-2 μm wide) with a smooth, unbranched structure. It lacks prominent sheaths or heterocysts, and its cells are cylindrical, dividing via binary fission. This species is adapted to terrestrial or semi-aquatic environments, often forming mats on wet surfaces, and its morphology suggests resilience to environmental fluctuations, though detailed morphological data remain limited (Heidari et al., 2018).

This molecular identification is essential to accurately interpret the ecological behavior and functional potential of the strains, particularly regarding their capacity to form stable and productive biofilms in wastewater treatment systems. These genera have been reported in the literature to contribute to surface attachment and biofilm establishment, resilience in fluctuating environments such as wastewater and to facilitate nutrient removal and biomass accumulation (Mukherjee et al., 2022; Thomas et al., 2019).

4.6 Functional Performance of Microalgal Isolates

4.6.1 Pollutant Removal Efficiency: Total Nitrogen (TN) and Total Phosphorus (TP) Analysis

The twenty-four microalgae isolates were tested for the ability to remove nitrogen and phosphorus pollutants from synthetic wastewater. Table 4.4 shows the physical observations of microalgae isolates after seven days of incubation in synthetic wastewater.

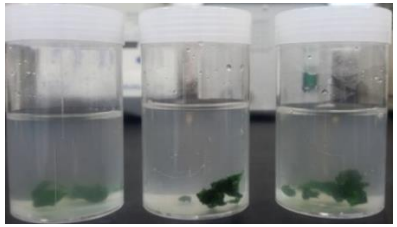
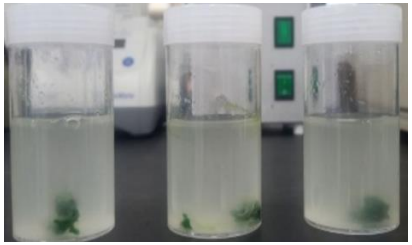
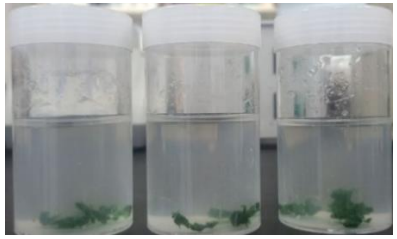
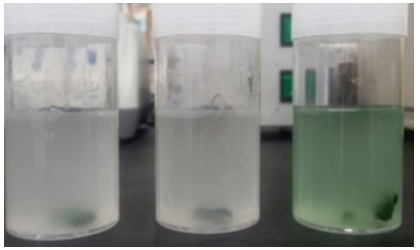
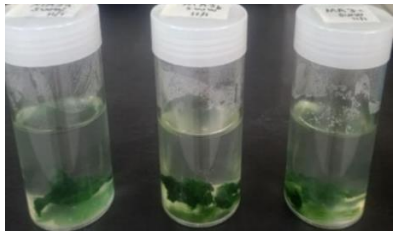
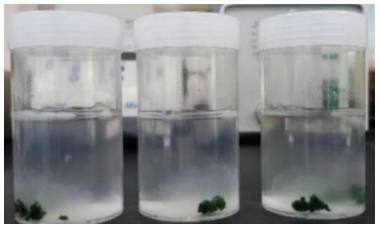
After seven days of incubation in synthetic wastewater, notable physical changes were observed across the different microalgal isolates. The microalgae isolates were observed based on the colour changes of the media, biomass growth, cloudiness or turbidity and growth of filaments (Jumiarni & Anggraini, 2021; Miranda et al., 2017). The varying levels of green coloration in the medium, ranging from pale to deep green, were observed in MA5, MA8 and MA19. This suggests various levels of chlorophyll accumulation and active photosynthetic activity among the isolates. Most of the

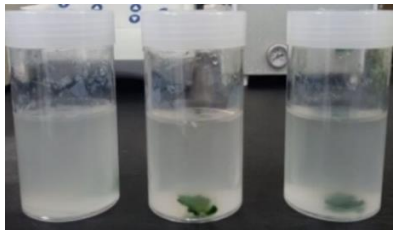
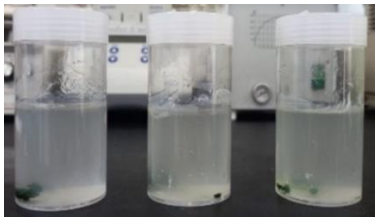
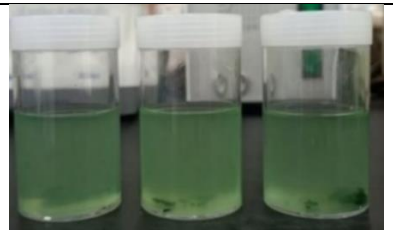
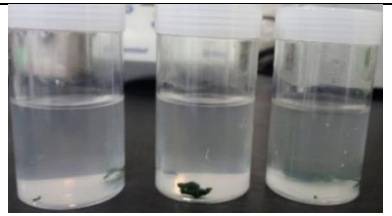
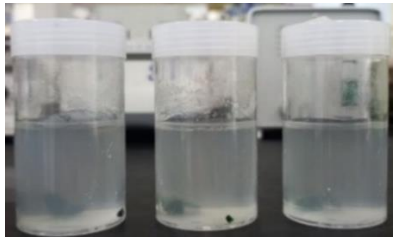
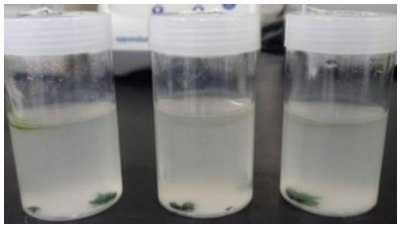
cultures' media became noticeably cloudy, indicating increased cell density due to active suspended cell growth, while others remained relatively clear, as seen in MA3 and MA16.

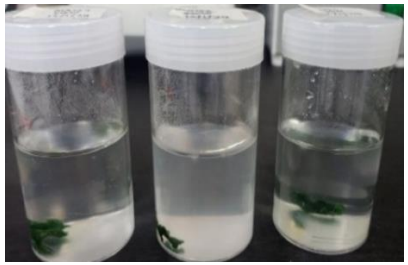
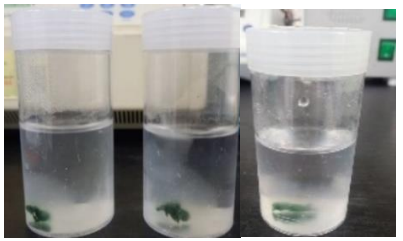
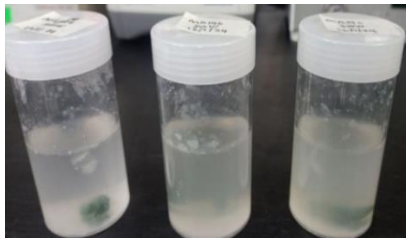
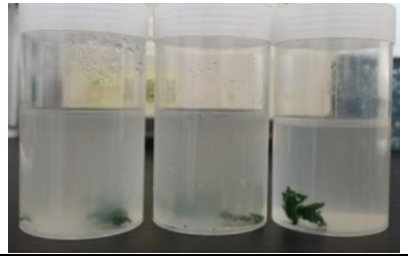
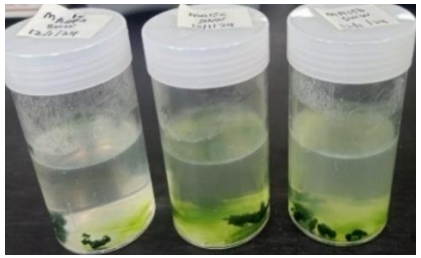
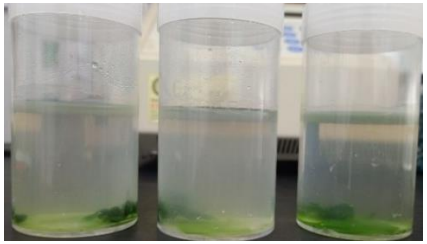
On the side of the culture bottle walls of MA4, MA10 and MA12, the formation of transparent and green films was observed. This may suggest early biofilm development. Biomass accumulation varied among the isolates, with growth either settling at the bottom or attaching to the container walls, as observed in MA3, MA15, MA18, and MA22. The attached biomass may suggest biofilm formation, and the settled biomass could reflect denser cell structures or gravity-induced settling. A few isolates formed surface pellicles, notably observed in MA6, MA7, and MA9, while occasional gas bubbles were present in MA4, indicating possible metabolic activity or gas exchange.

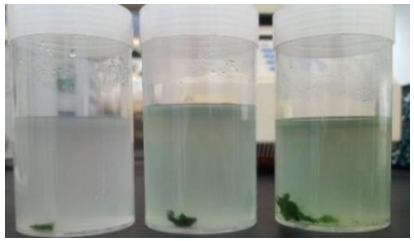
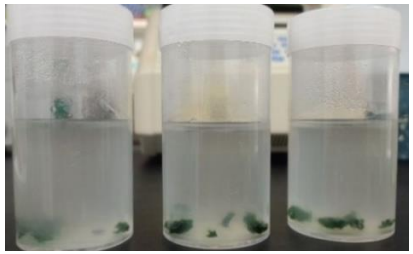
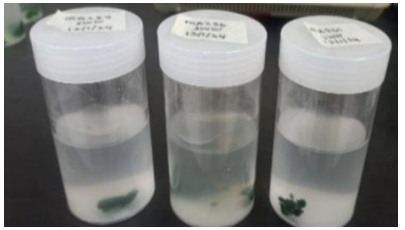
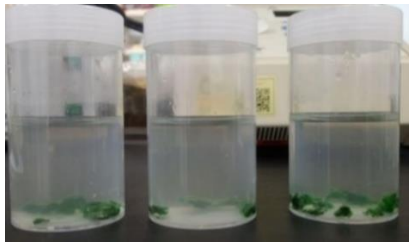
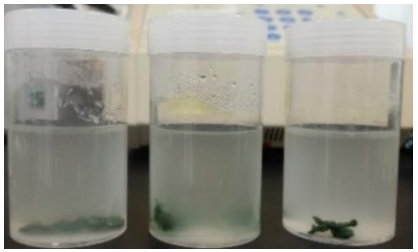
These changes suggest varying adaptation rates to the synthetic wastewater environment among the isolates, possibly influenced by differences in nutrient uptake efficiency, stress tolerance or inherent growth characteristics (Abdur Razzak et al., 2024).

Table 4.4
Comparative Observations and Characteristics of Microalgae Isolates in Synthetic Wastewater

Samples	Observation	Characteristics	Samples	Observation	Characteristics
MA1		• Cloudy media • No gas bubbles • No biomass growth • No presence of white filaments	MA4		• Cloudy media • Presence of gas bubbles • Formation of slimy green films • No biomass growth • Presence of white filaments
MA2		• Cloudy media • No gas bubbles • No biomass growth • No presence of white filaments	MA5		• Cloudy and green media • No gas bubbles • No biomass growth • Presence of white filaments • Formation of scum mats
MA3		• Clear media • No gas bubbles • Biomass growth • Presence of white filaments • Microalgae settled to the bottom of flask	MA6		• Slightly cloudy media • No gas bubbles • No biomass growth • Visible presence of white filaments • Formation of scum mats

MA7		• Cloudy media • No gas bubbles • No biomass growth • Presence of white filaments • Formation of scum mats	MA10		• Cloudy media • No gas bubbles • Formation of slimy transparent films on the surface • No biomass growth • Presence of white filaments • Formation of scum mats
MA8		• Green media • No gas bubbles • No biomass growth • No presence of white filaments	MA11		• Cloudy media • No gas bubbles • No biomass growth • No presence of white filaments
MA9		• Cloudy media • No gas bubbles • No biomass growth • Presence of white filaments • Formation of scum mats	MA12		• Cloudy media • No gas bubbles • Formation of slimy green films on the surface • No biomass growth • No presence of white filaments • Formation of scum mats

MA13		• Slightly cloudy media • No gas bubbles • No biomass growth • No presence of white filaments	MA16		• Slightly cloudy media • No gas bubbles • No biomass growth • Visible presence of white filaments
MA14		• Cloudy media • No gas bubbles • No biomass growth • Presence of white filaments • Formation of scum mats	MA17		• Cloudy media • No gas bubbles • No biomass growth • Presence of white filaments
MA15		• Slightly cloudy media • No gas bubbles • Appearance of biomass growth • Presence of white filaments • Microalgae settled to the bottom of flask	MA18		• Cloudy media • No gas bubbles • Appearance of biomass growth • Presence of white filaments • Microalgae settled to the bottom of flask • Formation of scum mats

MA19		• Cloudy to pale green media • No gas bubbles • No biomass growth • Presence of white filaments	MA22		• Cloudy media • No gas bubbles • No biomass growth • Presence of white filaments • Microalgae settled to the bottom of the flask
MA20		• Cloudy media • No gas bubbles • No biomass growth • Presence of white filaments • Formation of scum mats	MA23		• Cloudy media • No gas bubbles • No biomass growth • No presence of white filaments • Formation of scum mats
MA21		• Cloudy media • No gas bubbles • No biomass growth • No presence of white filaments	MA24		• Cloudy media • No gas bubbles • No biomass growth • Presence of white filaments • Formation of scum mats

Images adapted from experimental observations of microalgae isolates growth in synthetic wastewater.

Figure 4.7 shows the efficiency of microalgae isolates in removing TN and TP in synthetic wastewater after seven days of incubation.

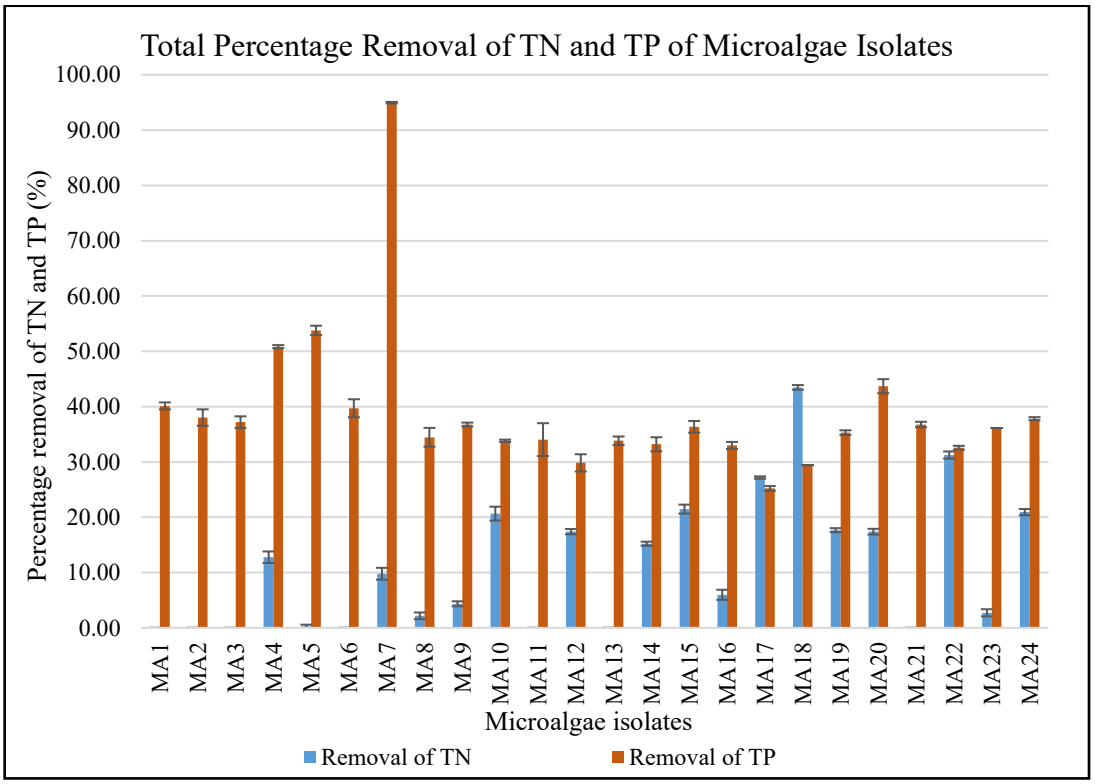


Figure 4.7 Removal of TN and TP by the Isolated Microalgae

Error bars indicate the standard error of the means.

