

Tomás Marques Jorge

Bioremediation of greenhouse effluents using locally isolated microalgal strains



UAlg FCT

UNIVERSIDADE DO ALGARVE
FACULDADE DE CIÊNCIAS E TECNOLOGIA

2024

Tomás Marques Jorge

Bioremediation of greenhouse effluents using locally isolated microalgal strains

Mestrado em Biologia Molecular e Microbiana

Trabalho efetuado sob a orientação de:

Supervisor: Prof. Dr. João Carlos Serafim Varela (UAlg and CCMAR)

Co-supervisora: Dra. Mariana Carneiro (Necton S.A.)



UAlg FCT

UNIVERSIDADE DO ALGARVE
FACULDADE DE CIÊNCIAS E TECNOLOGIA

2022/2024

Bioremediation of greenhouse effluents using locally isolated microalgal strains

Declaração de autoria de trabalho

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Copyright

A Universidade do Algarve reserva para si o direito, em conformidade com o disposto no Código do Direito de Autor e dos Direitos Conexos, de arquivar, reproduzir e publicar a obra, independentemente do meio utilizado, bem como de a divulgar através de repositórios científicos e de admitir a sua cópia e distribuição para fins meramente educacionais ou de investigação e não comerciais, conquanto seja dado o devido crédito ao autor e editor respetivos

Resumo

Dada a escassez de recursos de água devido ao impacto humano, quer para agricultura, uso industrial ou uso doméstico, o tratamento de águas contaminadas é de grande prioridade, de modo a certificar que recursos de água potável não sejam consumidos mais rapidamente do que são recuperados, através da adoção de métodos e tecnologias mais sustentáveis para o planeta.

De especial interesse é a poluição de águas doces por nitratos com a principal fonte de contaminação sendo o uso de fertilizantes na agricultura, com cerca de metade do azoto usado globalmente sendo para esse fim. As contaminações de água com nitratos têm como possíveis consequências a perda de biodiversidade e eutrofização. Adicionalmente, os fosfatos são um recurso finito que deve ser reciclado para ser sustentável a longo termo.

De modo a reduzir o impacto ambiental da agricultura, novos métodos têm sido adotados de modo a reduzir o consumo de água e aumentar a eficiência da produção de culturas. Um desses métodos é a hidropónica, uma forma de agricultura sem solo em que as raízes das plantas são expostas diretamente a uma solução de nutrientes. O método é mais sustentável, uma vez que há uma maior eficiência no uso de água, espaço e nutrientes. Apesar de haver uma melhor eficiência no uso hidráulico, há ainda uma parte da água usada, rica em nutrientes, que não é usada.

Existem vários métodos de tratamento de água, sendo a biorremediação uma deles. A biorremediação foca-se no uso de organismos, por exemplo microalgas, no tratamento de águas, uma vez que usam estes nutrientes em excesso para o seu crescimento. A biorremediação tem vantagens relativamente a outros métodos como não precisar de energia e o benefício adicional de produção de biomassa para outros fins como biocombustíveis, alimentação de animais ou fertilizantes.

Por isso, a biorremediação de efluentes agrícolas, especificamente remoção de nitratos, é de especial interesse. Especificamente, o uso de microalgas isoladas localmente para remoção de nitratos, é um método de tratamento de águas que já se mostrou eficaz quer em outros estudos, quer em aplicações em grande escala como em estações de tratamento de águas residuais.

O objetivo desta tese é medir as capacidades de biorremediação de microalgas isoladas de efluentes hidropónicos para determinar quais as mais eficazes. E depois, determinar as

características filogenéticas e bioquímicas das mesmas, para elucidar futuros esforços de uso delas.

Das amostras de água de drenagem recolhidas de uma produção agrícola em hidropônica, foram isoladas e identificadas sete estirpes de microalgas através da extração e sequenciação de ADN e inferência filogenética, com árvores filogenéticas feitas para todas as estirpes, determinando a ordem e, para algumas, o género a que pertencem.

As capacidades de crescimento e remoção de azoto das sete estirpes foram comparadas através de um ensaio de crescimento durante 8 dias, mostrando que duas estirpes, *Chlorella* sp. (CHL) e *Pseudochlorella* sp. (PSE) foram significativamente melhores ($p < 0,05$) que as outras em termos de crescimento, com uma densidade ótica final superior às restantes (1,04 e 1,08, respetivamente em comparação com uma densidade ótica de 0,31 para a estirpe com o crescimento mais baixo), e em termos de remoção de nitratos, com concentrações de azoto inferiores às outras (0 mM em comparação com 4,09 mM para os mais elevados, ou 100% e 13.89% de remoção respetivamente). Para a comparação ser fiável, foram todas crescidas em condições idênticas, mas como a taxa de crescimento e remoção de nitratos depende de fatores como a temperatura e luminosidade, serão necessários mais estudos para determinar qual as melhores condições para crescimento, caso a biorremediação de nitratos com estas espécies seja adotada em maior escala.

Foram feitos ensaios de crescimento durante 8 dias para comparar pré-tratamentos do meio de crescimento, testando o uso de radiação UV por 20 minutos, filtração a 0,2 μm e tratamento com cloro (1 mL/L de hipoclorito de sódio por 30 minutos), nas duas espécies selecionadas. Em termos de crescimento, o pré-tratamento com radiação UV foi o mais eficaz para ambas as espécies e melhorou tanto o crescimento (0.69 OD final vs 0.53) quanto a remoção de nitratos (0 mM vs 2.98 mM, ou remoção de 100% e 36.73% do azoto respetivamente), significativamente comparado ao controlo e ao filtro.

Adicionalmente, um ensaio de co-cultivo foi realizado durante 8 dias, comparando as duas estirpes selecionadas com a espécie *Tetrademus obliquus*, uma estirpe conhecida como sendo eficaz na biorremediação de nitratos. Ambas as estirpes foram mais eficazes em termos de crescimento (a CHL atingindo 8.9 em comparação com 3.9×10^6 células/mL da *T. obliquus* e a PSE atingindo 5.6 relativamente às 2.9×10^6 células/mL dela), a diferença sendo significativa para a CHL, relativamente a *T. obliquus*, demonstrando que

as microalgas obtidas localmente tendem a ser melhores para o tratamento das próprias águas.

Por fim, as duas estirpes foram cultivadas em fotobiorreatores de escala laboratorial de 1L para a produção de biomassa durante 9 dias, sendo monitorizadas quanto à densidade ótica, concentração celular e consumo de nutrientes, sendo o crescimento significativamente diferente entre elas (densidade ótica de 0.91 para CHL e 1.07 para PSE) ($p < 0.05$), e ambas com completa remoção de nitratos do meio.

A biomassa produzida foi avaliada na sua composição bioquímica, em que as duas estirpes demonstraram um conteúdo semelhante em termos de proteínas (entre os 14-15%), lípidos (16.8% e 17.94%) e monoinsaturados (20 e 23%), mas diferentes em termos de ácidos gordos saturados (17.85% para CHL, mas 36.68% para PSE) e de ácidos gordos polinsaturados (32% para CHL, mas 47% para PSE). Notavelmente, a estirpe *Chlorella* sp apresentou 23.39% de ácidos gordos ómega-3, um ácido gordo essencial uma vez que este não é sintetizado pelos humanos e que não foi detetado em PSE

Tudo isto serve como prova adicional à eficácia de biorremediação de nitratos usando algas locais em vez de as não locais. Esta tese serve também para elucidar as características das algas que podem ser isoladas de efluente hidropónicos, quer filogeneticamente, em termos de composição nutricional e capacidades de biorremediação. Servindo como um possível passo inicial para bioprospecção futura destes mesmos efluentes hidropónicos.

Palavras-chave: Microalgas, Biorremediação, Bioprospecção, Hidropónica, Nitratos

Abstract

Given increasing scarcity of water resources, the treatment of contaminated waters and its efficiency is of high priority, specially removing pollutants from it. A promising methodology is bioremediation, where microalgae are used for the removal of nutrients, being even considered one of the most promising strategies.

Samples from hydroponic drainwater were collected from which seven microalgal strains were isolated and identified by DNA extraction, sequencing and phylogenetic inference. Their growth was compared via competition assay, in which *Chlorella* sp. (CHL) and *Pseudochlorella* sp. (PSE) strains were the most effective and were selected for further studies.

In terms of growth, we found that the two selected strains showed a significantly higher ($p < 0.05$) growth, assessed through optical density (OD=1.03-1.08 when compared to those of other isolated strains (lowest OD=0.31).

Several water pretreatment methods were tested: UV radiation, chlorine treatment and filtering, with UV proving to be the most effective. UV radiation was found to improve growth when compared to control group ($p < 0.05$), showing a peak OD of 0.69 for CHL and of 0.58 for PSE, with a 99% nitrate removal for PSE and 62% for CHL, the highest for each strain in both cases. Additionally, the strains were also co-cultivated with *Tetradismus obliquus*, a microalgal strain previously studied regarding nutrient removal. Both novel strains were more effective in the bioremediation process, having a higher growth performance than *Tetradismus obliquus* reaching higher cell concentrations. Finally, they were cultivated in photobioreactors for scale-up and biomass production. Both strains presented similar growth and nitrate removal. The produced biomass was evaluated for biochemical composition, with both strains presenting similar protein content (14-15%) and PUFAs (42-47%) relative to other microalgae, which may be useful in feed industries.

All this adds to the existing body of evidence for the effectiveness of bioremediation using locally bioprospected microalgal strains.

Keywords: Microalgae, Bioremediation, Bioprospection, Hydroponics, Nitrates.