The results highlight significant TN and TP removal efficiencies across the tested microalgal isolates, reflecting differences in their nitrogen and phosphorus uptake capabilities and adaptability to the wastewater environment. TN removal ranged from 2% to 43%. It was observed that only 16 out of 24 microalgae isolates were able to remove TN, while the remaining eight were unsuccessful. MA18 exhibited the highest TN removal efficiency at 43.5%, whereas MA1, MA2, MA3, MA5, MA6, MA11, MA13, and MA21 showed negligible or no significant TN removal. In contrast, TP removal consistently outperformed TN removal for most isolates, with several strains demonstrating exceptionally high phosphorus removal rates. The percentage of TP removal ranges between 25% to 95%. The highest TP removal was observed in MA7, with an excellent 95% removal efficiency. This is followed by MA5 and MA4 with 53.8% and 50.8%, respectively. This demonstrates an effective reduction of over half

of the TP content. The lowest removal efficiency was observed in MA17 at only 25.2% percentage removal. The majority of the isolates demonstrated moderate TP removal efficiency, ranging between 30% and 40%.

The superior TN removal observed in MA18 may be attributed to species-specific characteristics such as higher nitrogen assimilation rates and influenced by various environmental parameters, with temperature significantly affecting the growth, enzymatic activity and metabolism (Wu et al., 2024).

MA18 was identified as *Nodosilinea nodulosa* UTEX 2910, which is known for its ability in nitrogen fixation (Khomutovska et al., 2021; Zhang et al., 2019). In a study by Phyu et al. (2025), an isolate of *Nodosilinea* (strain S2), showed a total nitrogen (TN) removal efficiency in high-strength wastewater (HSW) at 38.3 ± 4.4%. Additionally, S2 showed strong biofilm-related traits, producing 1.8 µg/ml of polysaccharides and standing out among the strains for its high extracellular polymeric substances (EPS) production (13.5 µg/ml), highlighting its potential as an efficient flocculation and biofilm-forming strain. In this study, MA18 was observed to possess these traits as reflected in Table 4.2. Thus, *Nodosilinea sp.* excelled in producing EPS, which supports stable biofilm development, potentially enhancing nutrient retention and removal efficiency. These characteristics highlight its value in wastewater treatment systems through biofilm formation, despite moderate TN removal performance. The 43% TN removal by MA18 aligns closely with the 38.3% removal reported for *Nodosilinea sp.* (S2) by Phyu et al. (2025), suggesting consistent removal efficiency for this species.

When compared to existing studies, *N. nodulosa* specifically has not yet been widely reported for its nitrogen removal capabilities in wastewater treatment. However, related cyanobacteria, including *Leptolyngbya sp.* (Papadopoulos et al., 2021) and *Phormidium sp.* (Abd. Razak & Sharip, 2020) have been documented to remove nitrogen through biofilm formation and uptake mechanisms. These comparisons suggest that *N. nodulosa* may share similar nutrient assimilation traits with other filamentous cyanobacteria.

Nevertheless, several isolates in this study showed no or little TN removal ability. Microalgae usually need energy-intensive enzymatic activities for the assimilation of ammonium (NH_4^+), nitrate (NO_3^-), and nitrite (NO_2^-) to remove TN (Fallahi et al., 2021). Furthermore, factors including pH and light intensity, as well as

the availability of carbon sources, may affect nitrogen assimilation. The observed lower removal efficiencies may also be explained by the comparatively slower and more intricate nitrogen transformation routes, such as nitrification and denitrification (Yaakob et al., 2021).

In contrast, all the isolated microalgae demonstrated the ability to remove TP, indicating a broader capacity for phosphorus uptake across species. Phosphorus uptake varied based on species type, exposure duration, and treatment method, showing notable interaction effects among these factors (Patel et al., 2012). Among the isolates, MA7 demonstrated the highest TP removal efficiency at 95%, while MA4 and MA5 recorded moderate removal rates of 50.8% and 53.8%, respectively. Molecular identification revealed that both MA7 and MA4 correspond to *Leptolyngbya sp.* D1C10, while MA5 was identified as *Leptolyngbyopsis coimbrensis*. Although both MA7 and MA4 were molecularly identified as *Leptolyngbya sp.*, remarkable differences were observed in their abilities to remove TP. This variation is likely attributed to intraspecies genetic diversity, where strains within the same species may exhibit different metabolic profiles or nutrient assimilation efficiencies. *Leptolyngbya sp.* are recognized for their ability to exhibit phenotypic plasticity, allowing them to adjust their phosphorus uptake and biofilm formation in response to environmental factors such as nutrient levels, light, or temperature (Shimura et al., 2015). Additionally, differences in biofilm structure, particularly the amount and composition of EPS produced, may enhance phosphorus adsorption in one isolate over the other. Experimental factors such as initial biomass concentration or minor variations in culture conditions could also influence TP removal performance (Frau et al., 2018; Shimura et al., 2015).

Isolates MA7, MA4 and MA5 belong to the same family of filamentous cyanobacteria, Leptolyngbyaceae. Both genera, *Leptolyngbya sp.* and *Leptolyngbyopsis sp.*, exhibit subtle morphological and physiological differences that may contribute to the variation in phosphorus removal efficiency observed. *Leptolyngbya sp.* are generally characterized by cells that are usually longer than wide, while *Leptolyngbyopsis sp.* tend to have cells that are usually isodiametric and rarely longer or shorter than wide. These characteristics suggest that structural and metabolic traits inherent to each genus can influence their phosphorus uptake performance (Hentschke et al., 2024).

According to previous studies, *Leptolyngbya sp.* shows moderate to high

phosphorus removal efficiency, similar to the values observed in this study. A study by Khemka & Saraf (2015) investigated the phycoremediation of dairy wastewater using *Leptolyngbya sp.,* where the microalgae were able to remove 52.3% of TP. In another study by Patrinou et al. (2023), *Leptolyngbya sp.* were observed to remove phosphorus by 84.6% and 83.9% in discarded fruit juices wastewater. It can be concluded that *Leptolyngbya* and related genera typically achieve TP removal in the range of 50–85% as reported in previous studies. Notably, in this study, MA7 achieved a higher TP removal efficiency of 95%, indicating potentially superior performance under the tested conditions. The outstanding performance of MA7 could be due to higher EPS production or thicker biofilm formation. This provides more binding sites for phosphorus, or potentially more active phosphorus assimilation pathways. These observations highlight the importance of strain selection even within the same species for optimizing wastewater treatment performance using microalgal biofilms (Liang et al., 2025).

Isolates MA5 and MA4 demonstrated moderate TP removal rates, making them potential candidates for phosphorus-rich wastewater treatment applications. Notably, these values align with previously reported TP removal ranges for *Leptolyngbya* and related genera, which typically achieve phosphorus removal of between 50% and 85%. Although neither isolate reached the higher TP removal observed in MA7 (95%), their performance remains relevant. The ability of these isolates to consistently remove high levels of phosphorus underscores their potential for mitigating eutrophication risks in aquatic systems and recovering valuable resources from wastewater (Nishshanka et al., 2023). On the other hand, the remaining microalgae isolates showed an average TP removal rate of 25.2% to 40.1%. This reduced pollutant removal efficiency may be due to slower phosphorus absorption rates in these strains or less favourable environmental conditions that affect their activity.

Additionally, MA17 and MA22 exhibited more balanced removal performance for both TN and TP, with MA17 recording 27.2% TN and 25.2% TP removal, and MA22 showing 31.3% TN and 32.6% TP removal. These values are considered low compared to other isolates with higher single-pollutant removal rates. However, such balanced pollutant reduction is advantageous in integrated wastewater treatment systems where both nitrogen and phosphorus need to be addressed simultaneously. The

ability to remove both pollutants indicates that MA17 and MA22 likely possess metabolic traits or biofilm properties that facilitate efficient assimilation of both pollutant types. This suggests them as promising options either within multi-strain consortia or as independent agents in post-secondary wastewater treatment systems (Kumari et al., 2025). Therefore, a combined or consortia approach by combining complementary pollutant removal capacities of isolates, such as those excelling in TP (MA7) and TN (MA18), could further enhance treatment efficiency in wastewater.

Aside from the biological factors of the isolates, environmental factors such as nutrient availability, light intensity, pH and cultivation period (Li et al., 2025) likely influenced the removal performance, as these conditions directly affect microalgal metabolism and nutrient assimilation pathways.

The observed ability of all isolates to remove TN and TP suggests their potential for application in tertiary wastewater treatment processes, particularly for phosphorus management in Malaysian municipal wastewater settings where regulatory discharge limits are strict. However, it is important to acknowledge that these results were obtained under controlled laboratory conditions using synthetic wastewater, which may not fully replicate real-world scenarios.

4.6.2 Biomass Production

Next, the 24 microalgae isolates were screened for biomass production. The microalgae isolates were grown in synthetic wastewater for 7, 14, 21 and 28 days, and the biomass production was determined. The results are shown in Figures 4.8a and 4.8b.

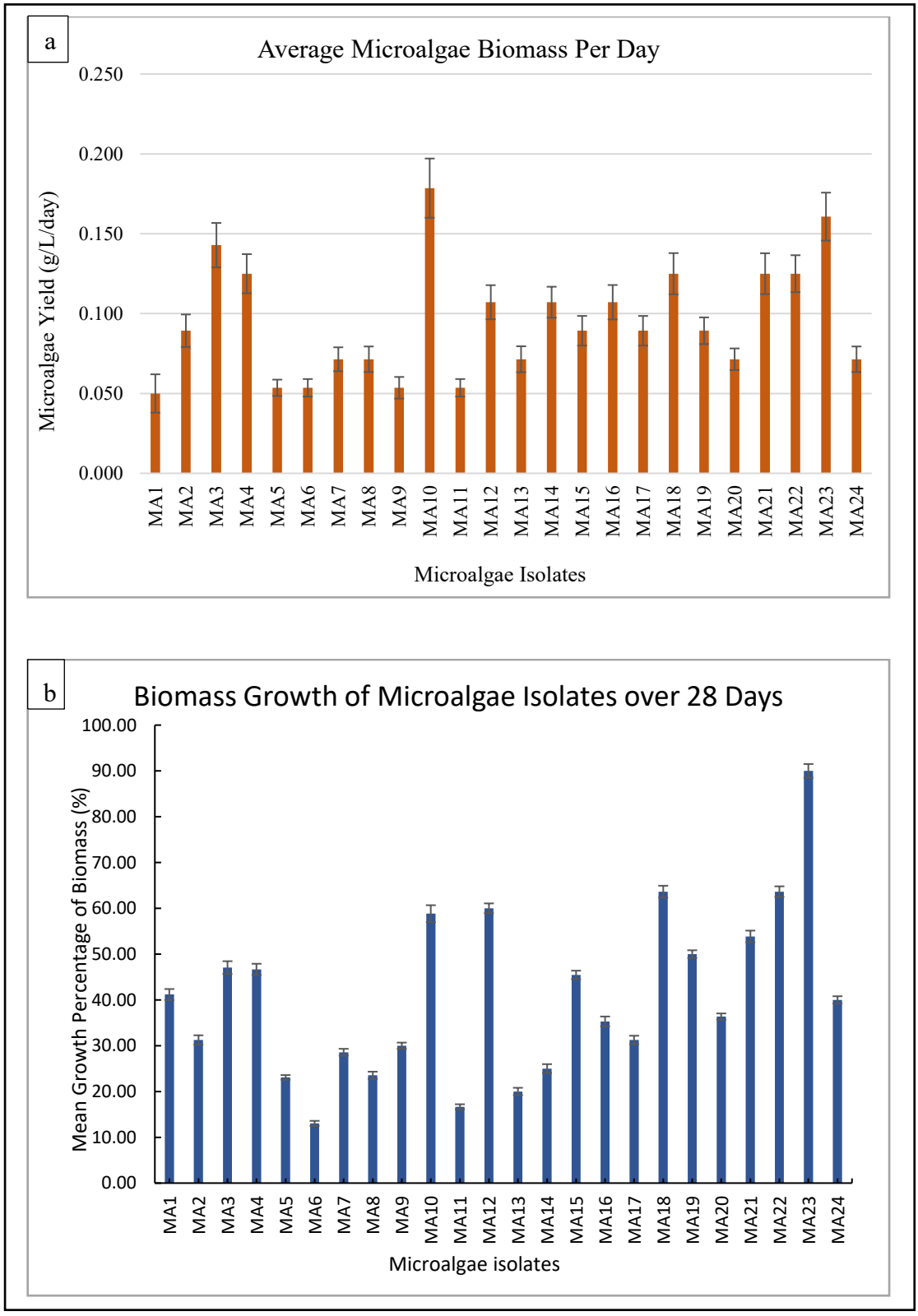


Figure 4.8 Biomass Production of the Isolated Microalgae (MA1-MA24). **(a)** Average microalgae biomass per day **(b)** Overall mean growth percentage of biomass

The graph demonstrates the growth percentages of biomass for 24 different microalgae isolates. All microalgae isolate show a varied growth pattern throughout the 28 days of incubation. Analysis of biomass production using both daily yield and total percentage growth revealed differing trends among the isolates. Based on yield per day, as shown in Figure 4.8(a), MA10 recorded the highest biomass accumulation rate of 0.179 g/L/day, indicating rapid initial growth and adaptation to the cultivation environment. However, when considering total biomass percentage after 28 days, MA23, with a total growth percentage of 90.0%, outperformed all other isolates, suggesting that it maintained steady growth over the entire cultivation period. The variation between these two metrics can be explained by differences in growth dynamics. Although MA10 achieved high productivity in the early phase, visual observations indicated that its cultures began to deteriorate after a certain period, likely due to nutrient depletion, self-shading or stress-induced aging (Chen et al., 2022; Udayan et al., 2021). This growth deterioration limited further biomass production. In contrast, MA23, although slower to establish growth, sustained biomass production throughout the cultivation period, resulting in the highest overall biomass yield. From an application perspective, MA10 may be better suited for short-term, high-output batch cultivation, whereas MA23 would be advantageous for long-term production cycles where sustained growth is desired.

When aligning these findings with the objectives of wastewater treatment, biomass recovery and subsequent lipid production, long-term growth stability is more advantageous. This is because prolonged nutrient uptake is essential for effective wastewater remediation, and sustained biomass productivity ensures optimal feedstock quality for lipid extraction (Arrojo et al., 2022). Therefore, MA23, MA18 and MA22 appear to be the most suitable candidates for integrated wastewater treatment–biomass–lipid production systems, while MA10, MA23 and MA3 remain valuable for rapid, short-term biomass harvesting in targeted applications.

The overall biomass growth of the microalgae isolates is shown in Figure 4.8(b), which illustrates the percentage increase in biomass over the 28-day cultivation period for each isolate. The biomass growth of all microalgal isolates demonstrated considerable variation, ranging from 10% to 90%. Notably, seven isolates, including MA10, MA12, MA18, MA19, MA21, MA22 and MA23, exhibited biomass growth

exceeding 50%. The highest biomass growth was observed in MA23, which achieved a 90% increase, while MA6 recorded the lowest growth at 13%. The high peaks in biomass growth were observed for isolates MA23, MA22 and MA18, with growth percentages exceeding 60% by day 28. Both MA18 and MA22 exhibited 63.6% biomass growth percentage more than the initial growth. In contrast, isolates MA5, MA6, MA8, MA13 and MA14 show relatively lower growth, with percentages of 23.1%, 13%, 23.5%, 21% and 25%, respectively. Overall, all isolates demonstrated gradual biomass increase over time, although the growth rates and final biomass yields varied among isolates.

While all isolates are capable of growing and producing biomass, some show more robust and faster growth than others. This likely relates to species-specific traits, genetic differences, and the adaptability of each strain to factors like light exposure and nutrient availability. The varying growth patterns also suggest certain isolates have more efficient photosynthetic, nutrient uptake and metabolism, making them better suited for high biomass production in synthetic wastewater conditions.

Molecular identification revealed that MA23 was *Leptolyngbya sp. Leptolyngbya sp.*, a genus of filamentous cyanobacteria, which is recognized for its ability to produce substantial biomass across diverse cultivation conditions (Lee et al., 2024). As mentioned in 4.5.1, *Leptolyngbya sp.* are known for their flexible traits that enable them to adapt based on changing environmental conditions like nutrient availability, light intensity or temperature (Shimura et al., 2015). This aligns with previous reports that highlight *Leptolyngbya*'s robust growth capabilities. According to earlier studies by Patsialou et al. (2024), *Leptolyngbya sp.* demonstrated biomass productivity ranging from 55 to 234.8 mg L^{-1} day^{-1} in batch and semi-batch bioreactors. Similarly, Gongi et al. (2022) reported *Leptolyngbya sp.* generated a notable quantity of 1.35 g L^{-1} of biomass and 2.15 g L^{-1} of extracellular polymeric substances (EPS) after nine days of cultivation. Lee et al. (2024) further demonstrated *Leptolyngbya sp.* KIOST-1 achieved high protein content exceeding 66% of the dry weight while maintaining stable growth in large-scale outdoor systems. Additionally, Assobhi et al. (2024) reported *Leptolyngbya gelatinosa* reached up to 4 g/L of dry biomass concentration in optimized conditions. These findings show that *Leptolyngbya sp.* exhibits strong growth potential and substantial EPS production under controlled

conditions. This highlights its adaptability and potential for biomass production and industrial applications such as wastewater treatment or biofuel.

In addition, MA22 and MA18 isolates were both observed to possess a moderate ability to grow biomass in synthetic wastewater. In this study, MA22 was molecularly identified as *Klisinema persicum*. Since the biomass production of *K. persicum* has not been extensively studied in the literature to date, this observation may be a potentially novel contribution to understanding its growth potential. While direct comparisons are unavailable, other filamentous cyanobacteria, such as *Leptolyngbya* and *Nodosilinea* have been reported to produce equivalent or greater biomass yields under similar conditions (Aravindh et al., 2023; Gongi et al., 2022). This suggests that *K. persicum* may share functional similarities with related taxa in terms of biomass production capacity. In contrast, MA18 was identified as *N. nodulosa.* A study by Aravindh et al. (2023) reported that *N. nodulosa* can produce high biomass concentrations, suitable for biodiesel applications. Among 90 marine microalgal isolates tested, *N. nodulosa* SNMVBTAR001 achieved one of the highest biomass yields at 294 ± 2.0 mg L^{-1}. The study also noted that this strain showed a high lipid content of 59%, further supporting its potential for biodiesel production. Another study conducted by Yadav et al. (2021) observed a maximum dry biomass production of *N. nodulosa* DS_1B_I1 at 0.52 g L^{-1} under pH 7, which is found to be consistent with the present study that also recorded substantial biomass growth (63.6% increase from initial weight) for *N. nodulosa,* MA18, under similar pH conditions. This suggests that pH 7 may be an optimal growth condition for *N. nodulosa*, supporting both high biomass yield and growth efficiency across different strains and experimental setups.

The high yield of biomass by MA23 highlights its exceptional potential for industrial-scale applications. High biomass production translates to effective nutrient removal, contributing to environmental sustainability (Lee et al., 2024; Nishshanka et al., 2023). The ability of seven isolates to achieve more than 50% biomass growth emphasizes the strength of local isolates in adapting to synthetic wastewater conditions. High-growth isolates like MA23, MA22 and MA18 could be prioritized for bioresource recovery, wastewater treatment, or biofuel production due to their superior biomass yields. MA23 and other productive isolates may help to close the loop in wastewater management by recovering valuable resources while treating pollutants. In the future,

culture conditions such as light intensity, nutrient composition and temperature can be considered to be optimized to enhance the productivity of these isolates further (Lee et al., 2024; Nishshanka et al., 2023).

In contrast, isolates such as MA6 and MA11 exhibit significantly lower growth percentages. This may indicate possible limitations in nutrient utilization or sensitivity to environmental stressors. Their genetic or physiological limitations could also be studied to boost their growth.

These findings in this study reveal notable differences in growth capacity between isolates, which emphasizes the need to select appropriate strains tailored to specific uses.

4.6.3 Lipid Production and Fatty Acid Methyl Esters (FAMEs) Profiling

The lipid production from the 24 microalgal isolates was assessed using FAME analysis on a GCMS. The FAME standard reference chromatogram is presented in Figure 4.9 and Table 4.5.

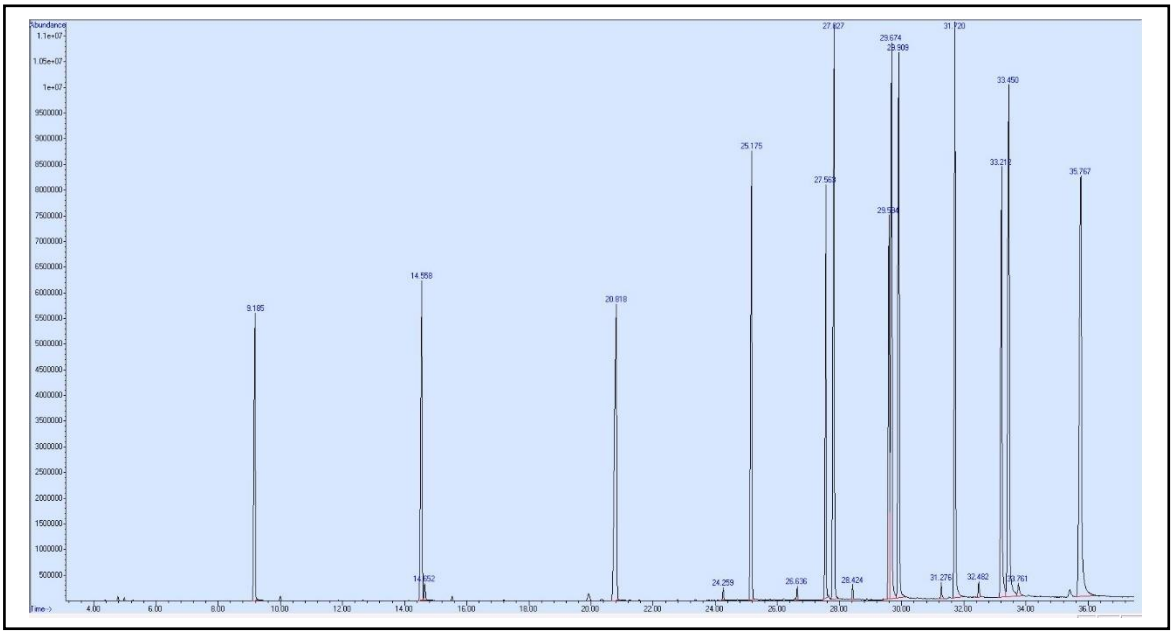


Figure 4.9 The Chromatogram of FAME Standards

Table 4.5
Identified FAMEs in Standard Mix Based on GC-MS Analysis

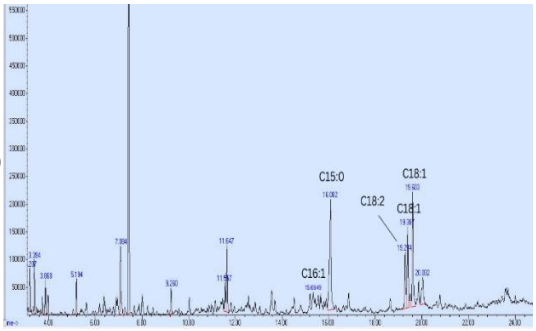
PK	RT	Library/ID

1	9.0942	Octanoic acid, methyl ester
2	14.4376	Decanoic acid, methyl ester
3	20.6254	Dodecanoic acid, methyl ester
4	25.0713	Methyl tetradecanoate
5	27.4821	9-Hexadecenoic acid, methyl ester, (Z)-
6	27.7307	Hexadecanoic acid, methyl ester
7	29.5169	11,14-Octadecadienoic acid, methyl ester
8	29.5801	9,12,15-Octadecatrienoic acid, methyl ester
9	29.8204	Methyl stearate
10	31.6352	Eicosanoic acid, methyl ester
11	33.1232	13-Docosenoic acid, methyl ester, (Z)-
12	33.3439	Docosanoic acid, methyl ester
13	35.6037	Tetracosanoic acid, methyl ester

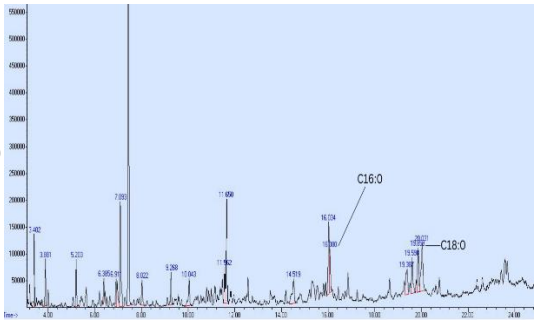
The compounds were identified using the NIST mass spectral library, with match quality indicating the degree of similarity between the sample spectra and reference spectra. This table serves as a reference for comparing FAMEs in microalgal lipid extracts.

The chromatogram of the FAMEs standard shown in Figure 4.9 revealed a clear series of sharp peaks corresponding to known methyl esters of fatty acids. Table 4.5 shows a total of 13 peaks were identified, with retention times ranging from 9.09 to 35.60 minutes. These included key biodiesel-relevant compounds such as methyl esters of octanoic acid, decanoic acid, dodecanoic acid, hexadecanoic acid (palmitic acid), stearic acid, and eicosanoic acid. The high match quality values (96–99%) confirm accurate identification against the library. This standard profile serves as a reference for identifying FAMEs in microalgal biodiesel extracts. The presence of a range of saturated and unsaturated fatty acid methyl esters is essential for evaluating fuel quality, with compounds such as hexadecanoic and octadecenoic acid methyl esters commonly contributing to optimal cetane number, oxidative stability, and cold flow properties in biodiesel. The results of lipid production from the microalgae isolates are summarised in Figure 4.10.

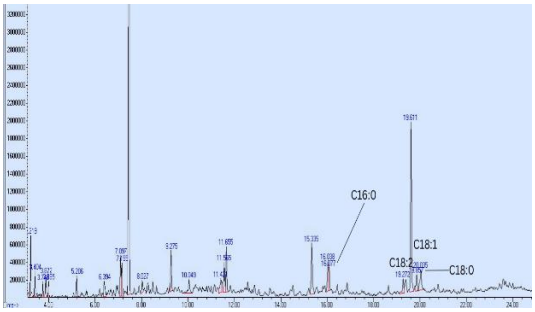
A)


C15:0
C18:1
C18:2
C18:1
C16:1

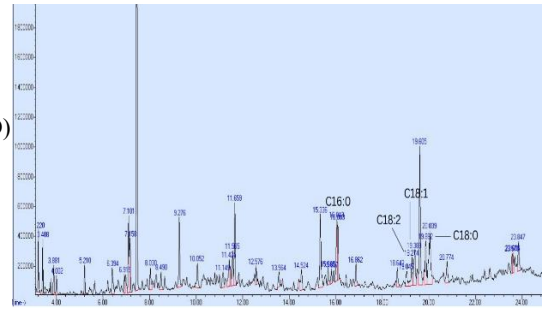
B)


C16:0
C18:0

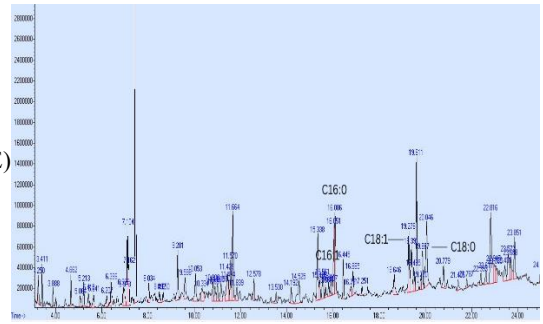
C)


C16:0
C18:1
C18:2
C18:0

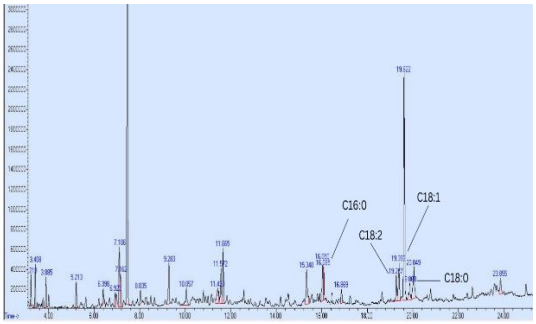
D)


C16:0
C18:1
C18:2
C18:0

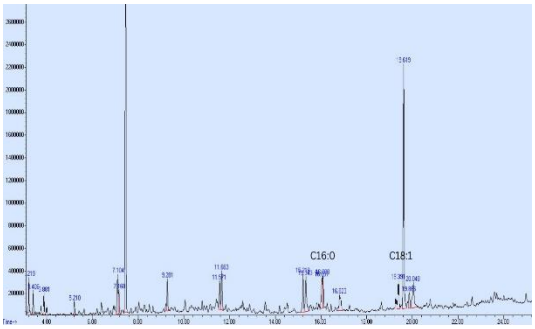
E)


C16:0
C18:1
C18:0
C16:1

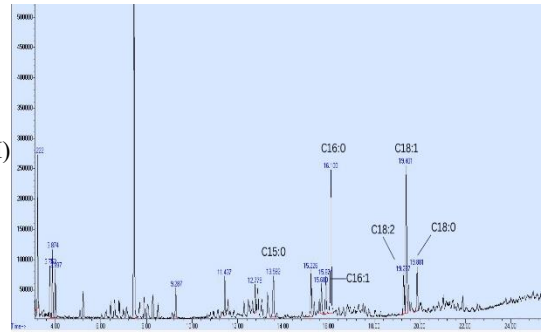
F)


C16:0
C18:1
C18:2
C18:0

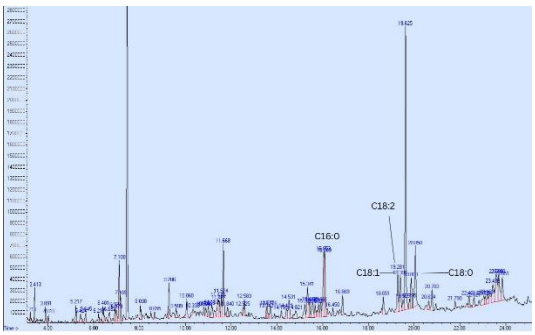
G)


C16:0
C18:1

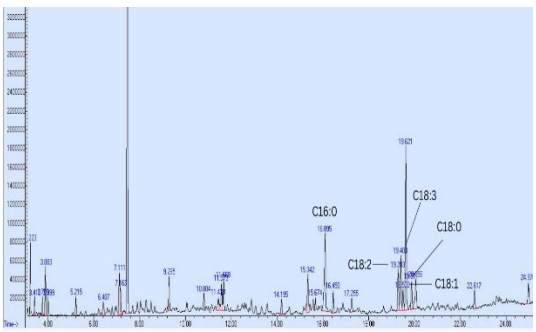
H)


C16:0
C18:1
C18:2
C18:0
C15:0
C16:1

I)


C18:2
C16:0
C18:1
C18:0

J)


C18:3
C16:0
C18:0
C18:2
C18:1

K)

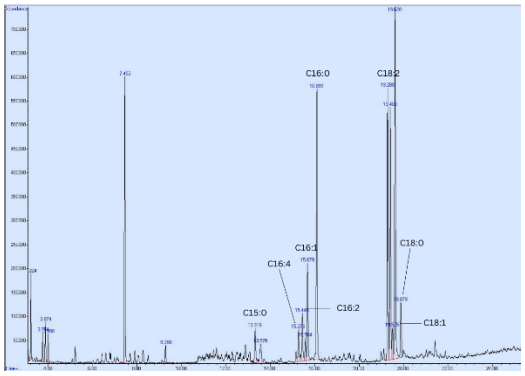


Figure 4.10 GC-MS Chromatograms of Fatty Acid Methyl Esters (FAMEs) Obtained From Different Microalgal Samples

A) MA2, B) MA4, C) MA7, D) MA9, E) MA10, F) MA12, G) MA13, H) MA14, I) MA15, J) MA17 and K) MA23. Chromatograms are presented with labelled major fatty acid methyl esters (C15–C18) according to carbon chain length and degree of unsaturation.