Index

| | |
|---|----|
| RESUMO | 4 |
| ABSTRACT | 7 |
| INDEX | 8 |
| GLOSSARY | 10 |
| 1. INTRODUCTION | 12 |
| 1.1. IMPACTS OF AGRICULTURE | 12 |
| 1.2. MITIGATION OF WATER CONTAMINATION | 13 |
| 1.3. MICROALGAE | 14 |
| 1.4. BIOPROSPECTION | 20 |
| 2. OBJECTIVES | 24 |
| 3. MATERIALS AND METHODS | 25 |
| 3.1. ISOLATION | 25 |
| 3.1.1. TRADITIONAL METHODS | 25 |
| 3.1.2. FLOURESCENT ACTIVATED CELL SORTING (FACS) | 26 |
| 3.2. IDENTIFICATION | 26 |
| 3.2.1. MICROSCOPY | 27 |
| 3.2.2. DNA EXTRACTION AND PCR AMPLIFICATION | 27 |
| 3.2.3. SEQUENCING | 30 |
| 3.2.4. BLAST AND TREE | 30 |
| 3.3. SCALE UP | 30 |
| 3.4. LAB-SCALE TRIALS | 31 |
| 3.4.1. STRAIN SELECTION | 31 |
| 3.4.2. DRAINWATER PRE-TREATMENT | 32 |
| 3.4.3. CO-CULTIVATION | 32 |
| 3.5. PRODUCTION OF BIOMASS IN 1-L BUBBLE COLUMNS | 33 |
| 3.6. CULTURE MONITORING | 34 |
| 3.6.1. OPTICAL DENSITY | 34 |
| 3.6.2. CELLULAR CONCENTRATION | 34 |
| 3.6.3. NUTRIENT CONSUMPTION | 35 |
| 3.7. BIOCHEMICAL COMPOSITION | 36 |
| 3.7.1. PROXIMAL COMPOSITION | 36 |
| 3.7.2. FATTY ACIDS PROFILE | 36 |
| 3.8. STATISTICAL ANALYSIS | 38 |
| 4. RESULTS AND DISCUSSION | 39 |
| 4.1. IDENTIFICATION | 39 |
| 4.1.1. PHYLOGENY TREES | 39 |

| | |
|---|----|
| 4.2. LAB-SCALE TRIALS | 48 |
| 4.2.1. GROWTH PERFORMANCE | 48 |
| 4.2.2. WATER TREATMENT | 50 |
| 4.2.3. COMPETION | 53 |
| 4.3. PRODUCTION OF BIOMASS IN 1-L BUBBLE COLUMNS | 54 |
| 4.3.1 BIOCHEMICAL COMPOSITION | 55 |
| 4.3.1.1 PROXIMATE COMPOSITION | 55 |
| 4.3.1.2 FATTY ACIDS | 55 |
| 5. CONCLUSION | 58 |
| 6. REFERENCES | 59 |
| 7.ANNEX I | 74 |

GLOSSARY

aLRT - Approximate Likelihood Ratio Test

a.u - Arbitrary Units

CC - Cellular Concentration

CHL - *Chlorella* sp.

CL+ - Chlorine treatment

COD - Chemical Oxygen Demand

CT - Control

DES - *Desmodesmus* sp.

DNA - Deoxyribonucleic acid

DWF - Filtered Drainwater

EU - European Union

FACS - Fluorescence-activated cell sorting

FAME - Fatty Acid Methyl Ester

FL3 - Channel measuring chlorophyll autofluorescence

FSC - Forward Scatter, an estimator of cell size

HEPES - (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

ITS - Internal Transcribed Spacer region

MUFA - Monounsaturated Fatty Acids

NB+ - NutrientBloom+

NCBI - National Center for Biotechnology Information

n.s.- Not specified

OD - Optical Density

PBR - Photobioreactors

PCR - Polymerase Chain Reaction

PerCP-Cy5 - Channel measuring chlorophyll autofluorescence

PSE - *Pseudochlorella*

PUFA - Polyunsaturated Fatty Acid

Rpm - Rotations per minute

SFA - Saturated Fatty Acids

SNS - *Tetrademus obliquus*

SSC - Side scattering, an estimator of inner cell complexity

STI - *Stichococcus* sp.

SCE - *Scenesdemus* sp.

UV - Ultraviolet

WW - Wastewater

1. Introduction

1.1. Impacts of agriculture

European waters remain under pressure due to increased population and industrial activity, resulting in poor quality surface and groundwater, which impacts the ecological equilibrium of the surrounding ecosystems. This leads to non-compliance with the Water Framework Directive and the reduction of available drinking water (Kristensen, 2018). Several of these human activities lead to the production of inorganic compounds (J. Singh et al., 2020) like nitrites, nitrates, phosphates, and ammonium as well as heavy metals (Rekha Kathal, 2016). These inorganic compounds can have an extremely negative impact when released into the environment, leading to eutrophication (Carpenter, 2005), groundwater contamination, and destruction of biodiversity (Rashmi et al., 2022).

European Union (EU) farms used 157 million hectares of land for agricultural production in 2020, 38% of the total land area of the EU (Eurostat, 2022). Because of the extent of crop production, agriculture is the primary source of water contamination, as it leads to diffused pollution from nitrates and pesticides that leach to the groundwater, affecting over 18% of the total area of groundwater bodies (Kristensen, 2018). Additionally, the overuse of fertilizers also enriches the waters with ammonium, phosphate (Vaccari, 2009) and potassium (J. Singh et al., 2020) from non-organic fertilizers, as well as heavy metals such as Hg, Cd, As, Pb, and Cu and high radionuclide concentrations (J. Singh et al., 2020).

Anthropogenic nutrient pollution is mainly caused by agricultural fertilizers, where 81.7 million tonnes of nitrogen are used. From this, approximately half of all N currently used reaches global croplands (Martínez-Dalmau et al., 2021). With this extreme use, N pollution levels is expected to increase up to 150% of current levels by 2050, with the agricultural sector accounting for 60% of this increase.

Phosphorus is a finite resource and an essential nutrient for optimal biological functioning (Brownlie et al., 2023). A sizeable percentage of phosphorus also accumulates in soils due to excessive use of fertilizer, animal manure, or municipal waste application and becomes susceptible to transport via surface runoff, which can result in eutrophication in surface waters. Hence, phosphorus is a serious concern for most aquatic ecosystems and because of the nature of P-based compounds, unlike other nutrient cycles, transfer of this nutrient is slower from living organisms into soil, water, and sediment, potentially

taking millions of years for oceanic phosphorus to transform into sediments and rock formations. As a result of all of this, phosphate rock is a non-renewable resource that is being used at a distressing rate. It is estimated that the current phosphate rock and reserves will become exhausted during the next 50 years (Iqbal, 2023). Phosphorus' future scarcity and consequent increasing costs, which threatens food security, requires urgent measures and alternatives (Vaccari, 2009).

Agriculture accounts for approximately 70% of all freshwater withdrawals globally (Siebert et al., 2010), with expectations to increase by 20% to 30% by 2050 (Boretti & Rosa, 2019) due to a combination of population growth, urbanization, and climate change, with serious impacts on food production and security, water supply, industrial production, and energy production (Siebert et al., 2010).

The main way to address water scarcity and nutrient contamination is waste mitigation (lessening the amount of water and nutrients being used through more efficient methods), as well as through bioremediation and recovery, in which living organisms degrade and recycle polluting or contaminating substances.

Conventional farming started shifting towards more sustainable alternatives that lessen their impact on the environment such as soilless farming. This emerging technique consists of plants growing without soil, receiving a nutrient solution adequate for each growth phase. Soilless farming employs new techniques for decreasing the use of water and nutrients, including irrigation to complement soil moisture deficit, sub-surface drip irrigation (Camp, 1998), and water run-off capture (Sample & Liu, 2014).

Soilless farming includes systems like aeroponics and hydroponics. In aeroponic systems, plants are supported by plastic or polystyrene panels and arranged horizontally at the top of the growing container. In hydroponic cultivation, plants are grown without soil and their roots are exposed to a nutrient rich water-based solution (Arumugam, 2021). Currently, about 3.5% of the world's crops are produced using this system (Joshi et al., 2022), with 20% of agricultural production in most European countries being accomplished in hydroponic systems (García, 2018).

1.2. Mitigation of water contamination

Because of the above-mentioned challenges, it is important to apply methods to mitigate water contamination. Nitrate removal can be accomplished by the application of methods

such as: a) reverse osmosis, considered as a conventional method, where a partially permeable membrane is used to separate ions, unwanted molecules and larger particles from water by applying pressure to counteract the generated osmotic pressure, so that a treated permeate is produced (Warsinger et al., 2016); b) distillation, where water is evaporated and then subsequently condensed back into liquid in a different container, a technique used for desalinization via a direct contact membrane (Gryta et al., 2006; Boubakri et al., 2015); c) electrodialysis, also a conventional method (Shrimali & Singh, 2001), where the transport of ions through non-selective semipermeable membranes under the driving force of a direct current and an applied potential is used (Mostert & Frylinck, 2003); and d) bioremediation, where living organisms, primarily microorganisms such as microalgae, bacteria and fungi (Vidali, 2001) are often used to degrade polluting or contaminating substances into less toxic forms, or down to a concentration below the limits established by regulatory agencies (Lynch & Moffat, 2005).

Despite remediation methods not being new, interest in bioremediation solutions has increased exponentially (Pacheco et al., 2020; Breil et al., 2022). Microorganisms such as microalgae have been extensively used for wastewater treatment, being even considered one of the most promising bioremediation solutions used for removal and recovery of nutrients, such as nitrogen and phosphorus (Chatzissavvidis, 2014; Alazaiza et al., 2022; Abdelfattah et al., 2023). There has been a continuous increase in studies on this subject, where between 2011 and 2018, 2578 articles were published on this topic in comparison to only 250 between 2001 and 2010, showing an increased interest that is likely to continue.

1.3. Microalgae

Microalgae can be defined as photosynthetic microorganisms that are present within the majority of the world's ecosystems, and can convert carbon dioxide, water, and inorganic nutrients into oxygen and bio-compounds such as proteins like phycobilins, carbohydrates like starch, and β -glucans when grown photoauto- or mixotrophically (Ruane et al., 2010; Ekevwe, 2018; Tan et al., 2020) In contrast to higher plants, microalgae are typically unicellular, existing individually or in colonies, which can form chains, cenobia, or cell agglomerates with partial differentiation (e.g., *Volvox*; El-Bawab, 2020) or covered by mucilage (e.g., palmelloid cells of *Chlamydomonas* and *Euglena*; (Visviki & Santikul, 2000). They can be grown using non-potable water on non-arable

land (Dragone, 2010) and have higher CO₂ fixation and growth rates, when compared to higher plants (Kumar et al., 2010). About 50% of the total photosynthesis on the planet is done by microalgae, being thus pivotal in the environmental carbon sequestration worldwide.