This preliminary screening aimed to assess the lipid-producing potential of microalgal isolates by identifying the presence and types of lipids using GC, without quantifying total lipid content. The chromatographic analysis of FAMEs from individual microalgal isolates revealed that 11 out of 24 tested samples produced detectable amounts of lipids, as summarized in Figure 4.10. These lipids were identified based on retention time comparisons with known standards and library matches, focusing particularly on the most important fatty acid compounds in the production of biofuels such as methyl esters of palmitic acid (hexadecanoic acid), stearic acid (octadecanoic acid) and oleic acid (9-octadecenoic acid).

The majority of detected fatty acids were within the C16–C18 carbon range, consistent with previously reported lipid profiles of microalgae and is considered optimal for biodiesel production. The lipid profiles comprised a mixture of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA), indicating metabolic diversity among the isolates.

Among the isolates, MA23 displayed the widest range of FAME types, with the highest diversity of fatty acid methyl esters, encompassing multiple carbon chain lengths and degrees of saturation. This indicates their strong potential as biofuel-producing strains, encompassing saturated (C16:0, C18:0), monounsaturated (C16:1, C18:1), and polyunsaturated fatty acids (C16:2, C16:4, C18:2). The presence of both C16 and C18 carbon chain lengths, together with varying degrees of unsaturation,

indicates a metabolically versatile lipid biosynthesis pathway. This compositional diversity is advantageous for biofuel applications, as saturated and monounsaturated fatty acids contribute to improved cetane number and oxidative stability, while polyunsaturated fatty acids enhance cold-flow properties (Kim et al., 2024). Therefore, MA23 demonstrates strong potential as a biofuel-producing strain due to its balanced and diverse FAME profiles.

The lipid profiles were characterised by a predominance of C16 and C18 fatty acids, which are widely recognised as optimal for biodiesel production due to their favourable cetane number, oxidative stability, and cold-flow behaviour (Knothe, 2014). Methyl palmitate (hexadecanoic acid methyl ester) was consistently identified in all isolates, excluding MA2 and MA15, indicating its common occurrence as a fundamental constituent of the microalgal lipid profile. Similarly, methyl stearate (octadecanoic acid methyl ester) was repeatedly detected in the majority of the isolates, except for MA4, highlighting its prevalence among the selected strains. Notably, the dominance of monounsaturated methyl esters, particularly 9-octadecenoic acid (C18:1), highlights strong biodiesel suitability, as monounsaturated fatty acids provide an effective balance between oxidative stability and fuel fluidity (Ikechukwu et al., 2022). Furthermore, the detection of C18 fatty acids such as methyl stearate (C18:0) and 9,12-octadecadienoic acid (C18:2) indicates the formation of biofuel-relevant lipid profiles, with polyunsaturated components contributing to improved cold-flow properties and combustion efficiency (Knothe, 2014; Paschou et al., 2025). Unsaturated fatty acids contribute positively to biodiesel quality by improving cold-flow properties and combustion efficiency. The presence of double bonds introduces structural kinks that reduce molecular packing, resulting in lower melting points and better fuel fluidity at low temperatures. In addition, their higher chemical reactivity supports improved atomization and more complete combustion (Folayan et al., 2019). However, excessive levels of unsaturated fatty acids may reduce oxidative stability and increase susceptibility to fuel degradation during storage (Huang et al., 2021). Therefore, a balanced proportion of saturated and unsaturated FAMEs is generally preferred for optimal fuel performance.

The identification of key saturated and unsaturated FAMEs supports the suitability of these microalgal strains for biodiesel feedstock (Ravanipour et al., 2021).

Particularly, strains producing both saturated and monounsaturated FAMEs may yield a balanced biodiesel composition that is both stable and efficient under various climatic conditions (Tian et al., 2022). Moreover, the high occurrence of C16–C18 chain lengths is aligned with European biodiesel standards (EN 14214) and US ASTM D6751 requirements, which further supports the biofuel potential of these isolates (Ravanipour et al., 2021). The moderate presence of polyunsaturated compounds also indicates the possibility of using these microalgae for co-products in nutraceutical or pharmaceutical applications, depending on downstream processing preferences (Qin et al., 2023).

The lipid screening of local microalgal isolates in this study revealed the presence of several key fatty acid methyl esters, indicating the potential of these strains for biodiesel production. Among the identified compounds were methyl hexadecanoate (C16:0), methyl hexadeca-9-enoate (C16:1), methyl octadecanoate (C18:0) and methyl 9,11-trans-octadecadienoate (C18:2 isomers), which are commonly reported in biodiesel-producing microalgae. The presence of saturated and monounsaturated FAMEs suggests favorable fuel properties, such as higher cetane numbers, improved oxidative stability and better cold flow behavior (Awogbemi et al., 2021; Wang et al., 2024). Although polyunsaturated compounds such as methyl 4,7,10,13-hexadecatetraenoate and methyl 9,11-trans-octadecadienoate were also detected, which may negatively impact oxidation stability, their proportions were relatively low compared to C16 and C18 chains (Veillette et al., 2017).

This result aligns with the findings of Enwereuzoh et al. (2020), who investigated *Tetradesmus obliquus*, *Heterochlorella luteoviridis*, and *Chlamydomonas reinhardtii* cultivated in both unmodified and modified fish farm wastewater (FFW). In their study, FFW supported the accumulation of desirable C16–C18 fatty acids, comprising up to 95% of total FAMEs, with over 60% unsaturated fatty acids and more than 22% saturated fatty acids in all samples. Furthermore, biodiesel produced from microalgae cultivated in FFW exhibited fuel properties comparable to those grown in standard modified tris-acetate-phosphate (TAP) media and met the ASTM D6751-02 and EN 14214 standards. This supports the current results, emphasizing the role of nutrient-rich media in enhancing the production of high-value lipids and further affirming the suitability of locally isolated strains for sustainable biofuel production.

Additionally, some uncommon lipids were detected in isolates, including

pentadecanoic acid (C15:0), methyl hexadeca-9-senoate (C16:1), methyl 4,7,10,13-hexadecatetraenoate (C16:4) and methyl 9,11-trans-octadecadienoate (C18:2). Pentadecanoic acid (C15:0), a saturated odd-chain fatty acid, contributes to oxidative stability but is less commonly emphasized in biodiesel feedstocks due to its lower abundance and reduced cold-flow performance. On the other hand, methyl hexadeca-9-enoate (C16:1) was particularly relevant due to its monounsaturated structure, which supports both fuel stability and favorable cold-flow properties, making it a valuable component in biodiesel formulation. Methyl 4,7,10,13-hexadecatetraenoate (C16:4) and methyl 9,11-trans-octadecadienoate (C18:2), both detected in MA23, may contribute to broader fuel properties and hint at polyunsaturated fatty acid (PUFA) metabolism. However, C16:4 has poor oxidative stability, limiting its suitability for large-scale fuel production, while C18:2 is better known for its nutraceutical value than for fuel application, with limited benefits for biodiesel quality (Monika et al., 2023; Paschou et al., 2025). Their presence reflects metabolic diversity among isolates, though more stable saturated and monounsaturated FAMEs remain preferable for high-quality biodiesel. The differences in lipid profiles could be attributed to species-specific lipid metabolism, environmental tolerance or the inherent variation in desaturase and elongase enzyme activity among the microalgal isolates (Morales et al., 2021; Qin et al., 2023).

However, several isolates did not exhibit detectable levels of FAMEs when subjected to gas chromatography. Despite the presence of chromatographic peaks, none of these peaks corresponded to the retention times or mass spectra of the standard FAME compounds. This suggests that the isolates either lack significant lipid accumulation or produce lipid profiles that do not match conventional methyl esters used for biodiesel applications. This could be due to inconducive growth environment for lipid production, low lipid content that falls below the detection limit of the GC, predominance of non-methylated or structurally different lipids such as hydrocarbons, alcohols or complex polar lipids not targeted in the standard FAME profiling or inefficient transesterification, possibly due to high water content, cell wall resistance or improper reaction conditions during lipid extraction and methylation (Paschou et al., 2025; Ravanipour et al., 2021).

Overall, the lipid profiling results demonstrate that microalgal isolates and

consortia predominantly produce biofuel-relevant C16–C18 fatty acids, with MA23 exhibiting notable compositional diversity. These findings indicate that selected microalgal strains can be suitable for lipid production, highlighting the importance of strain screening and optimization for lipid-rich phenotypes.

4.7 Microalgal-Consortium Formation and Performance Evaluation

4.7.1 Microalgal-Microalgal Consortium

Based on the results of this study, a total of ten microalgal consortia were customised with the objective to improve performance in producing biomass, efficiently removing TN, TP and subsequently, lipid production. These microalgal consortia were developed with different combinations of selected microalgae isolates, as shown in Tables 4.6 and 4.7, and were then tested for their efficiency when cultured together.

Table 4.6
Selected Microalgae Strains Based on Their Overall Performance

Isolates	TN removal (%)	TP removal (%)	Biomass (%)	Capacity
MA18	**43.5***	29.4	63.6	Highest reduction of TN
MA7	9.8	**95.0***	28.6	Highest reduction of TP
MA23	2.7	36.1	**90.0***	Highest biomass production
MA4	12.8	50.8	46.7	Good reduction of TP
MA5	0.3	53.8	23.1	Good reduction of TP
MA12	17.4	29.8	60.0	Good biomass production
MA19	17.7	35.3	50.0	Good biomass production
MA22	21.3	32.6	63.6	Good biomass production

*= best performing isolates

Table 4.7
Consortia Formation Based on the Isolates' Ability to Reduce Pollutants (TN, TP) and Biomass Production

Consortia	TN	TP	Biomass
1	MA18	MA7	MA23
2	MA18	MA7	MA12
3	MA18	MA7	MA19
4	MA18	MA7	MA22
5	MA18	MA5	MA12
6	MA18	MA5	MA19

7	MA18	MA5	MA22
8	MA18	MA4	MA12
9	MA18	MA4	MA19
10	MA18	MA4	MA22

The consortia combinations were arranged by grouping selected microalgal strains based on their pollutant removal performance, biomass production and lipid profiling. The purpose of combining these strains was to encourage synergistic interactions, where the microorganisms can support each other and form a more stable biofilm. Mixed-strain consortia were used instead of single strains because they are generally more robust and better able to tolerate changes in environmental and nutrient conditions (Bian et al., 2023). Overall, the consortia were designed to improve biofilm development and treatment performance rather than simply to increase species diversity.

The ten formulated consortia varied in their combinations of TP reducers and biomass producers while maintaining MA18 as the TN remover. This design allowed a comparative analysis of how different TP- and biomass-focused isolates influenced overall performance in a consortium.

MA18 was selected as the core strain across all consortia due to its being the only isolate superior in TN removal ability. MA7, which demonstrated excellent TP removal efficiency, was incorporated into Consortia 1 to 4, while MA23 was included in Consortia 1 due to its high biomass productivity. In subsequent consortia, moderately performing isolates were included in various configurations to investigate potential synergistic interactions.

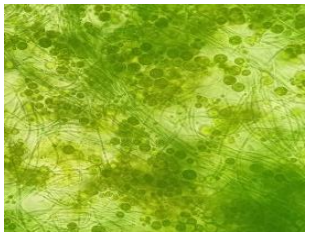
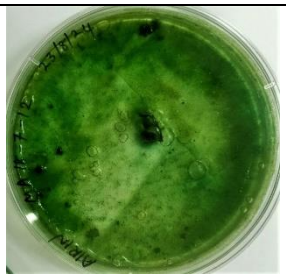
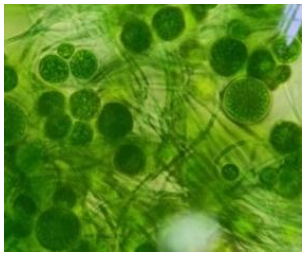
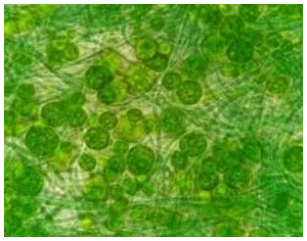
This study also included MA5 and MA4 (moderate TP reducers), as well as MA12, MA19, and MA22 (moderate biomass producers). Moderately functional isolates were deliberately included in the consortia to simulate natural microbial diversity and exploit potential synergistic effects. In complex communities, such strains often provide critical support functions such as extracellular matrix production, quorum sensing modulation, or synergistic metabolic activity that cannot be observed in monoculture assays (Abu Hasan et al., 2021; Liu & Hong, 2021). Incorporating this diversity within the consortium may also offer complementary traits, such as enhanced tolerance to environmental fluctuations, contributing to increased ecological stability and long-term performance under wastewater conditions. Other than that, some

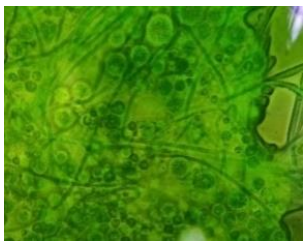
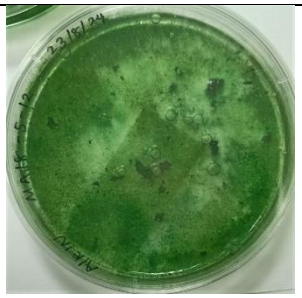
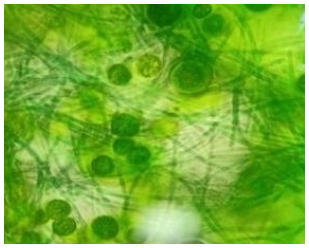
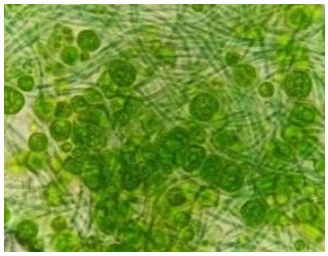
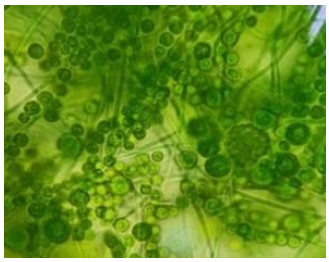
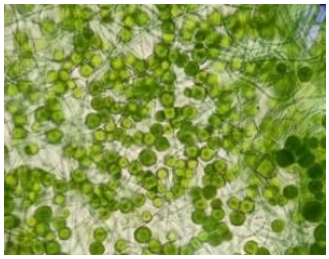
moderately efficient strains may contribute structural or adhesive properties, supporting biofilm stability and nutrient retention (Liang et al., 2022; Thomas et al., 2019).