Among microalgae, the phylum Chlorophyta (green algae), which contain chlorophyll *a* and chlorophyll *b* and store energy as starch (Hoek, 1995) is one of the most relevant taxa. Within Chlorophyta, there are two main freshwater classes, namely Chlorophyceae, with common Scenedesmaceae genera such as *Desmodesmus* and *Tetradesmus*, and Trebouxiophyceae. a class with genera like *Chlorella* (family *Chlorellaceae*) and *Stichococcus* (family *Stichococaceae*) (Guiry et al, 2024).

Chlorophyceae algae show a wide variety of cell organizations. When unicellular, they can present coccoid cells and colonial morphologies; when multicellular, they can occur as simple flattened thalli, displaying unbranched and branched filaments (Leliaert et al., 2012). The flagellar basal bodies of most chlorophycean green algae can be arranged in a clockwise direction or are directly opposed (Lemieux et al., 2015). More recently, Chlorophyceae that have been studied for their bioremediation capabilities include *Scenedesmus* (Pham & Bui, 2020) or *Desmodesmus* (Pandey et al., 2019).

Trebouxiophyceae algae are typically coccoid or ellipsoid unicells, filaments, blades and colony-forming species of marine or land green algae (Muggia et al., 2018), with some having parasitic heterotrophic lifestyles after having lost their photosynthetic capacity (Lemieux et al., 2014) and a few such as *Chloroidium*, which have had their bioremediation properties studied (Aketo et al., 2020).

Microalgal biomass can be employed in various industries, such as food and feed, cosmetics, nutraceuticals, pharmaceuticals, among many others (Sreekala, 2019). The carotenoids extracted from algal biomass can be used as colourants, antioxidants, nutritional supplements, and nutraceuticals by the aquaculture, cosmetic, food, and ornamental fish industries (Varela et al., 2015).

Additionally, microalgae require high carbon, nitrogen, phosphorus, and water consumption. As wastewater is an abundant source of these, the use of microalgae for water treatment has been increasing, especially since using these waters as a culture medium helps to reduce production costs. At the end of the bioremediation process, two valuable products are obtained: microalgal biomass and clean water. This represents a

sustainable process in which valuable metabolites and products are obtained within the biomass and can be refined from it (Carpenter, 2005). Using microalgae or fungi over other forms of bioremediation also has advantages, such as their ease of operation, potential lower operation costs due to the production of additional useful biomass (Silva et al., 2019), having a high adsorption efficiency and utilizing readily available remediators (Abdelfattah et al., 2023). As a result, microalgae bioremediation has had a high potential for wastewater treatment, including hydroponic effluent (Renuka et al., 2018).

A literature review of bioremediation studies testing microalgae growth and nitrate (and in some cases other nutrients) removal from wastewater is presented in Table 1.1. Among recent reports, 7 studies were on hydroponic wastewater (Larsdotter et al., 2010; Saxena & Bassi, 2013; Lee et al., 2018; Salazar et al., 2021; Delrue et al., 2021; Yousif et al., 2022; Salazar et al., 2023) and 6 focused on effluent from municipal wastewater treatment plants, because those facilities typically use biological degradation for the removal of polluting inorganic elements such as nitrogen (Abdel-Raouf et al., 2012). However, all 23 studies measured the removal rate of nitrates, with a removal rate greater than 40% with 11 of them reporting removal rates greater than 95% (Phang et al., 2000; Lee, 2002; Beuckels et al., 2015; Pandey et al., 2019; Tossavainen et al., 2019; Arif et al., 2020; Tamil Selvan et al., 2020; Delrue et al., 2021; Yousif et al., 2022; and Salazar et al., 2023).

Table 1.1 - Examples of the bioremediation using microalgae, for different wastewater treatment. COD: Chemical Oxygen Demand; WW: wastewater; n.s.: not specified

| Microalgae | Wastewater source | Contaminant | Removal rate | Volume Used (L) | Average growth rate | Source |
|--|-----------------------------------|---|----------------------------|------------------------|----------------------------|-----------------------|
| <i>Arthrospira platensis</i> | Digested sago starch factory | COD Ammoniacal-nitrogen phosphate | 98.0% 99.9% 99.4% | 213 | 0.51 | Phang et al., 2000 |
| <i>Chlorella</i> sp. | Municipality | Ammonium Phosphorus COD | 82.4% 90.6% 83% | 0.3 | 0.41 0.43 0.34 | Wang et al., 2010 |
| <i>Chlorella</i> sp. <i>Desmodesmus</i> sp. <i>Scenedesmus</i> sp. | Domestic and dairy effluent WW | COD Nitrate Total phosphorus | 87.85% 97.75% 91.95% | 16.8 | 0.23 0.14 0.20 | Pandey et al., 2019 |
| <i>Chlorella kessleri</i> | Urban WW | Nitrogen | 98.6% | n.s | ~0.1 | Lee, 2002 |
| <i>Chlorella sorokiniana</i> | Coffee cassava flour, industrial | Nitrogen Phosphorus COD | 90.4% 57% 80% | 1.5 | ~0.1 | Melo et al., 2022 |
| <i>Chlorella vulgaris</i> | Hydroponic WW | Nitrogen Phosphorus | 98.5% 99% | 50 | 0.15 | Yousif et al., 2022 |
| <i>Chlorella vulgaris</i> <i>Scenedesmus obliquus</i> | Urban WW | Nitrogen | 100% | 4 | n.s. | Beuckels et al., 2015 |

| | | | | | | |
|--|-----------------------------|---|------------------------------|-------|--------------|--------------------------|
| <i>Chlorella vulgaris</i> <i>Scenedesmus obliquus</i> | Urban WW | Total nitrogen Total phosphorus COD | 84%-95% 92%-95% 36-63% | 150 | n.s | Gouveia et al., 2016 |
| <i>Chlorella sorokiniana</i> <i>Parachlorella kessleri</i> | Urban Treatment Plant WW | Total nitrogen Total phosphorus | ~100% | 0.3 | 0.15 0.16 | Arif et al., 2020 |
| <i>Chlorococcum</i> sp. <i>Selenastrum</i> sp. | Hydroponic WW | Nitrate Phosphate | 18-52% 40-98% | >1000 | 0.36-0.76 | Salazar et al., 2021 |
| <i>Chloroidium</i> sp. <i>Parachlorella kessleri</i> <i>Saccharophilum</i> sp. <i>Tetraselmis</i> sp. | Municipal WW | Nitrogen Phosphorus | 60–100% | 2.9 | n.s | Aketo et al., 2020 |
| <i>Dunaliella salina</i> | Hydroponic WW | Nitrates | 80% | 2.5 | 0.2 | Saxena & Bassi, 2013 |
| <i>Euglena gracilis</i> <i>Selenastrum</i> sp. | Aquaculture WW | Nitrates Phosphate | 98.9–99.5% 98.4–99.8% | 294 | 0.27-0.38 | Tossavainen et al., 2019 |
| <i>Haematococcus pluvialis</i> , <i>Monoraphidium griffithii</i> , <i>Selenastrum</i> sp. | Aquaculture system WW | Phosphate Nitrate | 99% 40% | 250 | 0.47 | Calderini et al., 2021 |
| <i>Monoraphidium</i> sp. | Hydroponic WW | Nitrogen | 80% | 534 | n.s | Larsdotter et al., 2010 |

| | | | | | | |
|-----------------------------------|------------------|--|---------------------------------|--------|----------|---------------------------|
| <i>Neochloris aquatica</i> | Tannery effluent | Nitrate Phosphate | 97.89% 97.5% | 2 500L | n.s | Tamil Selvan et al., 2020 |
| <i>Paracercomonas saepenatans</i> | Hydroponic | Nitrogen | 80% | 0.25 | n.s | Lee et al., 2018 |
| <i>Scenedesmus</i> sp. | Agriculture WW | Ammonium Nitrate Phosphate Total phosphate COD | 93% 84% 97% 96% 93% | 2.4 | 0.3-0.38 | Pham & Bui, 2020 |
| <i>Scenedesmus obliquus</i> | Hydroponic WW | Nitrate Phosphate | 98.2% 7.1 | 0.5 | 0.19 | Delrue et al., 2021 |
| <i>Scenedesmus</i> sp. | Swine WW | Nitrates | 71.81% | 30L | n.s | Zhao et al., 2022 |
| <i>Tetradesmus obliquus</i> | Hydroponic WW | Nitrogen Phosphorous | 100% | 65 | 0.13 | Salazar et al., 2023 |
| <i>Tetraselmis</i> sp. | Urban WW | Nitrogen Phosphorus | 100% | 4 | n.s | Schulze et al., 2017 |

1.4. Bioprospection

One important aspect of microalgal bioremediation is the use of local strains isolated from the water source. For this purpose, a process referred to as **bioprospection** allows for the isolation of naturally occurring microalgae species from within a given area (Bohutskyi et al., 2015). This process is more advantageous since these strains are easier to obtain due to being locally sourced. Moreover, they are already adapted to the surrounding ecosystems and abiotic conditions, resulting in higher productivity, relative to non-local strains (Sanchez Rizza et al., 2017). Additionally, the use of autochthonous microalgae species prevents the introduction of new species into that environment, which can have unintended consequences on the local ecosystem. Some examples of the application of a locally sourced microalga is *Tetraselmis striata* CTP4, which was isolated at CCMAR (Faro, Portugal) from Ria Formosa (Pereira et al., 2016) and demonstrated its wastewater treatment capabilities by Schulze et al. (2017); and two microalgal strains, *Micractinium* sp., and *Scenedesmus* sp., isolated from local wastewater environment, ranked among the top strains in terms of maximal grow rate and biomass productivity by Zhou et al. (2011).

Bioprospection can be broken down into several steps, namely i) collection of environmental samples; ii) isolation and identification of microalgae cells; iii) physiological analysis, where the strains are evaluated for their capabilities of growth and nutrient removal; and iv) biochemical analysis, where the composition of their biomass is studied to determine their use and commercialization.

Traditional techniques for isolating microalgae include serial dilutions (Singh et al., 2016), wherein the freshwater sample is repeatedly diluted in a series of wells using liquid medium (Figure 1.1). In a second step, the final suspension is plated on an agar plate containing the same medium, resulting in isolated colonies. Each colony can then be expanded via streaking on agar plates (Figure 1.2). Other methods for isolation include micropipette washing technique, centrifuge washing and streak plating technique (PARVIN et al., 2007).