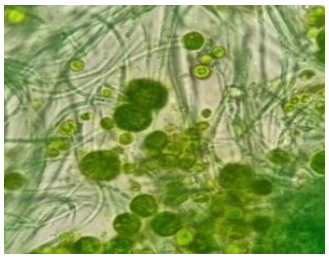
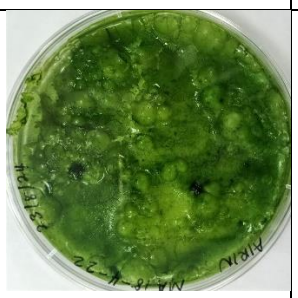
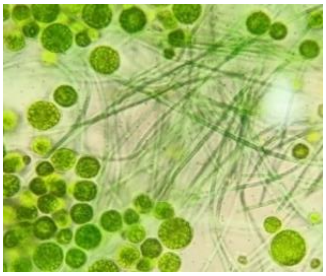
Consortia 1–4 featured MA7, which is a highly effective TP reducer, paired with various biomass producers, MA23, MA12, MA19 and MA22, to test the optimal combination for high pollutant removal and productivity. On the other hand, consortia 5–7 included MA5 (moderate TP removal), which offered a test of whether slightly lower individual performance could still yield strong consortia outcomes when combined with different biomass producers. In addition, consortia 8–10 featured MA4, another moderate TP reducer, in combination with moderate biomass-producing strains.

These combinations help to assess potential synergistic or antagonistic effects in co-culture environments, particularly when treating post-treatment wastewater, where functional diversity is advantageous (Zhang et al., 2020).

Table 4.8
The Consortia Formed from Different Combinations of Microalgae Isolates After 28 Days of Cultivation

Consortia	Sample	Macroscopic	Microscopic
1	MA18-MA7-MA23		
2	MA18-MA7-MA12		
3	MA18-MA7-MA19		

4	MA18-MA7-MA22		
5	MA18-MA5-MA12		
6	MA18-MA5-MA19		
7	MA18-MA5-MA22		
8	MA18-MA4-MA12		

9	MA18-MA4-MA19		
10	MA18-MA4-MA22		

Macroscopic and microscopic views of different microalgal consortia.
All consortia formed visible biofilms with varying structural integrity and pigmentation.
Microscopic images reveal differences in cell morphology, density, and extracellular polymeric substance distribution.

Table 4.8 displays the macroscopic and microscopic observations of consortia formed by different combinations of microalgae isolates. All consortia incorporated MA18 as the primary isolate, in combination with varied secondary isolates such as MA7, MA5 and MA4, and a range of tertiary isolates, namely MA12, MA19, MA22 and MA23. Good biomass density at the screening stage was evaluated based on visual macroscopic and microscopic indicators rather than quantitative measurements. These included the extent of surface coverage, apparent biofilm thickness, biomass coloration intensity, and uniformity of growth. Microscopic criteria included cell or filament abundance, structural compactness, and attachment strength (Winklbauer et al., 2025). Combinations exhibiting denser and more continuous biomass structure were classified as having higher biomass density.

In general, all microalgal consortia exhibited dense and uniform surface growth, characterized by a deep green pigmentation indicative of healthy and active proliferation. The top view of each cultivated consortium showed near-complete surface coverage with minimal clear zones, suggesting successful colonization and robust biofilm development across the substrate.

The macroscopic evaluation of Consortium 1 revealed a thick, dark green biofilm with an uneven surface. The darker pigmentation suggested higher chlorophyll

content and likely greater photosynthetic activity, aligning with potential for higher biomass and nutrient uptake (Saini et al., 2023). Microscopically, this combination showed a diverse population of cell morphologies, including filamentous structures, along with unicellular and colonial clusters. The mixed morphology contributed to a well-integrated biofilm network, with observable EPS-like matrix between cells, suggesting enhanced adhesion and inter-species interaction.

Next, Consortium 2 exhibited a smooth green film on the medium surface. Microscopic observation shows large spherical cells dominating the structure, with some visible clumping but moderate EPS presence. Although still forming a complete surface layer, it showed less biomass density. The presence of large spherical cells and moderate EPS indicates partial compatibility, possibly due to less cooperative interactions or a slower growth rate of MA12.

Additionally, Consortium 3 displayed a diffuse biofilm with patchy surface coverage, produced a minimal cohesive biofilm, with lower pigmentation and more irregular coverage. Under the microscope, smaller cells with lower EPS and a more open structure were observed. The microscopic image confirmed weaker structural integrity and lower EPS, which may result in reduced biofilm resilience and nutrient absorption capacity. However, MA19 may still play a role in enhancing productivity or targeting specific nutrients.

In contrast, Consortium 4 exhibited dense, well-developed green biomass with a rich filamentous structure and uniform surface coverage. The deep green, cohesive layer was visible, indicating strong biofilm formation (Bozan et al., 2024). Microscopically, the sample showed high cell density with chloroplast-rich cells, including both filamentous and unicellular forms. The observed clear cell clustering and layering suggested substantial EPS production, supporting structural integrity. Most cells appeared intact and metabolically active, indicating good physiological compatibility (Cheah & Chan, 2021).

On the other hand, Consortium 5 formed a moderately dense, green, and semi-uniform biofilm with some patchiness. The biomass is thinner and less cohesive. Microscopically, it showed a moderate cell density, mainly rounded cells with minor filamentous forms. Despite moderate synergy and cohesion, its adaptive surface attachment and ease of harvesting suggest potential for mid-tier biofilm-based

wastewater treatment applications.

Consortium 6 is observed to be rich in green color with moderate surface biomass. They were somewhat patchy but evenly spread. Under the microscope, the consortium appeared as well-formed filaments and rounded cells, indicating a healthy consortium interaction (Cheah & Chan, 2021). The dense and evenly spread green layer observed suggests that this isolate exhibits a strong ability to form cohesive biofilms, which is a key trait for immobilized microalgal systems (Mantzorou & Ververidis, 2019).

Furthermore, Consortium 7 formed thick green biomass with a biofilm-like structure. It is a highly compact, dense matrix with uniform cell distribution. This may indicate strong synergistic interaction between the cultivated isolates within the consortium. The consortium likely benefited from spatial complementarity as the cells occupied different physical spaces or niches within the biofilm. The vibrant pigmentation also signified a high chlorophyll content, often associated with active photosynthesis and robust metabolic activity (Mantzorou & Ververidis, 2019).

Consortium 8 is observed to form deep green biomass, but with thinner patches and less dense surface coverage. When observed under the microscope, the cells were seen to be dispersed with some aggregates and a moderate presence of filamentous forms. This indicates weaker interaction among the strains. They may have competed for similar resources. The lack of structural integrity may have hindered biomass development.

In addition, Consortium 9 formed a medium to dense green biofilm with visible patchiness. The consortium exhibited healthy cell morphology, with both rounded cells and filamentous features. The presence of filamentous structures indicates improved cohesion and possibly better nutrient sharing.

Visual observation of Consortium 10 showed dark green biomass, visibly structured and thick. Under the microscope, it is observed to be highly organized with diverse microalgae morphology, such as filaments and chlorophyll-rich spherical cells. The consortium was denser than most other consortia, indicating robust biomass accumulation. This may be due to the strong interactions and compatibility of the isolates when grown together.

Based on Table 4.8, the consortia demonstrating the highest visual biomass

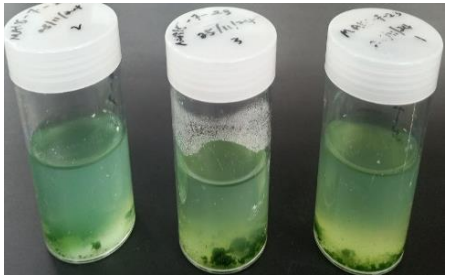
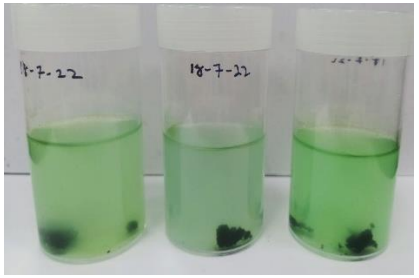
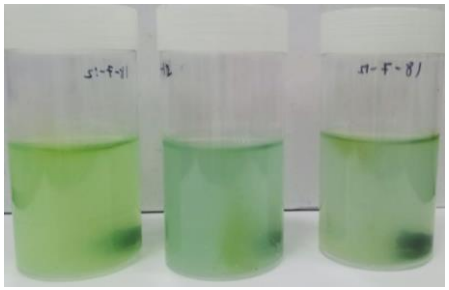
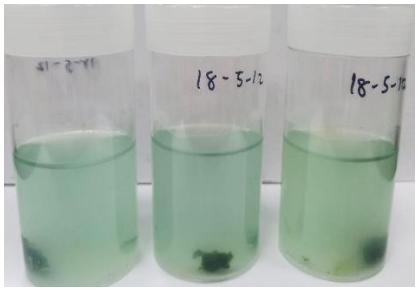
density were Consortium 2 (MA18–MA7–MA12), Consortium 4 (MA18–MA7–MA22), Consortium 7 (MA18–MA5–MA22), and Consortium 10 (MA18–MA4–MA22). The combination of MA7, MA5, or MA4 with MA12 or MA22 likely enhances growth by adding structural variety, supporting each other's metabolism, and boosting EPS production. These consortia combinations exhibited strong macroscopic biomass, characterized by a thick, cohesive biofilm structure (Zhang et al., 2020). Microscopically, they demonstrated healthy and dense microalgae populations, highlighting strong overall performance across various indicators. Although Consortium 1 demonstrated observable growth during the initial screening stage, it was not categorized among the highest biomass density groups based on visual evaluation. Compared with other consortia, Consortium 1 showed relatively lower coverage and cell structural density, while several other combinations exhibited more compact and extensive biomass accumulation.

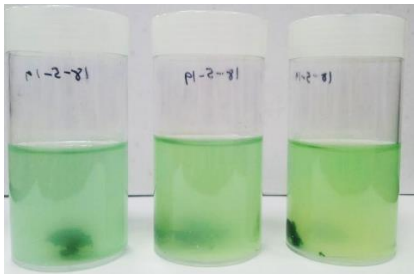
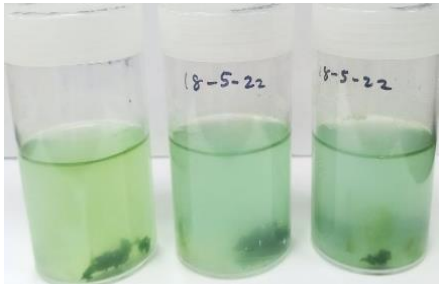
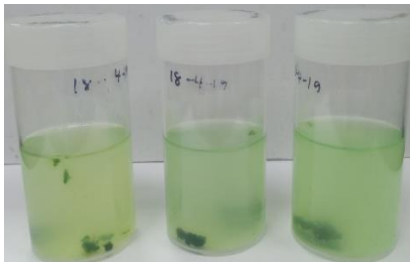
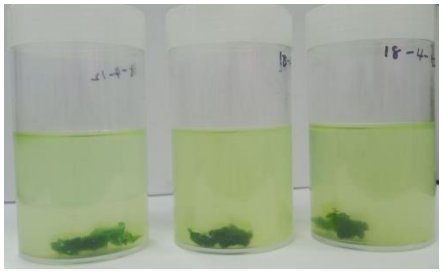
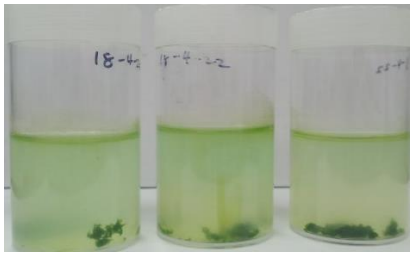
Successful consortium growth is driven by synergistic interactions that enhance both biomass and biofilm structure. Filamentous species and EPS production strengthen attachment and retention, while ecological balance among high and moderate-yield isolates ensures stability (Ling et al., 2019). These findings highlight that strong performance depends not only on dominant strains but also on the complementary roles of all isolates. Consortia with both productivity and structural integrity are crucial in optimizing microalgae use in wastewater treatment and bioresource recovery.

4.7.2 Pollutants Removal Efficiency: TN and TP Analysis

The ten microalgal consortia were then tested for the ability to remove TN and TP. Table 4.9 shows the visual observations of microalgae isolates after seven days of incubation in synthetic wastewater.

Table 4.9
Observation of Microalgal Consortia in Synthetic Wastewater

Consortia	Observation	Characteristics	Consortia	Observation	Characteristics
1		• Green cloudy media • Biomass growth	4		• Green cloudy media • Formation of green films on the walls of the bottle
2		• Green cloudy media • Formation of green films on the walls of the bottle	5		• Green cloudy media

3		• Green cloudy media	6		• Green cloudy media
7		• Green cloudy media • Formation of green films on the walls of the bottle	9		• Pale green cloudy media • Formation of green films on the walls of the bottle
8		• Pale green cloudy media • Formation of green films on the walls of the bottle	10		• Pale green cloudy media • Formation of green films on the walls of the bottle

Based on visual observations of Table 4.9, several microalgal consortia demonstrated enhanced growth characteristics compared to others in synthetic wastewater. Among the consortia, only Consortia 3, 5 and 6 stood out as consortia with no growth of green films, unlike the other consortia. Although they displayed bright green media, no visible growth of biomass or biofilms was observed by these consortia. The other consortia exhibited vivid pale to deep green coloration of the media with high turbidity and prominent green films on the container walls, which are signs of strong biofilm formation.

The green coloration of the synthetic wastewater is primarily due to the proliferation of microalgae, which contain photosynthetic pigments such as chlorophyll-a that reflect green light. The formation of green films along container surfaces results from the adhesion of microalgal cells facilitated by EPS, which anchors the cells and supports the development of structured biofilms (Abu Hasan et al., 2021; Mantzorou & Ververidis, 2019). Hence, leading to enhanced biomass production and visible film formation.

When compared to the previously studied monocultures grown in synthetic wastewater, the consortia exhibited higher photosynthetic activity, as evidenced by noticeable changes in the media's color intensity, which ranged from pale to deep green across all consortia. In contrast, only a few monocultures, specifically MA5, MA8 and MA19, showed similar green coloration, indicating limited photosynthetic performance when grown in isolation.

The enhanced activity observed in consortia is likely due to synergistic interactions among the different strains in a consortium. The inclusion of diverse morphologies, including filamentous and colonial forms, may contributes to higher light capture and spatial organization, enabling more efficient use of resources (Moreno Osorio et al., 2021). This allows consortia to outperform monocultures in terms of growth, pigment production and biomass development in synthetic wastewater environments.

Next, these consortia were then tested for the ability to remove TN and TP. Figure 4.11 shows the removal rate of TN and TP in synthetic wastewater by microalgal consortia after seven days of incubation.