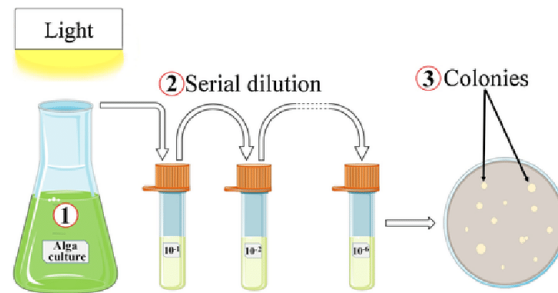


Figure 1.1-Serial dilution of algal cultures to get isolated colony-forming units on an agar plate (Dong et al., 2020).

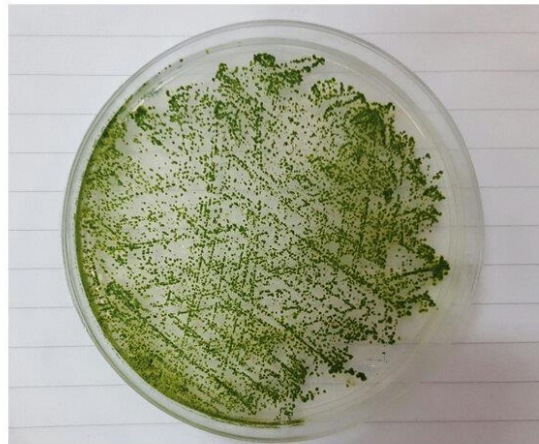


Figure 1.2- Streaking of a microalga on an agar plate containing medium suitable for autotrophic growth.

For micropipette washing technique, a micropipette is employed to pick up an individual cell through repeated trial and error. Ten drops of sterile medium are placed in the groove of a glass slide, then a drop of the microalgal sample will be added and observed under an inverted microscope. Having confirmed the species of the target organism, one drop of the sample will be transferred to the next medium (PARVIN et al., 2007). Centrifuge washing involves a volume of microalgal sample being taken especially from an enrichment culture in at least four centrifuge tubes. These tubes would be centrifuged at 3000 rpm for 15 minutes. After removing the supernatant, the cells would be suspended in fresh sterile water using a vortex mixer until a homogeneous suspension is obtained. The process can be repeated up to 6 times, after which the samples will be transferred to an agar plate (PARVIN et al., 2007.). A more modern and high-throughput methodology is fluorescence-activated cell sorting (FACS), which is capable of concurrent measuring of singular cells. Light scatter angles and fluorescence intensity can be assessed in separate channels creating distinctions between different cell clusters with differing characteristics (Figure 1.3).

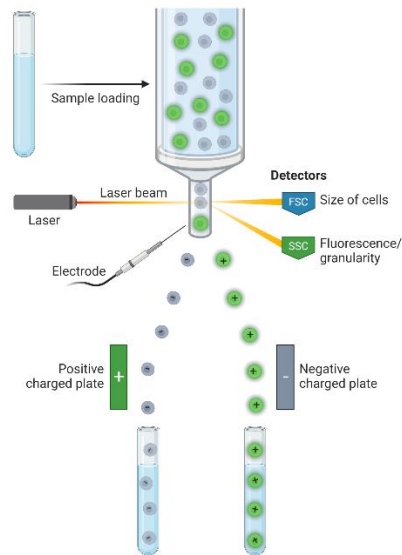


Figure 1.3- Fluorescent activated cell sorting (FACS). The simplified diagram shows a laser beam exciting the sample, resulting in it being attracted based on the resulting charge to either a positive or negatively charge plate. Graphic made with Biorender; by ATT Bioquest.

The resulting information can be visualized in two-dimensional plots combining two of the variables at a given time, such as relative cell size, estimated via forward scatter (FSC); inner cell complexity, estimated via side scatter (SSC) and fluorescence emission by chlorophyll (FL3) channel at 695/40 nm (Pereira et al., 2011; Pereira et al., 2018).

After isolation, a common method for eukaryotic algal identification is the extraction of DNA, followed by 18S rDNA amplification using polymerase chain reaction (PCR). As very often this gene is not enough phylogenetic signal to obtain a classification down to the species level, the internal transcribed spacer region (ITS) region is preferred for complementing the information given by the previous locus (Ghosh, 2019). This analysis is possible because both loci possess conserved and hypervariable regions. The ITS is located between the 18S, 5.8S and 28S rRNA genes and has a higher degree of sequence variation (Ghosh, 2019). As a result, it can be used to better distinguish between different species of the same genus.

After isolation, the strains are typically inoculated in growth media to obtain enough biomass for further analysis. Optimal growth temperature for most algae was found to be at 23 °C and broadly within the range of 15 to 26 °C (Kumar et al., 2010). Other factors influencing algae growth include light, nitrogen source, pH (González-Camejo et al., 2022) and should thus be standardized across samples when performing trials.

The growth rate for a given microalga can greatly vary depending on the treatment applied to the wastewater used for growth, as it can alter parameters like nitrate content and thus the growth of the algae (Kalana et al., 2016). Because of that, it is worth comparing wastewater treatment methods and their effects on the growth rate of a strain to determine which is most optimal when combined with bioremediation, especially due to the possibility of the combination of both methods resulting in either synergy, antagonism, or redundancy.

2. Objectives

The goal of this thesis was to bioprospect and identify novel microalgal strains from a soilless plant production facility to determine which strains showed the highest potential to grow in the drainwater of this facility and their ability for nitrate removal. The final goals were to determine the conditions that maximized bioremediation, while generating treated water, and produced the highest amount of microalgal biomass for further use and/or commercialization.

To achieve these goals, specific objectives were established, namely: 1) evaluate several strains of microalgae that were isolated from drainwater samples by means of several trials in order to compare growth between the isolated samples and different water treatment strategies and then determine which strain and strategy were the most effective; 2) perform a co-cultivation trial to evaluate how the isolated strains were able to compete with an established strain; 3) carry out a laboratory scale growth trial in 1-L bubble columns under all the previously selected conditions to perform a final optimization step; and 4) evaluate the biochemical composition of the strains to determine the quality of the produced biomass.

3. Materials and Methods

3.1. Isolation

Drainwater samples were collected from water deposits at a soilless production company, Grupo Hubel (Tavira, Portugal) to isolate autochthonous microalgae strains.

Microalgae strains gathered from Hubel's hydroponic effluents were received in the form of both liquid and solid media, in 250 mL Erlenmeyers and agar plates from company Necton S.A. The former were then transferred onto solid media using agar plates made using autoclaved tap water (TW), agar powder in a 15 g/L concentration with an additional 1 mL/L of NutriBloom® Plus (NB+) algal medium, as well as liquid media in the form of 250 mL Erlenmeyers' flasks containing ~50 mL of freshwater and NB+ in the same concentration. Several methods of isolation were used for assessment of the strains present in this environment.

3.1.1. Traditional methods

The collected samples were transferred to Erlenmeyer's flasks of 100 mL and grown in an incubation shaker (Jeiotech IST-3075R, Daejeon, Korea) under constant temperature (20 °C), constant mixing (120 rpm) and constant light (50 $\mu\text{mol}/\text{m}^2/\text{s}$). Additionally, the samples were also inoculated in agar plates (15 g/L), supplemented with NB+ at a final nitrate concentration of 4 mM. Serial dilutions were also prepared in 24-well plates, until a final dilution of 1:32 of the original sample concentration (Figure 3.1).

Before inoculation, all materials were sterilized in an autoclave at 121 °C for contamination prevention. When microalgae colonies started to develop in the solid medium, they were transferred onto new agar plates and, if isolated, into Erlenmeyer's flasks using an inoculation loop and placed back into the incubation shaker.

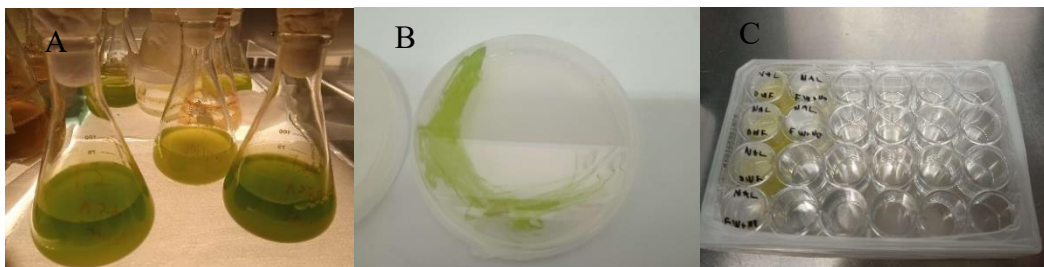


Figure 3.1- Example of methods applied for locally isolated microalgae. (A) Environmental sample in liquid medium; (B) Agar plate containing a streaked isolate and (C) 24-well plate used for serial dilution.

3.1.2. Fluorescence-activated cell sorting (FACS)

To isolate microalgae using fluorescence-activated cell sorting, i.e., FACS (Figure 3.2), several events were distributed in 96-well plates containing algal growth medium solidified with 1.5% agar. Samples of 100, 10 and 1 event, with a total of 32 wells were taken. Events exhibiting chlorophyll autofluorescence were separated from unwanted events (mostly non-photosynthetic bacteria and fungi) as well as cell and growth medium debris (Pereira et al., 2018). Figure 3.3 shows an example of the combination of the channel for detecting autofluorescence emission by chlorophyll (PerCP-Cy5) and inner cell complexity (SSC) in order to better distinguish prokaryotic from eukaryotic cells.



Figure 3.2-Flow cytometer BD FACSAria II (Becton Dickinson, USA) (Left) used to gather samples in a 96-well plate (right).

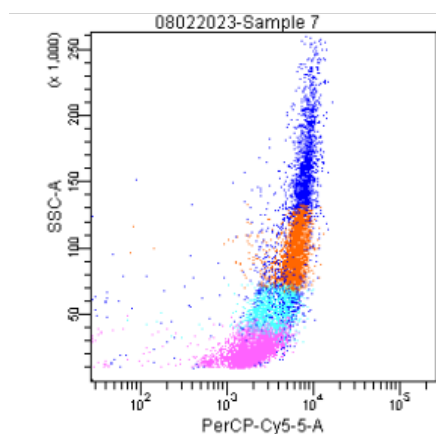


Figure 3.3- Example of FACS results, clusters indicate different strains, which have different absorbance. Side scatter (FSC) measures inner cell complexity. Cy-5 measures fluorescence emitted by pigment phycocyanin.

The strains that were successfully isolated were placed onto 96-well plates and upon demonstrating growth were transferred to solid medium in the form of agar plates and later inoculated in liquid medium in 100-mL Erlenmeyer's flasks. Cells were incubated in a growth chamber at the temperature of 20°C and constant luminosity (50 $\mu\text{mol}/\text{m}^2/\text{s}$).

3.2. Identification

3.2.1. Microscopy

The isolated strains were observed by optical microscopy, and pictures were taken using an optical Axioscope 5 (Zeiss, Germany) microscope at $\times 100$ amplification with immersion oil, using a Colibri 3 attachment for taking micrographs of the samples (Figure 3.4).



Figure 3.4- Axioscope 5 (Zeiss, Germany) microscope.

3.2.2. DNA extraction and PCR amplification

For DNA extraction and strain identification, 18S rDNA sequencing was performed. For DNA extraction, the Quick DNA miniprep kit (Zymo Research, USA) was used, following the manufacturer's procedure. Briefly, 2 mL of sample were taken of each strain, along with colonies collected with an inoculation loop from the agar plates and placed in a microtube. The microtubes were centrifuged ($7000 \times g$, 5 minutes) and the supernatant was discarded. Genomic lysis buffer was added, the solution was centrifuged at maximum rotation ($20,000 \times g$, 5 minutes), and the supernatant was transferred to a spin column with a collection tube. The samples were again centrifuged at maximum rotation for 1 minute, and afterwards the DNA prewash buffer was added, centrifuged ($7000 \times g$, 1 minute) and the supernatant discarded. In a later step, DNA wash buffer was added, and a new centrifugation occurred ($7000 \times g$, 1 minute). Finally, the spin column was transferred to a microtube, elution solution was added, and a final centrifugation was performed at maximum rotation ($20,000 \times g$) for 30 seconds, so that the extracted DNA was transferred to a new microtube.

For DNA amplification, a polymerase chain reaction (PCR) was performed. For the PCR, a master mix (Table 1.2) was prepared, and 2 μL of each sample were added to 23 μL of mastermix.

Table 1.2: Ingredients used to create a Mastermix for 18s DNA replication.

| Ingredients | $\times 1$ (μL) |
|-------------------------------------|---|
| MilliQ water | 13.3 |
| 5x colorless GoTaq Flexi Buffer | 5 |
| MgCl ₂ solution (25mM) | 2 |
| Primer 18S UniF (10 μM) | 1 |
| Primer 18S UniR (10 μM) | 1 |
| dNTPs (10mM) | 0.5 |
| GoTaq G2 Flexi DNA Polymerase | 0.2 |

The samples were then placed in a Biorad Laboratories thermocycler (Bio-Rad Laboratories, USA) for PCR reaction (Figure 3.5), using the program described in Table 3.1.



Figure 3.5-Thermocycler MyCycler (Bio-Rad Laboratories, USA) used for polymerase chain reaction.

Table 3.1: Thermocycler program used for polymerase chain reaction.

| Program step | Time (minutes) | Temperature (°C) | N° of cycles |
|----------------------|-----------------------|-------------------------|---------------------|
| Initial denaturation | 5 | 94 | 1 |
| Denaturation | 0.5 | 95 | |
| Annealing | 0.5 | 55 | 35 |
| Elongation | 1 | 72 | |
| Final elongation | 10 | 72 | 1 |
| Hold | - | 10 | |

To understand if the PCR was successful, a gel electrophoresis was performed of the resulting samples. Electrophoresis was performed in a 1% agarose gel in 1× TAE Buffer at 120 volts for 45 minutes. The gel was loaded with 5 µL of sample, combined with 1 µL gel red (1:1000 dilution) and 2 µL of Green GoTaq Buffer. The samples were compared to 6 µL of 1k base pairs (bp) NZYDNA Ladder I (NZYTech, Portugal) with 2 µL of Green GoTaq Buffer.

Simultaneously to this, DNA extraction for ITS2 sequence analysis was also performed as part of an internship related to workplan of this thesis, with the results aiding in the interpretation of data derived from the 18S marker gene analysis in this thesis.

Briefly, DNA for ITS2 analysis was also extracted using the Quick DNA miniprep kit (Zymo Research, USA) following the manufacturer's procedure. For DNA amplification, a polymerase chain reaction (PCR) was performed using a primer already designed based on differences of the nucleotides of the second internal transcribed spacer (ITS2), ITS055R (CTCCTTGGTCCGTGTTTCAAGACGGG) and CrN1F (CTGCCAGTAGTCATATGCTTGTCTC) (Marin & Melkonian, 2010). For the PCR, a master mix was prepared, and 2 µL for the resulting samples were added to 23 µL of Mastermix (Table 3.2).

Table 3.2: Ingredients used to create a Mastermix for PCR amplification of the ITS2 marker locus

| Ingredients | x1 (µL) |
|-----------------------------------|----------------|
| MilliQ water | 13.3 |
| 5x colorless GoTaq Flexi Buffer | 5 |
| MgCl ₂ solution (25mM) | 2 |
| Primer CrN1F (10 µM) | 1 |
| Primer ITS055R (10 µM) | 1 |
| dNTPs (10mM) | 0.5 |
| GoTaq G2 Flexi DNA Polymerase | 0.2 |

The samples were then placed in a Biorad Laboratories thermocycler (Bio-Rad Laboratories, USA) for PCR reaction (Figure 3.5), using the same thermocycler program previously described (Table 3.1). A gel electrophoresis was also performed on ITS2 DNA fragments to determine if the PCR was successful, using the same methods as that described for the 18S marker gene.

3.2.3. Sequencing

The DNA sequencing was performed at CCMAR's Sequencing Platform, using an Applied Biosystems SeqStudio Genetic Analyzer and BigDye[®] Terminator v3.1 chemistry. Forward and reverse strands of 18S and ITS2 amplicons were sequenced.

3.2.4. BLAST and Tree

Upon the arrival of the DNA forward and reverse sequences, a consensus alignment employing CLC Genomics Workbench v. 21 software was performed. Afterwards, the National Centre for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) was used to acquire closely related sequences of 18S. In the case of ITS2, the Wuerzburg university ITS2 database (Ankenbrand et al., 2015) was employed.

Sequences were downloaded by means of the CLC Genomics Workbench v. 21 software and, after the 18S or ITS2 coding regions of those sequences being trimmed, they were aligned with the same software. For phylogenetic inference, the Phylogeny.fr platform (Dereeper et al., 2008) was employed for alignment curation, using Gblocks v.0.91b (Castresana, 2000), and a maximum-likelihood phylogenetic tree was generated via PhyML (Guindon et al., 2010) using an SH-like approximate likelihood ratio test (aLRT) to estimate branch support values. Initial tree rendering was carried out with TreeDyn v.198.3 (Chevenet et al., 2006). At a later stage, the tree was rendered using

FigTree v. 1.4.4. Adobe Illustrator 2024 was then used to enhance the placements of the branch support values.

3.3. Scale-up

The scale-up process of the isolated strains took place in an incubator with shaking, as described in section 3.4.1 (Figure 3.6). All cultures were grown in freshwater supplemented with NB+ with 4 mM of nitrates, in a working volume of 50 mL. When cultures reached a high cellular concentration, they were transferred to higher volumes, with replenishment of the culture medium. This process was repeated until 1 L of culture was obtained.

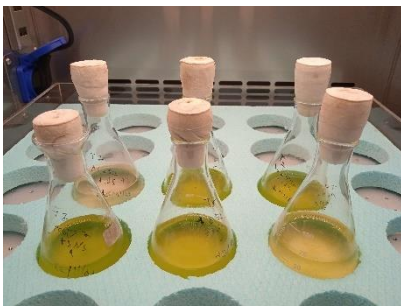


Figure 3.6- Scale-up process of isolated strains, with Erlenmeyer's flasks in an incubated shaker.

3.4. Lab-scale trials

The following lab-scale trials were conducted in an incubator with shaking (Jeitech IST-3075R, Daejeon, Korea), at 20 °C and 120 rpm with a continuous light source (50 $\mu\text{mol}/\text{m}^2/\text{s}$). HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) at 5.95 g/L was added to the culture media and the pH was adjusted to 7.5. The cultures were inoculated with an initial optical density of 0.2 and the nitrate concentration of the cultures was adjusted to be the same as the nitrate concentration present in the drainwater. All samples had an initial working volume of 60 mL in 100 mL Erlenmeyer's flasks. All trials were done in triplicate.

3.4.1. Strain selection

For strain selection, seven isolated strains were selected, and their growth was compared between two different culture media: drainwater and freshwater supplemented with NB+, used here as the control medium (Figure 3.7). The cultures were grown with an initial nitrate concentration of 3.3 mM, which was the nitrate concentration present in the drainwater. The trial had a duration of 7 days and was performed under conditions

outlined in subsection 3.4, during which the cultures had their optical density (OD) measured daily at 540 nm and 750 nm to determine growth. Nitrate concentrations were measured at the beginning and end of the trial. The two best performing strains were selected for ongoing trials.