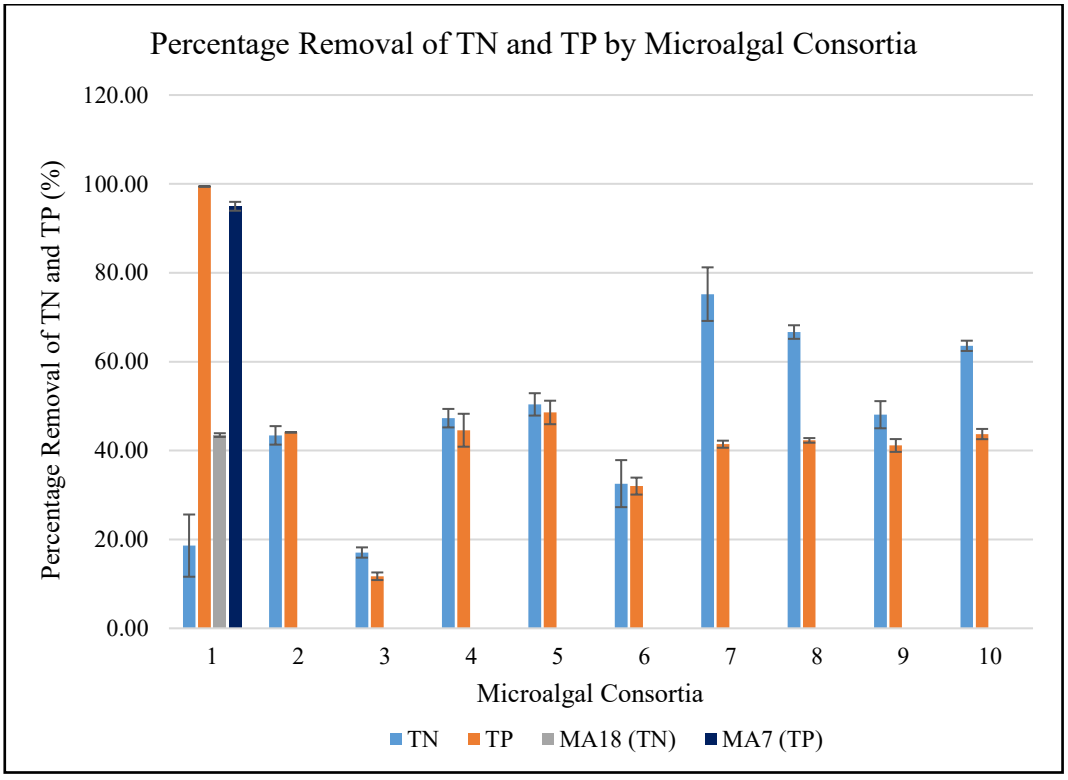


Figure 4.11 Removal of TN and TP by the Consortia

MA18 and MA7 serve to compare the removal of TN and TP between individual and microalgal consortia.

Based on Figure 4.11, all ten microalgal consortia demonstrated varying rates of removing TN and TP from synthetic wastewater. The TN removal ranged from approximately 17 - 75%, with Consortium 7 showing the highest TN removal, achieving approximately 75.2%, compared to the other consortia ($p < 0.05$), indicating highly active nitrogen metabolism and uptake. This may be potentially due to synergistic interactions between its constituent microalgal strains. In contrast, Consortium 3 exhibited the lowest TN removal efficiency, at roughly 17.1%, suggesting minimal nitrogen assimilation by the microalgae isolates within that group. Other high-performing consortia, including Consortium 8 and Consortium 10, achieved TN removal efficiencies at 66.7% and 63.6%, respectively. The average TN removal across all consortia was approximately 46.3%, which signifies moderate to strong potential for nitrogen reduction in wastewater treatment applications. These results show a consistent nitrogen removal performance across several consortia combinations, indicating that certain species pairings may have promoted synergistic nutrient uptake or enhanced growth dynamics.

For TP removal, the consortia also showed varying efficiencies, with removal

percentages ranging from around 10% to 99%. Consortium 1 stood out significantly ($p < 0.01$) by achieving the highest TP removal at approximately 99.4%, which suggests highly efficient phosphorus uptake. In contrast, Consortium 3 again showed the lowest removal at only 11.7%. This may reflect either a lack of phosphorus-utilizing species or limited metabolic activity. The majority of the remaining consortia showed moderate TP removal, generally ranging from 30% to 50%. The average TP removal across all consortia was calculated to be approximately 44.9%, slightly lower than the average of TN removal. These results imply that while phosphorus removal was highly efficient in selected consortia, notably in Consortium 1, the overall phosphorus uptake among the consortia was more variable and possibly more sensitive to the specific microalgal species present or environmental factors in the media.

As shown in Figure 4.11, all ten microalgal consortia exhibited varying efficiencies in removing TN and TP from synthetic wastewater. Generally, consortia that performed well in TN removal did not always show equally high TP removal, highlighting possible differences in nutrient uptake mechanisms or inter-strain metabolic roles.

The microalgal consortia demonstrated significant potential in removing TN and TP from synthetic wastewater, with removal efficiencies exceeding those of individual monocultures. Among the tested consortia, Consortium 7 (MA18-5-22) exhibited the highest TN removal rate at 75.2%, significantly ($p < 0.01$) surpassing the performance of MA18 in single culture. This is followed by Consortium 8 (MA18-4-12) and Consortium 10 (MA18-4-22), with removal rates of 66.7% and 63.6%, respectively. These values significantly exceeded the performance of MA18 as a monoculture ($p < 0.05$) and were substantially higher ($p < 0.05$) than the TN removal rates of MA5 (0.27%), MA4 (12.8%), MA12 (17.4%), and MA22 (31.3%). The markedly improved removal efficiency in the consortium suggests a synergistic interaction among the strains, enhancing nitrogen uptake and utilization compared to their individual performances. This result also indicates that the inclusion of MA4 and MA22 in consortia may notably enhance nitrogen removal efficiency, likely due to synergistic interactions and functional complementarity between strains.

The individual role of *Nodosilinea* (MA18) in biomass production and nutrient removal has been highlighted earlier in Section 4.6. As for *Leptolyngbya* (MA5), it is a versatile cyanobacterium known for its diverse applications, including alloprotein production, biological nitrogen fixation, use as green manure, and as a nutrient regulator

in rice cultivation (Bai et al., 2023). Notably, previous studies reported nitrogen (N_2) fixation rates as high as 10.17 ± 2.86 μmol cm^{-2} h^{-1} by *Leptolyngbya* colonies, the highest among species in the same study (Mukherjee et al., 2022). However, limited information is available on *Klisinema* (MA22), particularly regarding its role in nitrogen fixation. Despite this, its inclusion in high-performing consortia, Consortia 7 and 10, suggests a potentially valuable yet underexplored contribution to nitrogen removal, possibly through complementary metabolic interactions.

Conversely, the lowest TN removal was observed in Consortium 3 (MA18-7-19), with only 17.1%, lower than other consortia. Although *Leptolyngbya* is present in both Consortium 3 and Consortium 10 (MA18-4-22), which achieved a much higher TN removal rate of 63.6%, the difference may be attributed to strain-specific capabilities and interactions. Individually, MA7 exhibited a poor TN removal rate of just 9.8%, suggesting limited nitrogen uptake efficiency. This highlights the importance of strain compatibility and functional complementarity within consortia. While *Leptolyngbya* species are generally known for nitrogen fixation, not all strains contribute equally. In Consortium 10, the pairing of MA5 with *Klisinema* (MA22) and *Nodosilinea* (MA18) may have created synergistic interactions enhancing nutrient uptake, whereas in Consortium 3, the metabolic cooperation among MA18, MA7, and MA19 might have been weak or competitive, limiting TN removal efficiency.

For TP removal, the highest efficiency was recorded in Consortium 1 (MA18-7-23), achieving a remarkable 99.4% removal rate ($p < 0.01$). This performance exceeded that of the individual monoculture, which are *Nodosilinea* (MA18) at 29.4%, and *Leptolyngbya* strains MA7 and MA23 at 95.0% and 36.1%, respectively. The exceptionally high removal by MA7 suggests its dominant role in phosphorus uptake, while the inclusion of MA23 likely reinforced *Leptolyngbya*-specific metabolic strengths. Although MA7 already performed well individually, its pairing with *Leptolyngbya* MA23 and *Nodosilinea* (MA18) in a consortium may have enhanced phosphorus assimilation through improved biofilm formation, surface area for uptake, or cooperative nutrient processing (Bai et al., 2023; Liang et al., 2025). This result supports the idea that even high-performing strains like MA7 can benefit from consortial arrangements by stabilizing or amplifying their metabolic activity in the presence of compatible partners. It also underscores the potential of leveraging intra-genus variability (MA7 and MA23, both *Leptolyngbya*) and inter-genus complementarity (MA18, *Nodosilinea*) to achieve near-complete phosphorus removal.

In contrast, the lowest TP removal was observed in Consortium 3 (MA18-7-19), with only 11.7% efficiency. This poor performance was consistent with its TN removal (17.1%), indicating generally low nutrient removal capacity. Although the consortium includes *Nodosilinea* (MA18) and *Leptolyngbya* (MA7), both of which performed moderately well in TP removal as monocultures (MA18: 29.4%, MA7: 95%), the inclusion of MA19 appears to have disrupted the overall performance. In the previous part, individually, MA19 was observed to have a moderate efficiency in removing TP at 35.3%. The limited removal may be due to several factors, such as metabolic incompatibility among the strains, lack of functional complementarity or competitive interactions that reduced nutrient uptake efficiency (Abdelaziz et al., 2014; Abdelfattah et al., 2023). It is also possible that the high individual efficiency of MA7 was suppressed by unfavorable interactions in the consortium, such as nutrient competition or inhibitory signaling. This further emphasizes that the presence of efficient strains alone does not guarantee high consortium performance. The compatibility, synergistic behavior and balanced nutrient demands are key determinants in achieving optimal phosphorus and nitrogen removal. In a study by Gonçalves et al. (2017), they demonstrated that a synthetic consortium of *Chlorella* sp. and *Scenedesmus* sp. achieved high nutrient removal from primary-treated municipal wastewater, with TN and TP removal efficiencies ranging from 88.6–96.4% and 99.7–99.9%, respectively.

The observed trends underscore the importance of strain compatibility and functional complementarity in enhancing nutrient removal performance in consortia systems. This enhanced performance can be attributed to the synergistic interactions within the consortium, which facilitated the uptake and assimilation of nutrients into biomass.

4.7.3 Biomass Production

The consortia were then evaluated for their ability to produce biomass in synthetic wastewater over 28 days. The results are as shown in Figure 4.12.

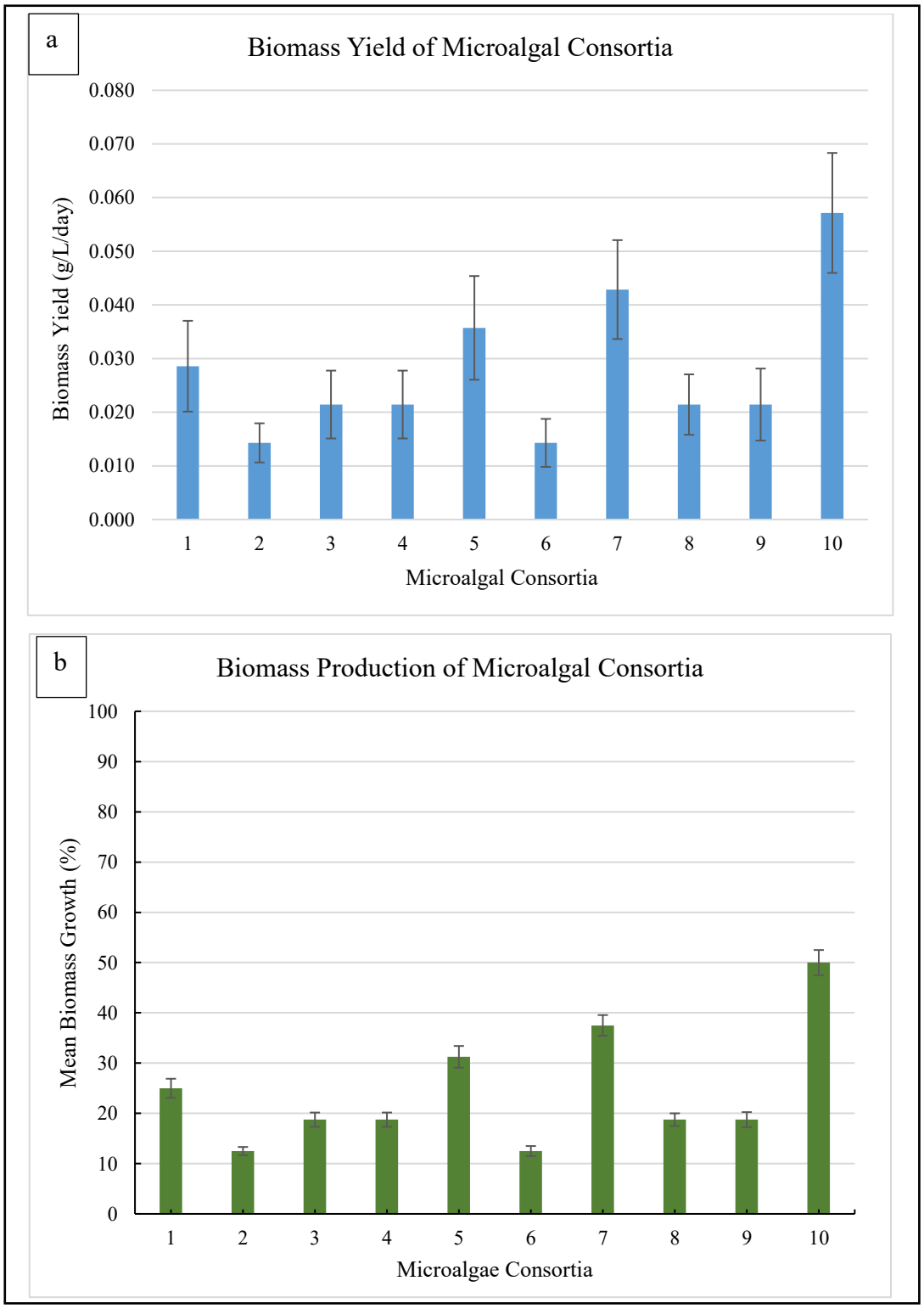


Figure 4.12 Biomass Production of Microalgal Consortia Over Time

(a) Daily biomass yield of ten microalgal consortia (b) Total biomass growth percentage of each consortium on day 28. Error bars represent standard deviation from triplicate measurements.

The mean biomass growth percentages of all ten consortia showed a distinct range between 10% and 50%. The highest biomass productivity was recorded in

Consortium 10, reaching approximately 50%, which significantly outperformed the other consortia ($p < 0.05$). This is followed by Consortia 7 and 5 producing 37.5% and 31.3% of biomass, respectively. In contrast, Consortia 2 and 6 exhibited the lowest mean biomass growth, both around 12.5%, indicating limited biomass accumulation. The average biomass growth across all consortia was 24.4%, reflecting varied performance levels among different microalgal combinations. Notably, all consortia demonstrated a steady increase in biomass throughout the 28-day cultivation period. This suggests active cell proliferation and adaptation to the experimental environment, including synthetic wastewater conditions (Renuka et al., 2013).

The biomass growth trends observed across the microalgal consortia highlight the influence of strain compatibility and synergistic interactions on productivity. Consortium 10 displayed superior growth performance, suggesting specific microalgal combinations promote enhanced biomass production, potentially due to mutualistic interactions, improved resource utilization, or differential tolerance to environmental stressors (Gonçalves et al., 2017; Serejo et al., 2019). On the other hand, Consortia 2 and 6, both with the lowest biomass productivity, may lack these synergistic effects or possibly exhibit competitive behavior among strains that inhibits collective growth (Hu et al., 2021; Zhu et al., 2019). The lower biomass yields in other consortia suggest that not all combinations are equally effective. The broad range of biomass growth from 10% to 50% reflects the diverse physiological capabilities and interspecies relationships among the consortia tested. The observed variations in biomass production indicate that the selection of microalgal species within each consortium significantly influences growth performance. This also highlights the importance of species-specific interactions in optimizing biomass production (Qin et al., 2016).

Despite having the same strain composition of *Leptolyngbya sp*., Consortium 10 and Consortium 1 exhibited slightly different biomass production levels, with 50.0% and 25% yield, respectively. This variation may be attributed to differences in initial inoculum ratios, where even slight shifts in cell concentrations can influence the dominance and functional roles of individual strains (Nguyen et al., 2020). Microalgae interactions within consortia are also dynamic and can vary based on environments (Abdelfattah et al., 2023), potentially leading to stronger cooperative behaviors in Consortium 10 compared to Consortium 1. Furthermore, in biomass retention, the formation and stability of the biofilm structure play a crucial role (Ennaceri et al., 2023). Consortium 10 was observed to form a denser and more cohesive biofilm, as shown in

Table 4.8, enhancing surface attachment and biomass production. Additionally, physiological adaptability among isolates could cause variable responses to microenvironmental factors, despite having the same genetic makeup (Abdelfattah et al., 2023). This emphasizes that successful biomass production depends not only on choosing compatible strain combinations but also on maintaining consistent inoculation methods and stable environmental conditions to ensure reliable and optimal results.

Previous studies have demonstrated that microalgal consortia often outperform monocultures in wastewater treatment due to synergistic interactions, niche complementarity and enhanced resilience under environmental stress and invasive species (Gonçalves et al., 2017; Zhu et al., 2019). These cooperative dynamics allow for improved nutrient uptake and biomass accumulation compared to single-species cultures (Renuka et al., 2013). In line with this, Consortium 10, comprising *N. nodulosa* (MA18), *Leptolyngbya sp.* (MA4), and *K. persicum* (MA22), exhibited one of the highest biomass yields among all tested groups.

Previously, individually, MA18 showed superior TN removal efficiency, while MA4 demonstrated moderate TP removal, and MA22 contributed moderately to biomass production. Interestingly, while MA18 and MA22 individually produced 63.6% more biomass and MA4 achieved 46.7% biomass growth, their combination in Consortium 10 resulted in the highest biomass production among all consortia at 50%. However, this was still lower than the biomass yields of MA18 and MA22 in monoculture, suggesting that while synergistic interactions in consortia can enhance performance relative to other combinations, they do not always surpass the yields of the best-performing monocultures. Certain isolates may perform better independently, possibly due to reduced competition or more favorable growth conditions in monoculture (De Francisci et al., 2018). Although existing literature has reported on the individual capabilities of *Nodosilinea* and *Leptolyngbya* species in bioremediation and biofilm formation (Phyu et al., 2025; Shimura et al., 2015), there is limited research involving this specific strain combination.

Although this study did not quantify the biochemical composition of biomass, previous research highlights the potential of *Nodosilinea* species as high-value contributors in microalgal consortia. For instance, in a study by Hajiyeva et al. (2024), *Nodosilinea sp.* has been reported to yield high phycocyanin content (26.8 ± 0.85 mg/g), while a study by Sri et al. (2023) *Nodosilinea sp.*-II showed the highest total carbohydrate content at 44.6%. In addition, *N. nodulosa* LEGE 06104 was found to

produce zeaxanthin as the dominant xanthophyll, with levels ranging from 52.7–192.9 mg/g (Rodrigues et al., 2024). These biochemical attributes suggest that *Nodosilinea sp.* is metabolically active and capable of contributing to biomass accumulation and quality (Aravindh et al., 2023). Although this study focused on general biomass productivity, the inclusion of *Nodosilinea* in consortia likely enhanced overall performance not only through growth support but also due to its underlying biochemical richness. *Leptolyngbya* and *Klisinema,* which previously demonstrated moderate biomass yield in monocultures, may have also contributed to the improved consortia performance due to their known adaptability and functional traits, as discussed in Section 4.6.2.

In addition, different microalgal strains have varying preferences for nutrient sources. This allows them to utilize available resources without intense interspecies competition efficiently (González-Camejo et al., 2018). This promotes enhanced nutrient removal and biomass productivity in consortial cultures. For instance, the potential of using microalgal consortia comprising *Chlorella sp.*, *Scenedesmus sp.* and C*hromochloris zofingiensis* to treat dairy wastewater has been evaluated, where higher COD removal (57.01–62.86%) and phosphorus removal (91.16–95.96%) were achieved by the consortium compared to the monoculture of *Chlorella sp.* (Qin et al., 2016; Zhu et al., 2019). These results highlight the benefits of consortia, where metabolic diversity and synergy between species enhance nutrient uptake and boost biomass productivity, as seen in Consortia 10, 7 and 5.

Conversely, some consortia, such as consortia 2 (MA18-7-12) and 6 (MA18-5-19), exhibited relatively lower biomass accumulation. This lower performance may be attributed to interspecies competition, inefficient nutrient assimilation or suboptimal growth conditions for the selected strains (Abdelfattah et al., 2023; González-Camejo et al., 2018). Despite the moderate to high biomass productivity of the individual isolates involved in Consortia 2 and 6, the overall performance of these consortia was unexpectedly low, with only a 12.5% increase in biomass from the initial weight. Previously, MA18 alone exhibited a 63.6% increase, MA12 reached 60.0%, and MA19 reached 50.0%, indicating their strong individual capabilities. However, when combined in consortia, the biomass yield for both consortia dropped significantly ($p < 0.05$). It is also possible that strains like MA7 and MA5, which showed relatively lower individual biomass production (28.6% and 23.1% respectively), may have introduced growth-inhibiting effects or failed to contribute positively to the consortium dynamic.

In particular, the presence of multiple high-performing strains does not necessarily guarantee enhanced biomass accumulation. Instead, the compatibility, ecological niche differentiation and metabolic complementarity of the strains are critical factors in determining the success of a consortium (Gonçalves et al., 2017).

The variation in biomass yield among consortia underscores the importance of selecting compatible and complementary strains to maximize productivity. These findings highlight the potential of microalgal consortia for enhanced biomass production, particularly for bioresource recovery applications such as biofuel generation and bioproduct synthesis. High-yielding consortia, such as Consortia 10, 7 and 5, warrant further investigation for their biomass productivity under varying cultivation conditions.

4.7.4 Lipid Profiling of Microalgal Consortia

The lipid production of all ten microalgal consortia was tested for the GC-MS. The standard reference is presented in Section 4.6.3. The results are summarized in Figure 4.13.

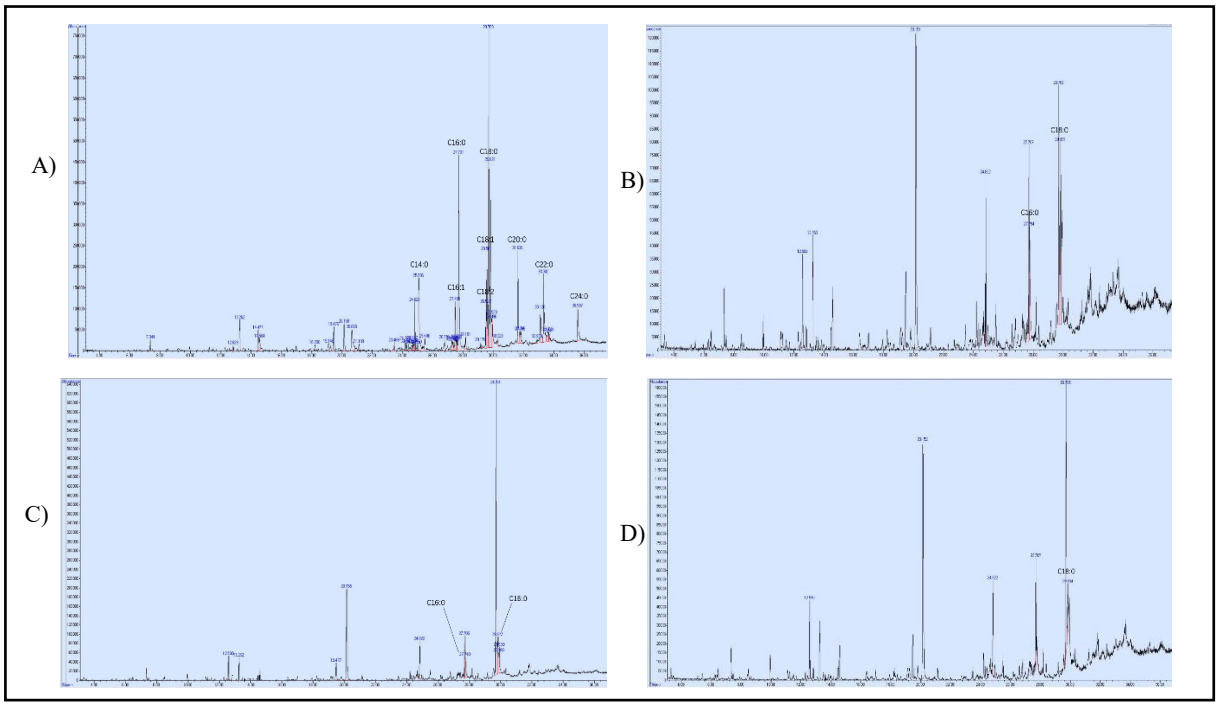


Figure 4.13 Representative GC-MS Chromatograms of FAMEs Produced by Microalgal Consortia

Chromatograms A) Consortia 1, B) Consortia 2, C) Consortia 3 and D) Consortia 4-10. No detectable targeted fatty

acids were observed for Consortium 6. Consortia 4 is presented as a representative profile for Consortia 4, 5, 7, 8, 9, and 10, as these consortia exhibited identical lipid profiles characterised by the presence of methyl stearate (C18:0) at a retention time of 29.83 min.

Nine out of the ten microalgal consortia analyzed through GC-MS produced detectable amounts of FAME, while no detectable FAMEs were identified for Consortia 6. Distinct lipid profiles were obtained for Consortia 1–4, as shown in Figure 4.13. In contrast, Consortia 4, 5, 7, 8, 9, and 10 exhibited identical chromatographic profiles, characterised by a single dominant peak corresponding to methyl stearate (C18:0) at a retention time of 29.83 min. Therefore, Consortia 4 was selected as a representative chromatogram for these consortia to avoid redundancy of data presentation.

Among the consortia, Consortia 1 exhibited the greatest diversity of FAMEs with a wide range of different fatty acid methyl esters. This includes hexadecanoic acid (methyl hexadecanoate; C16:0), methyl stearate (C18:0), 9-octadecenoic acid (oleic acid: C18:1), 9,12-octadecadienoic acid (linoleic acid: C18:2), eicosanoic acid (methyl eicosanoate; C20:0), docosanoic acid (methyl docosanoate; C22:0) and tetracosanoic acid (methyl tetracosanoate; C24:0). The most frequently detected lipid across the consortia was methyl stearate (C18:0), a saturated long-chain fatty acid, found as the sole component in Consortia 4, 5, 7, 8, 9 and 10. This result may suggest the dominance of specific microalgal strains in those consortia, leading to similar lipid profiles.

The presence of lipids in nine of the ten consortia demonstrates the lipid-producing potential of the microalgal isolates. The detection of saturated long-chain FAMEs such as methyl hexadecanoate (C16:0), methyl stearate (C18:0), methyl eicosanoate (C20:0), methyl docosanoate (C22:0) and methyl tetracosanoate (C24:0) across the consortia is highly relevant for biofuel production. These compounds are widely regarded as desirable components in biodiesel feedstock because of the lack of double bonds. This improves oxidative stability, extending fuel shelf life and reducing polymerisation during storage (Laemthong et al., 2025; Masudi et al., 2023). These FAMEs are also favorable due to their high cetane number, which improves ignition quality, suitable viscosity that supports efficient fuel injection and combustion and low volatility, which minimizes fuel losses during storage and handling (Masudi et al., 2023; Niemi et al., 2019). However, their relatively high melting points may negatively affect cold-flow properties when present in high proportions, potentially leading to crystallization at low temperatures (Sugami et al., 2017). Therefore, while these saturated FAMEs contribute positively to fuel stability, an excessive fraction may

reduce biodiesel performance under cold conditions.

Consortia 1 stands out for its broader range of FAMEs, reflecting a broad lipid biosynthetic capability (Wang et al., 2024). This also indicates a richer lipid metabolism possibly due to the synergy of multiple strains or greater biomass production. The detection of saturated long-chain such as methyl tetracosanoate, methyl hexadecanoate, methyl stearate, methyl eicosanoate and methyl docosanoate, and monounsaturated such as methyl trans-9-octadecanoate, further increases its relevance for biofuel, as these fatty acids strike a balance between fluidity and combustion efficiency (Alishah Aratboni et al., 2019; Khalaji, 2022).

Methyl hexadecanoate (palmitic acid methyl ester, C16:0) and methyl stearate (stearic acid methyl ester, C18:0) are particularly important in biodiesel formulation, as they provide a good balance between cetane number and cold flow properties (Chhandama et al., 2021; Rana & Prajapati, 2022). High cetane numbers ensure efficient combustion, while moderate chain length minimises excessive cloud and pour points (Rana & Prajapati, 2022). Methyl eicosanoate (C20:0), methyl docosanoate (C22:0) and methyl tetracosanoate (C24:0) contribute further to oxidative fuel stability, but their longer chains increase the pour point and can negatively affect cold flow performance, making them less suitable in colder climates (Hazrat et al., 2020). However, in tropical or warm regions, these very long-chain saturated FAMEs can enhance fuel density and combustion quality (Hazrat et al., 2020; Tian et al., 2022). These C20-C24 FAMEs may require blending with shorter-chain or unsaturated FAMEs to optimise cold flow characteristics for broader climatic suitability (Awogbemi et al., 2021). Overall, the presence of these saturated long-chain methyl esters in the microalgal consortia suggests strong potential for producing stable, high-cetane biodiesel, especially for applications in warm climates.

Methyl stearate (C18:0) was the most frequently detected compound. Its presence in nine out of ten consortia suggests that these microalgal communities possess strong biosynthetic capabilities for producing biofuel-suitable lipids (Qin et al., 2016; Zhu et al., 2023). The predominance of methyl stearate is significant, as C18:0 is commonly associated with good oxidative stability, a critical requirement in commercial biodiesel applications. Its consistent occurrence across multiple consortia indicates that these microalgal communities are inclined to produce saturated fatty acids, which improve the shelf life and combustion quality of biodiesel (Li et al., 2019).

Consortia 1 comprised MA18, MA7 and MA23. MA23 stood out as the top

individual isolate in terms of biomass productivity and FAMEs production, and this ability retained when it was part of Consortium 1, which exhibited the widest variety of FAMEs among all consortia. This suggests that MA23 not only maintains its lipid biosynthetic capacity in mixed cultures but may also contribute synergistically to the consortium's overall lipid diversity (Chhandama et al., 2021). Such strains are particularly valuable because they can deliver high biomass for increased total lipid yield while also providing a balanced FAME profile with both saturated and unsaturated components, ensuring better overall biodiesel quality (Sharma et al., 2020). Consortia 1 is the combination of MA18 (*N. nodulosa*), MA7, and MA23 (*Leptolyngbya*). Previous research Aravindh et al. (2023) reported that *N. nodulosa* SNMVBTAR001 possessed a favorable fatty acid profile, particularly rich in linoleic and other polyunsaturated fatty acids. This makes it suitable for biodiesel production, and the study is consistent with earlier findings by Knothe (2008). Similarly, Zainal Abidin et al. (2020) analyzed several *Leptolyngbya* species and observed the presence of both unsaturated and saturated fatty acids. Supporting this, Tiwari et al. (2019) found that *Leptolyngbya* sp. BTA 287 is a promising biodiesel feedstock, with a lipid profile comprising 34.09% unsaturated and 65.91% saturated fatty acids, dominated by oleic acid (C18:1) at 31.93%.