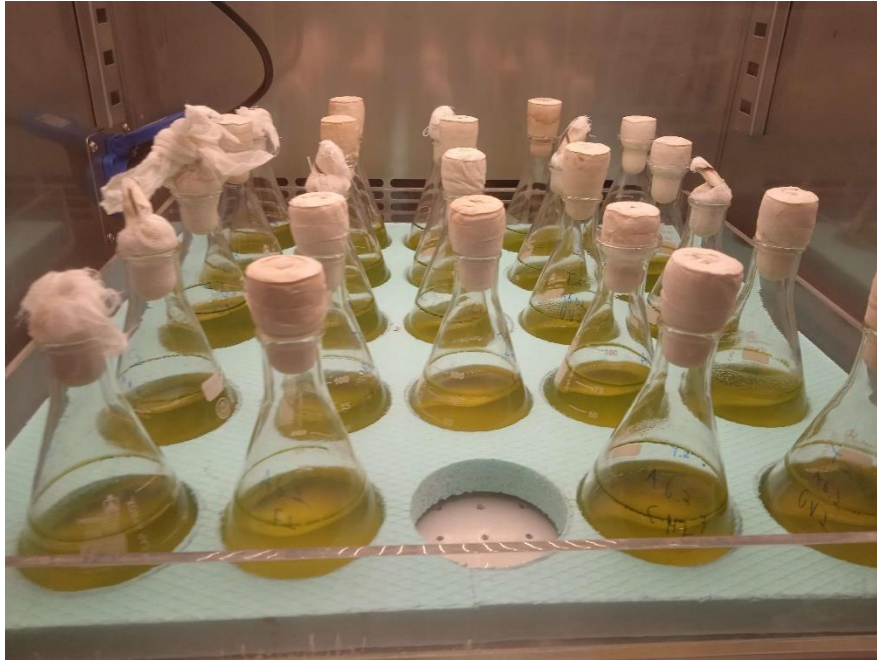


Figure 3.7- Microalgae strains used for lab-scale experiments. The experiments were performed in an incubator with shaking under constant temperature (20 °C), constant mixing (120 rpm) and constant light (Luminous flux: 50 $\mu\text{mol}/\text{m}^2/\text{s}$).

3.4.2. Drainwater pre-treatment

The two best performing strains from the previous trial were cultivated in triplicate in the conditions outlined in subsection 3.4, using four different conditions: **i)** control (drainwater with no treatment); **ii)** filtration using a sterile 0.2 μm filter; **iii)** chemical treatment using sodium hypochlorite, where 1 mL/L of sodium hypochlorite was added to the drainwater for 30 min, and neutralized afterwards with sodium thiosulphate at 0.5 mL/L; and **iv)** UV-treated drainwater, which was exposed to UV-light for 20 minutes. The trial had a duration of 8 days and OD was used for determination of growth. Nitrate concentrations were measured at the beginning and end of the trial.

3.4.3. Co-cultivation

Competition trials, or co-growth, are experimental trials in which the growth rate and other factors of two or more species are compared when they are growing in the same

medium. These assays allow to determine which strains can become dominant in the said medium. The advantage of competition assays is minimizing effects due to uncontrolled experimental variability and thus directly comparing the fitness of two different strains in the same niche at the same time (Visconti et al., 2022).

The two best performing strains were co-cultivated in triplicates, using two different combinations of strains. *Tetradismus obliquus* (SNS0120), a proven effective (Salazar et al., 2023) microalgal bioremediator, was grown together with each of the selected strains obtained from previous trials in the standard conditions described in subsection 3.4. The trial had a duration of 8 days and cellular concentration was used for monitoring growth. Nitrate concentrations were measured at the beginning and end of the trial.

3.5. Production of biomass in 1-L bubble columns photobioreactors

Microalgae biomass of the two selected strains was produced in triplicates using a 1-L bubble column photobioreactors (PBR) (Figure 3.8). Biomass concentration was adjusted by dilution to an OD of 0.2. Drainwater was used as the growth medium and was prepared by filtration using a sterile 0.2 μm filter, with pH adjusted to 7.5 and initial nitrate concentration at 4 mM. Growth was monitored via daily OD measurements, and nitrate measurements were performed every 2 days. At the end of the trial the samples were centrifuged at $7,000 \times g$, freeze dried and stored at $-20\text{ }^{\circ}\text{C}$ until further analysis.

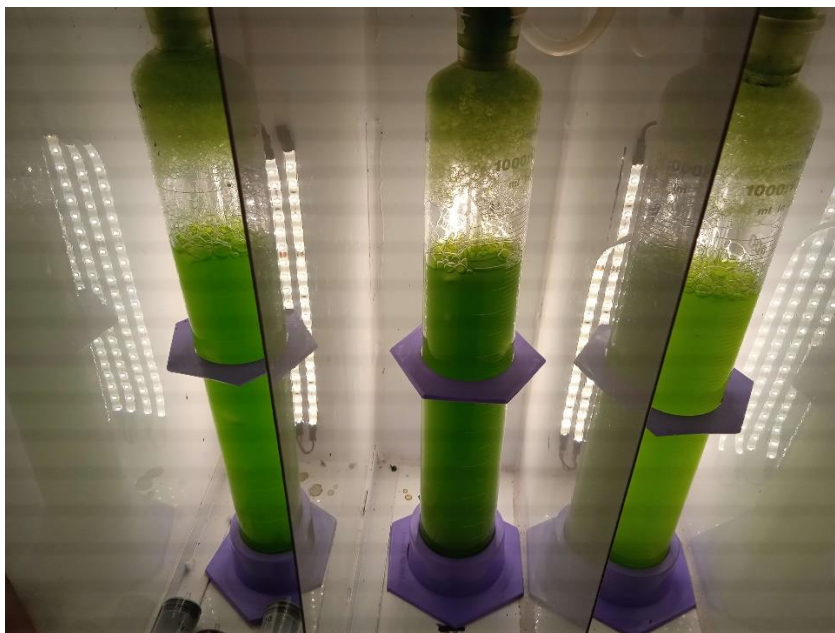


Figure 3.8- 1-L bubble columns photobioreactors (PBR) continuously illuminated and pressurized

3.6. Culture monitoring

3.6.1 Optical density

To measure OD of the cultures, 1-mL aliquots were taken from each sample and 200- μ L sub-aliquots were measured in triplicate, using a Gen 5 Microplate reader (Biotek, USA; Figure 3.9) at 540 nm and 750 nm. When the samples presented an OD measurement higher than 0.8, the samples were diluted and measured again.



Figure 3.9- Gen 5 Microplate reader(Biotek, USA)

3.6.2 Cellular concentration

Cellular concentration (CC) was determined by cell counting using an optical Axioscope 5 (Zeiss, Germany) microscope at $\times 40$ amplification. A 10- μ L aliquot of the cell suspension was placed into a Neubauer chamber according to the manufacturer's procedure. Dilutions were carried out as necessary to ensure between 30 and 300 cells per field. CC was obtained by the following formula:

$$CC \text{ (cells/mL)} = \text{number of counted cells} * (10^4) * \text{dilution}$$

3.6.3 Nutrient consumption

Nitrate concentration

To assess nitrate concentration, 2 mL of each sample was taken and centrifuged at $7000 \times g$ for 5 minutes. Falcon tubes were prepared with a blank solution, containing 9.8 mL of distilled water and 0.2 mL of HCl. For each sample, duplicates were prepared with 9.3 mL of distilled water, 0.2 mL of HCl and 0.5 mL of the supernatant of each sample. Absorbance was measured in quartz cuvettes at 220 and 275 nm using a Double Beam Spectrophotometer UH5300 (Hitachi, Japan) (Figure 3.10). The reading at the latter wavelength is mandatory to detect whether organic matter is also present, which can alter the reading of nitrate concentration (APHA 2000). In cases where absorbance was greater than 0.8, the sample was diluted with control solution. Nitrate concentration was calculated using a pre-established calibration curve between known concentrations of this ion and its absorbance values.



Figure 4.10- Double Beam Spectrophotometer UH5300 (Hitachi, Japan) used for determination of nitrate concentration.

3.7 Biochemical composition

The biomass produced in the 1-L bubble columns PBRs was centrifuged ($4000 \times g$, 10 minutes), frozen and later freeze-dried (Martin Christ Alpha 1-2 LDplus, Osterode am Harz, Germany). After this, the samples were stored at $-20\text{ }^{\circ}\text{C}$ until further analysis.

3.7.1 Proximal composition

3.7.1.1 Protein

Protein content was estimated via elemental analysis of nitrogen content by following procedure provided by the manufacturer, using a Vario EL III (Vario EL, Elemental Analyzer system, GmbH, Hanau, Germany), analysing 1 mg of biomass weighed and encapsulated in small aluminium caps. The final protein content was determined by multiplying the percentage of nitrogen by 4.97% (Templeton & Laurens, 2015).

3.7.1.2 Lipids

To determine lipid content, a modified protocol of the Bligh & Dyer (Bligh & Dyer, 1959) method was used (Pereira et al., 2011). Briefly, between 10-20 mg of biomass was weighed together with glass beads, and 0.8 mL of distilled water and 1 mL of methanol were added to the tubes. The samples were then homogenized in a Retsch MM 400 mixer mill at (30 Hz for 3 minutes). Afterwards, the samples were transferred to glass tubes and 1 mL of methanol and 1 mL of chloroform were added and homogenized in a vortex for 30 s. 1-mL samples of chloroform and 1-mL of distilled water were added to the samples, and between each addition, the samples were homogenized in a vortex for 30 s.

All the resulting tubes were then centrifuged for 10 minutes at room temperature and $2500 \times g$. The separated translucent chloroform was then transferred to new tubes with a Pasteur pipette; 0.7 mL of chloroform were then added to previously weighed tubes and placed in a dry bath at $60\text{ }^{\circ}\text{C}$ until the chloroform was totally evaporated, followed by 15 min in a desiccator until reaching room temperature. The resulting tubes being weighed in a precision scale once more.

The following formula was used to calculate the percentage of total lipids:

$$\% \text{total lipids} = \frac{\left[\frac{(\text{Final weight} - \text{Initial weight}) \times \text{total volume of chloroform}}{\text{Evaporated volume of chloroform}} \right]}{\text{weight of the dried sample}}$$

3.7.2 Fatty acids profile

Fatty acid composition was determined by using a modified protocol of (Lepage & Roy, 1984), as described in Pereira et al. (2012), which is a method based on direct

transesterification and later extraction of the lipidic phase. Freeze-dried biomass (10-20 mg) of each samples were mixed in a reaction vessel with a solution of methanol:acetyl chloride (20:1 v/v), and then homogenized using a Retsch MM 400 mixer mill (30 Hz for 3 minutes). The samples were then put in a VWR precision water bath (Figure 3.11) at 70 °C for 60 minutes (Figure 3.11-3.12) and cooled in ice afterwards.



Figure 3.11- VWR Precision Water Bath



Figure 3.12- Samples in a water bath at 70°C, for derivatization.

Then distilled water (1 mL) and *n*-hexane (4 mL) were added, and the samples were homogenised in a bead beater at maximum rotation ($20,000 \times g$) for two cycles of 30 seconds. Samples were then centrifuged at $2000 \times g$ for 5 minutes, at room temperature. Using a Pasteur pipette, the hexane fraction was transferred to new glass tubes. The centrifugation process was repeated until the hexane fraction was colourless. Anhydrous sodium sulphate was added in excess to precipitate any water that could be present in the hexane fraction, and the fractions were then filtered using 0.22- μm PTFE filters. The hexane was evaporated under a nitrogen gas flow until fully dried and resuspended in 500 μL of HPLC-grade hexane and re-filtered, with the addition of an internal standard (nonanoic acid C9:0) to each sample.

The FAME profiles were analysed using a Scion 456/GC Scion TQ MS (Bruker, USA) equipped with a 30-meter ZB-5MS capillary column (30 × 0.25 mm ID, 0.25 µm film thickness; Phenomenex). Helium was employed as the carrier gas, with a flow rate of 1 mL/min for elution. The injection temperature was maintained at 300 °C in split-less mode. The temperature program included the following steps: starting at 60 °C for 1 minute, ramping up at 30 °C/min to 120 °C, then at 4 °C/min to 250 °C, and finally at 20 °C/min to 300 °C, where it was held for 4 minutes. For the identification and quantification of FAMES, a Supelco® 37 component FAME Mix (Sigma-Aldrich, Sintra, Portugal) was used as the standard to create several calibration curves.

3.8 Statistical Analysis

Statistical analysis was performed using R Studio version 4.4.1. Data was tested for normality and homogeneity using the Shapiro-Wilk test and Levene test, two-way ANOVA and Tukey's HSD post hoc test were performed for the comparison of means of treatments with a confidence interval of 95%. For comparison of two variables (co-cultivation trials and production in 1-L bubble columns), a Student's *t*-test was applied. When normality or homogeneity were absent, non-parametric analysis were performed through the application of the Kruskal-Wallis test and Dunn's post hoc test.

4. Results & Discussion

4.1 Identification

Seven successfully isolated strains were selected for the optimization trials. These strains were observed under the microscope (Figure 4.1) and their morphology was analysed and described.

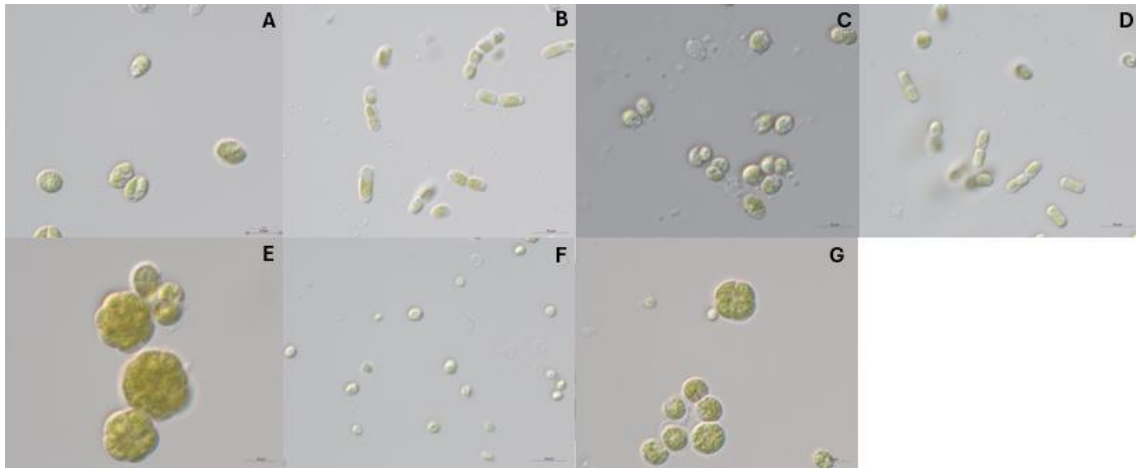


Figure 4.1-Microscopic observations of the isolated strains, using DIC and 100 × objective lens. A- Strain 1; B-Strain 2; C- Strain 3; D-Strain 4; E-Strain 5; F-Strain 6; G- Strain 7.

In terms of morphology, Figure 4.1A resembles a *Desmodesmus* as it is small, non-motile and consists of colonies of 2 cells arranged in sequence. This strain has similarities with that in Figure 4.1C. Figure 4.1B and 4.1D appear to be a *Stichococcus* given their cylindrical cell filaments, usually few-celled (2–4 cells) with rounded ends (Guiry & Guiry, 2020). Figure 4.1C has an appearance not incongruous with its later identification as *Desmodesmus*. Figure 4.1E appears to be a spherical aggregate of cells attached to one another organized in a spherical and free-floating coenobia, which is consistent with being a *Scenedesmaceae* microalga (Guiry & Guiry, 2020). The strain depicted in Figure 4.1F seems to have a *Chlorella*-like morphology as it shows spherical microscopic cells with 2–10 µm diameter (Safi et al., 2014), and Figure 4.1G demonstrates clustering that is exclusive to *Pseudochlorella* relative to *Chlorella*, indicating that this strain might belong to this genus (Darienko et al., 2016).

4.1.1 Phylogenetic Trees

To better identify the strains, phylogenetic trees were generated to infer their relatedness with other microalgal genera and species.

The resulting phylogenetic tree for Strain 1 can be seen in Figure 4.2. While the information is insufficient to determine the exact species, Strain 1 clusters with other *Desmodesmus* taxa with maximum support (1). Strain 1 clusters exclusively with *Desmodesmus* microalgae, as *Scenedesmus subspicatus* is viewed as synonym to *D. subspicatus*. As outgroups to this cluster, one can find a well-defined *Tetradesmus* clade, which branches off early with regard to the remaining *Scenedesmaceae* taxa.

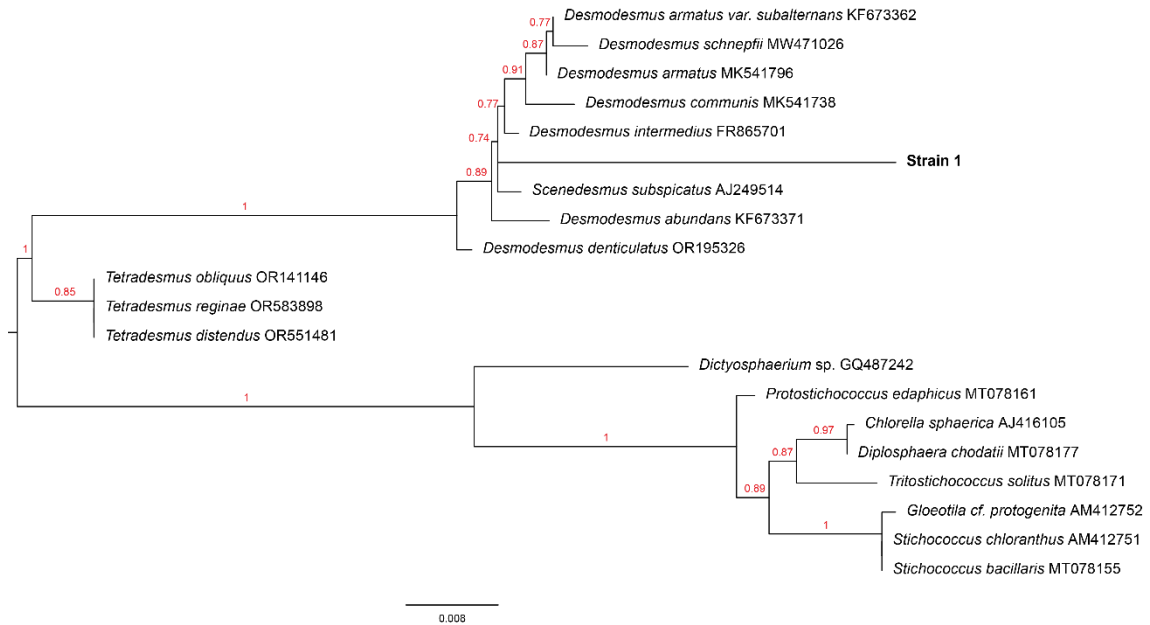


Figure 4.2- 18S Maximum Likelihood phylogenetic tree using the approximate likelihood-ratio test (aLRT) where strain 1 clusters together with a *Desmodesmus subspicatus* sequence and other *Desmodesmus* taxa with a branch support value of 0.72. Other taxa of other genera, including the one containing the outgroup (*Tetradesmus*), branch off this cluster with support values of 1.

It can be said with a high degree of certainty that Strain 2 belongs to the order Prasiolales, as it is grouped with a branch support value of 0.99 as opposed to the Sphaeropleales outgroup. In addition, given its morphology and these results, it likely belongs to the *Stichococaceae* family, as the genus is not possible to be ascertained with the present data.

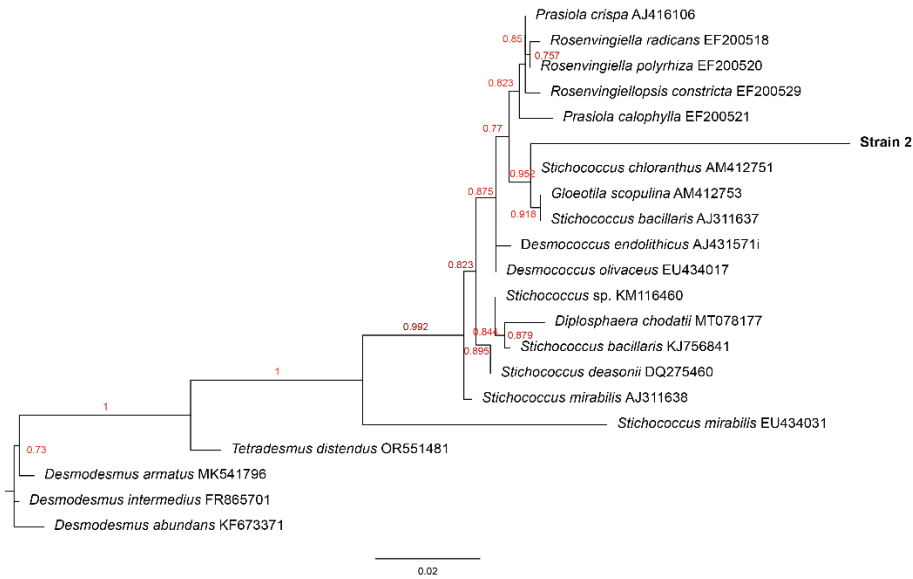


Figure 4.3-18S gene Maximum Likelihood phylogenetic tree using the approximate likelihood-ratio test (aLRT) where strain 2 clusters together with Prasiolales taxa with a branch support value of 0.99. Other taxa of other genera, including the one containing the outgroup (Sphaeropleales), branch off this cluster.