In contrast to monocultures, a consortium of these species may have produced complementing metabolic interactions that produced a wider range of FAMEs (Magdouli et al., 2016). This variety is beneficial for biodiesel applications since the presence of both saturated FAMEs, which is for oxidative durability and a high cetane number, and unsaturated FAMEs, which is for cold flow characteristics, might result in balanced fuel performance (Enwereuzoh et al., 2020; Hazrat et al., 2020; Li et al., 2019). Furthermore, this consortium is a viable target for additional strain selection and optimisation since the variety of lipid profiles also suggests potential environmental resilience, which might sustain steady biomass yields under a range of culture conditions (Zhu et al., 2023).

The absence of lipids in Consortia 6 could be due to multiple factors, including low biomass yield, poor lipid accumulation or extraction inefficiency. The species composition might favor strains optimized for nutrient removal or rapid growth of biomass rather than lipid accumulation, while competitive interactions or inhibitory metabolites could further suppress lipid biosynthesis in certain isolates of the consortium (Amaro et al., 2023; Huang et al., 2013). Additionally, the metabolic

activity under the given culture conditions may have prioritized carbon allocation toward protein or carbohydrate synthesis instead of lipid storage, resulting in an undetectable FAMEs profile (Gao et al., 2021).

Overall, the lipid profile analysis reveals that consortia containing saturated (C16–C24) and monounsaturated fatty acids (C18:1) are promising for biofuel applications. The detection of these FAMEs aligns with previous studies on microalgal biofuels, which yielded high levels of C16–C18 FAMEs suitable for biodiesel production (Chhandama et al., 2021; Qin et al., 2016).

CHAPTER 5
CONCLUSION

The present study demonstrated that targeted isolation and performance-based selection of biofilm-forming microalgae can create robust systems for wastewater remediation and bioresource recovery. The most frequent isolates that are able to form biofilm and thrive under laboratory conditions are various species of *Leptolyngbya sp.* Microalgal biofilms has the capability to remove considerable amounts of nitrogen from wastewater, with removal rates ranging from 2 to 43.0%. The capability to remove phosphorus is more pronounced, with the highest removal rate of 95.0%. Taken together, the microalgal biofilm demonstrated strong potential for the development of sustainable, green systems for removing residual levels of nitrogen and phosphorus from wastewater. *N. nodulose* and *Leptolyngbya sp*. emerged as leading isolates for nitrogen and phosphorus removal respectively. Biomass production by the investigated microalgae isolates demonstrated potentials, but will probably require more optimization to achieve higher yields suitable for commercial exploitation. Biomass productivity patterns showed two strategies: short-term, high-yield species like MA10, and long-term growers like MA23. For sustained wastewater treatment and consistent biomass recovery, long-term growers such as MA23 are more suitable for their stable productivity and robustness over time. Lipid profiling confirmed that the isolates predominantly produced C16–C18 fatty acids, with C16:0 and C18:0 being the most abundant, with blends of unsaturated fatty acids of C18:1 and C18:2. These are desirable for balanced biodiesel production.

By integrating the top-performing isolates, the combined microalgal consortia consistently outperformed individual strains in nutrient removal efficiency. Specifically, Consortium 7 (MA18-5-22) demonstrated the highest TN removal rate, while Consortium 1 (MA18-7-23) achieved exceptional TP removal. In biomass production, Consortium 10 (MA18-4-22) recorded the highest yield among the consortia. However, its performance was moderate when compared to the best-performing individual isolates in previous tests. Lipid profiling of Consortium 1 revealed the greatest diversity in fatty acid compositions, consisting saturated fatty acids ranging from C14 to C24, and monounsaturated fatty acids relevant for biodiesel applications. These findings emphasize the synergistic advantages of mixed cultures,

which can balance high pollutant removal efficiency, stable growth performance and the production of high-value biochemical compounds.

Future studies are recommended to expand the applicability and performance of microalgal biofilm systems beyond laboratory conditions. Scale-up experiments should be conducted using pilot-scale or continuous-flow reactors to evaluate treatment efficiency, biomass productivity, and operational stability under real environmental conditions. In addition, the performance of selected isolates and consortia should be tested using different types of real wastewater, such as municipal, agricultural, and industrial effluents, to assess robustness and adaptability across variable pollutant compositions. Further optimization of media composition and operational parameters is also recommended to enhance pollutant removal efficiency, biomass accumulation, and lipid yield. Parameters such as nutrient ratios, light intensity, photoperiod, carbon supplementation, and hydraulic retention time should be systematically evaluated. It is also recommended that future research investigate molecular and metabolic profiling to better understand interspecies interactions within microalgal consortia and their roles in nutrient removal and biofilm stability. Advanced analytical approaches such as metagenomics, transcriptomics, or metabolomics could provide deeper insight into functional contributions within the biofilm community.

This research contributes to the development of microalgal biofilm technology as a sustainable approach for wastewater treatment and bioresource recovery. The study demonstrates that selected microalgal isolates and consortia are capable of forming stable biofilms, supporting pollutant removal and producing valuable biomass and lipid profiles. This supports the concept of integrated treatment systems that simultaneously address environmental remediation and renewable resource generation. The findings also provide practical insights into isolate selection, consortium design, and biofilm-based cultivation strategies, which are important for improving system robustness and treatment performance. By highlighting the role of filamentous cyanobacteria and mixed consortia in structural stability and functional efficiency, this work adds to the growing knowledge base on engineered microalgal communities.

Overall, the study supports the transition toward greener and circular wastewater treatment models, where wastewater is converted into valuable biological resources. This has potential environmental, economic, and biotechnological impact, particularly in advancing low-energy and resource-recovery-focused treatment systems.

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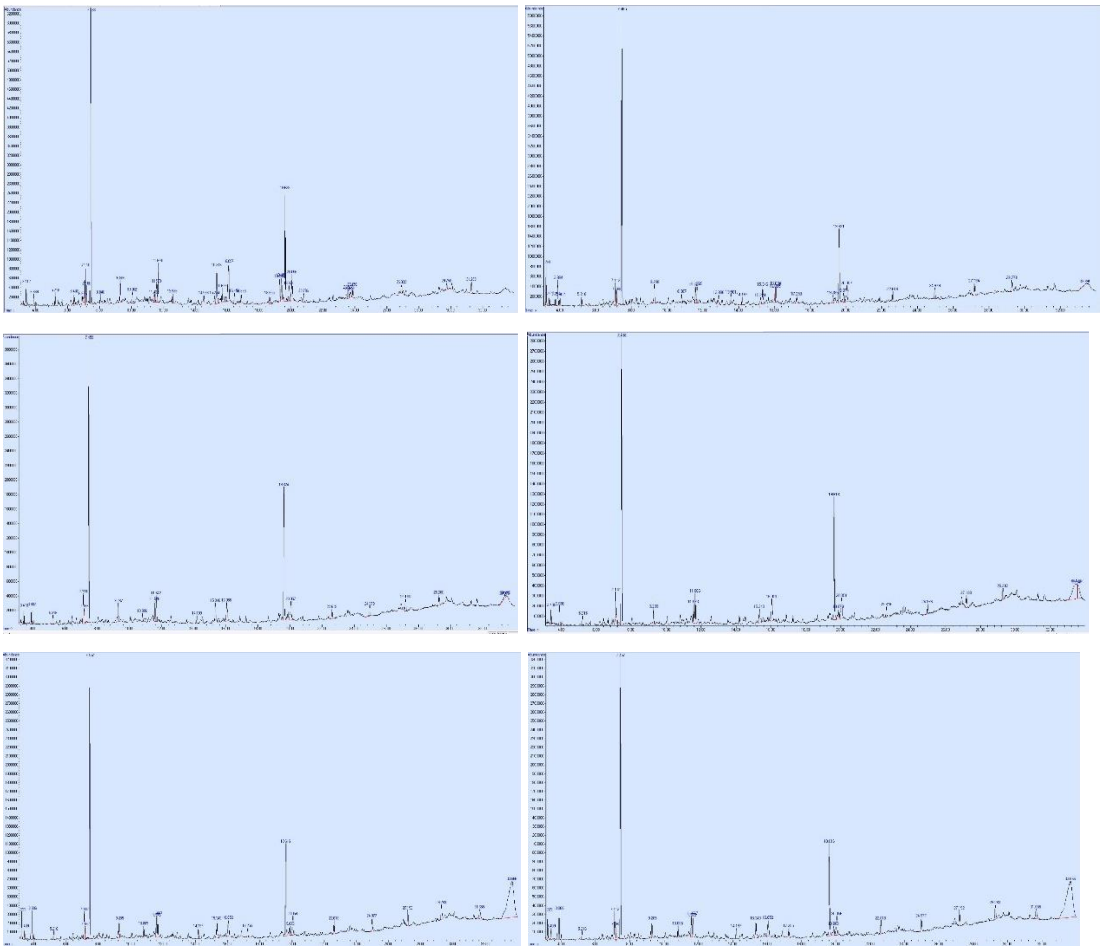
APPENDICES

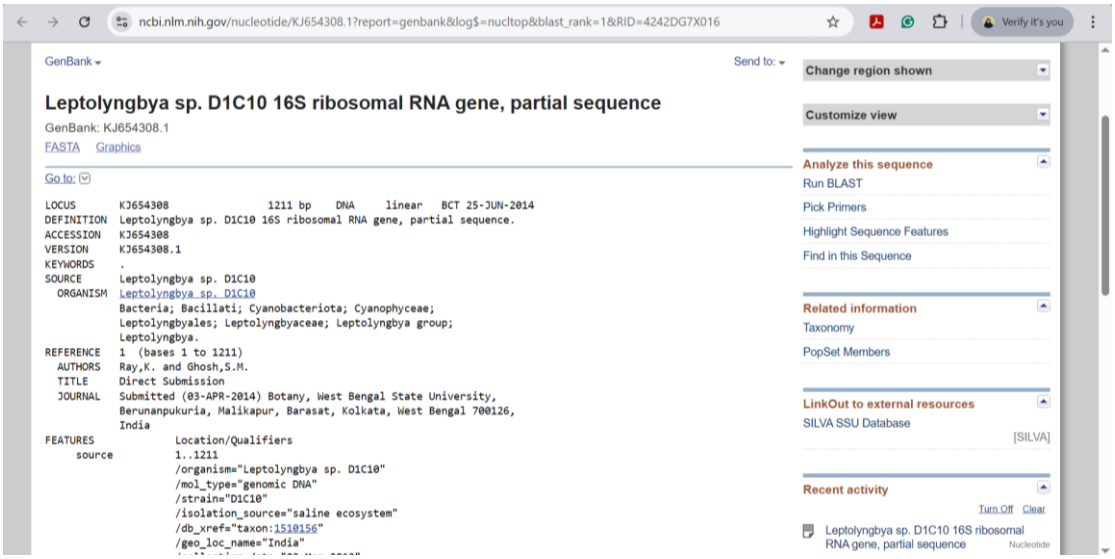
APPENDIX 1

Chromatograms of Microalgal Isolates



APPENDIX 2

GenBank Nucleotide BLAST of Sequenced Isolates



ncbi.nlm.nih.gov/nucleotide/KJ654308.1?report=genbank&log$=nucltop&blast_rank=1&RID=4242DG7X016

GenBank

Send to:

Leptolyngbya sp. D1C10 16S ribosomal RNA gene, partial sequence

GenBank: KJ654308.1

FASTA Graphics

Go to:

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ACCESSION   KJ654308
VERSION     KJ654308.1
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            Leptolyngbya.
REFERENCE   1  (bases 1 to 1211)
  AUTHORS   Ray,K. and Ghosh,S.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-APR-2014) Botany, West Bengal State University,
            Berunanpukuria, Malikapur, Barasat, Kolkata, West Bengal 700126,
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Change region shown

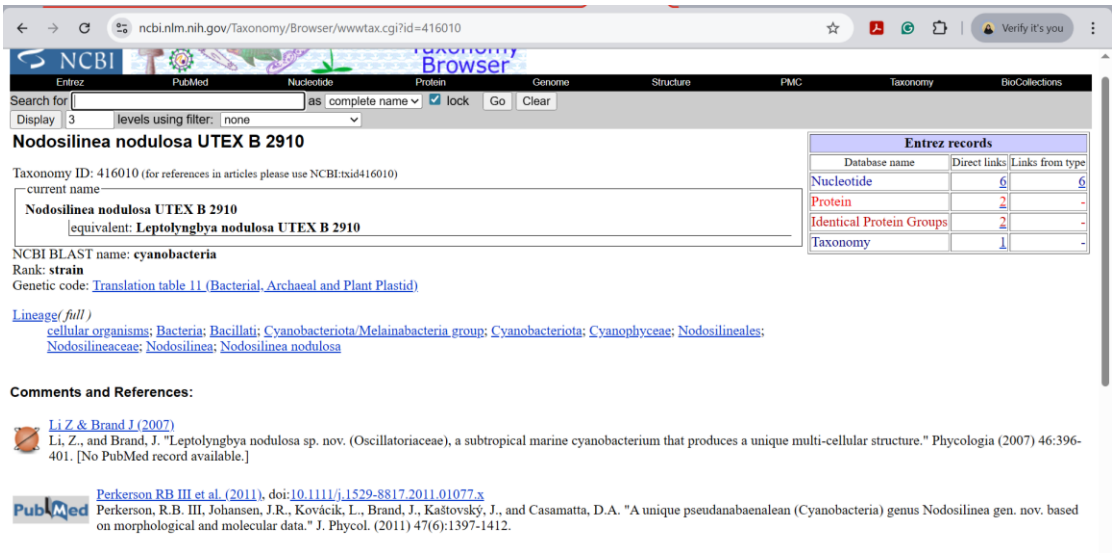
Customize view

Analyze this sequence
Run BLAST
Pick Primers
Highlight Sequence Features
Find in this Sequence

Related information
Taxonomy
PopSet Members

LinkOut to external resources
SILVA SSU Database [SILVA]

Recent activity
Turn Off Clear
Leptolyngbya sp. D1C10 16S ribosomal RNA gene, partial sequence Nucleotide



ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=416010

NCBI Taxonomy Browser

Entrez PubMed Nucleotide Protein Genome Structure PMC Taxonomy BioCollections

Search for as complete name lock Go Clear

Display 3 levels using filter: none

Nodosilinea nodulosa UTEX B 2910

Taxonomy ID: 416010 (for references in articles please use NCBI:txid416010)

current name
Nodosilinea nodulosa UTEX B 2910
equivalent: **Leptolyngbya nodulosa UTEX B 2910**

NCBI BLAST name: **cyanobacteria**
Rank: **strain**
Genetic code: Translation table 11 (Bacterial, Archaeal and Plant Plastid)

Lineage(*full*)
cellular organisms; Bacteria; Bacillati; Cyanobacteriota/Melainabacteria group; Cyanobacteriota; Cyanophyceae; Nodosilineales; Nodosilineaceae; Nodosilinea; Nodosilinea nodulosa

Entrez records		
Database name	Direct links	Links from type
Nucleotide	6	6
Protein	2	-
Identical Protein Groups	2	-
Taxonomy	1	-

Comments and References:

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Nik Airin Syazlin Azhan obtained a Bachelor of Science in Applied Microbiology (Hons.) from Universiti Teknologi MARA (UiTM) Shah Alam in 2023. She is currently pursuing a Master's degree in Molecular Biology. Her research interests include biofilm studies, microbiology, microalgae, environmental biotechnology, molecular biology and biochemistry.

LIST OF PUBLICATION:

Azhan, N. A. S., Abdullah, M. F. F., Abd Hamid, U. M., & Mohamad Jamil, N. (2025). The potential of local microalgae isolates in biotechnological applications. *Science Letters, 19*(2), 121–135. https://doi.org/10.24191/scl.v19i2.6908

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