The resulting phylogeny tree for Strain 3, can be seen in the Figure 4.4. While the information is not enough to confidently identify (0.76) a specific species, the genus *Desmodesmus* is likely to be the correct one, as the *Desmodesmus* clade has maximum support (1.0). Therefore, Strain 3 is tentatively identified as a microalga belonging to the genus *Desmodesmus*.

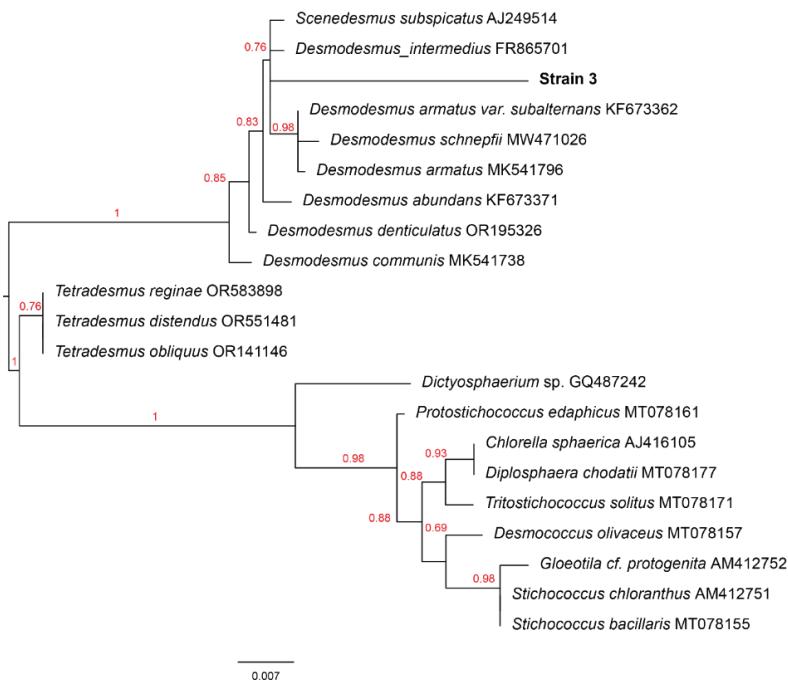


Figure 4.4- 18S Maximum Likelihood phylogenetic tree using the approximate likelihood-ratio test (aLRT) where strain 3 clusters together with a *Desmodesmus subspicatus* sequence and other *Desmodesmus* taxa with a branch support value of 0.755. Other taxa of other genera, including the one containing the outgroup (*Tetradismus*), branch off this cluster with maximum support (1).

It can be said with a high degree of certainty that Strain 4 belongs to the order Prasiolales as it clusters together with sequences of this taxon with maximum support (1) as opposed to the outgroup of Sphaeropleales. In addition, given its morphology and these results it likely belongs to the Stichococaceae family, although this is not strongly supported.

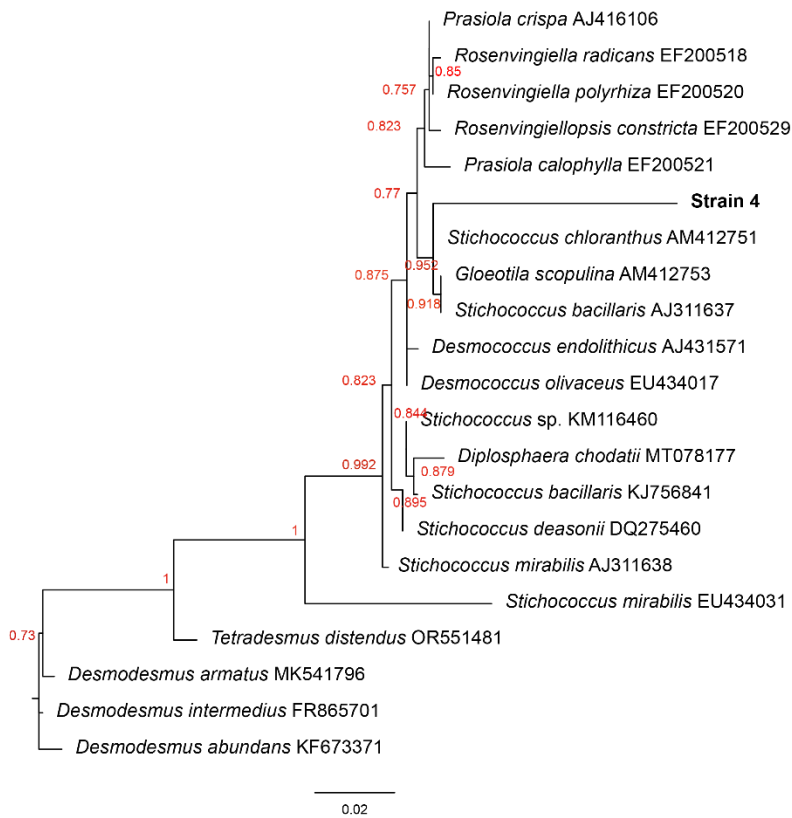


Figure 4.5- 18S Maximum Likelihood phylogenetic tree using the approximate likelihood-ratio test (aLRT) where Strain 4 clusters together with Prasiolales Order taxa with a branch support value of 0.99. Other taxa of other genera, including the one containing the outgroup (Sphaeropleales), branch off this cluster.

The phylogenetic tree for strain 5 is shown on Figure 4.6. Once again, Strain 5 clusters together with *Desmodesmus* spp. taxa in a cluster with maximum support. Unfortunately, due to the quality of the sequence obtained, it was not possible to determine the species within this genus, although these results suggest it to be closely related with *D. subspicatus*.

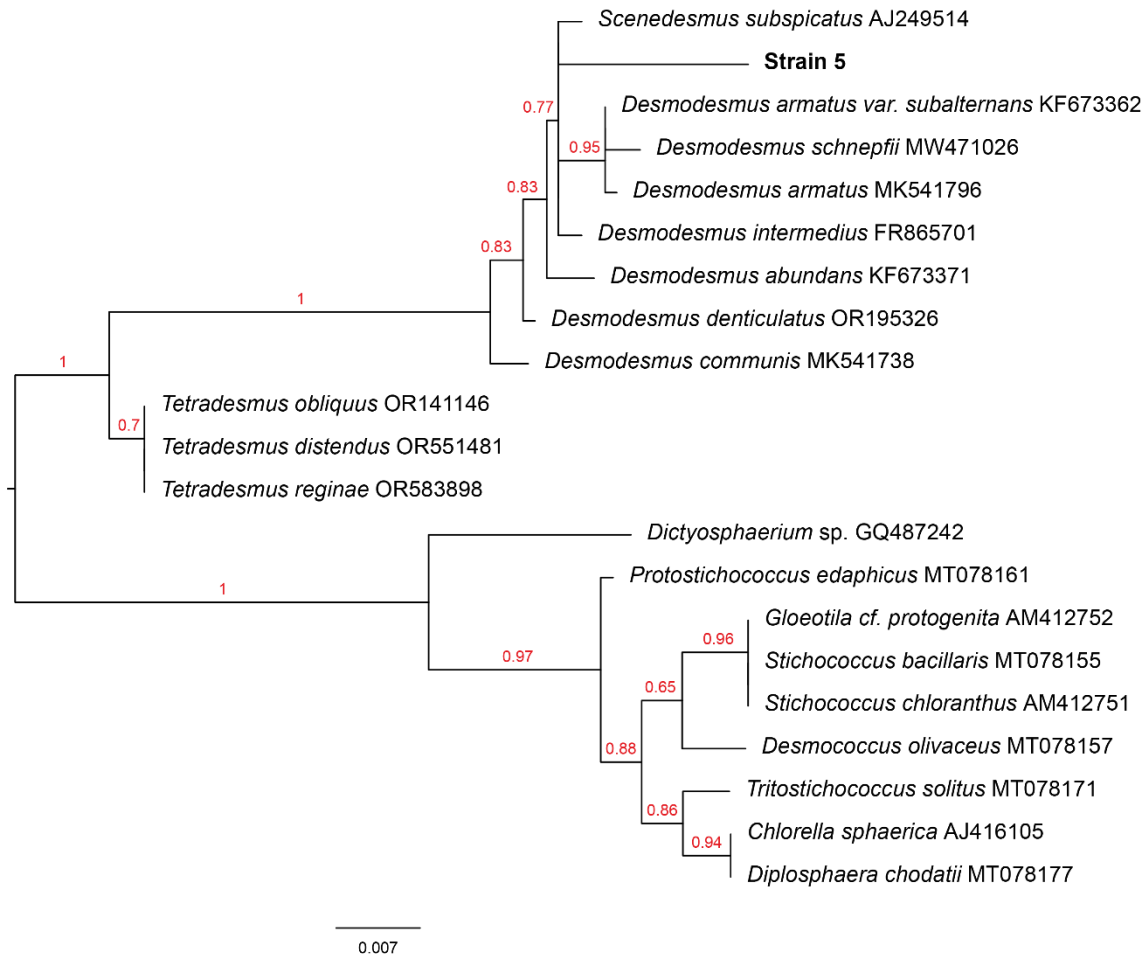


Figure 4.6-18s gene Maximum Likelihood phylogenetic tree using the approximate likelihood-ratio test (aLRT) where Strain 5 remains in its own branch with a support of 1. Likely insufficient data.

The phylogenetic tree for Strain 6 is presented in Figure 4.7. While its species or genus cannot be determined, as there is no isolated branch with only Strain 6 and a single species or genus, the results suggest that it clusters with almost maximum support (0.998) with other taxa belonging to the *Chlorella* clade, which includes genera such as *Didymogenes* and *Actinastrum* (Heeg & Wolf, 2015).

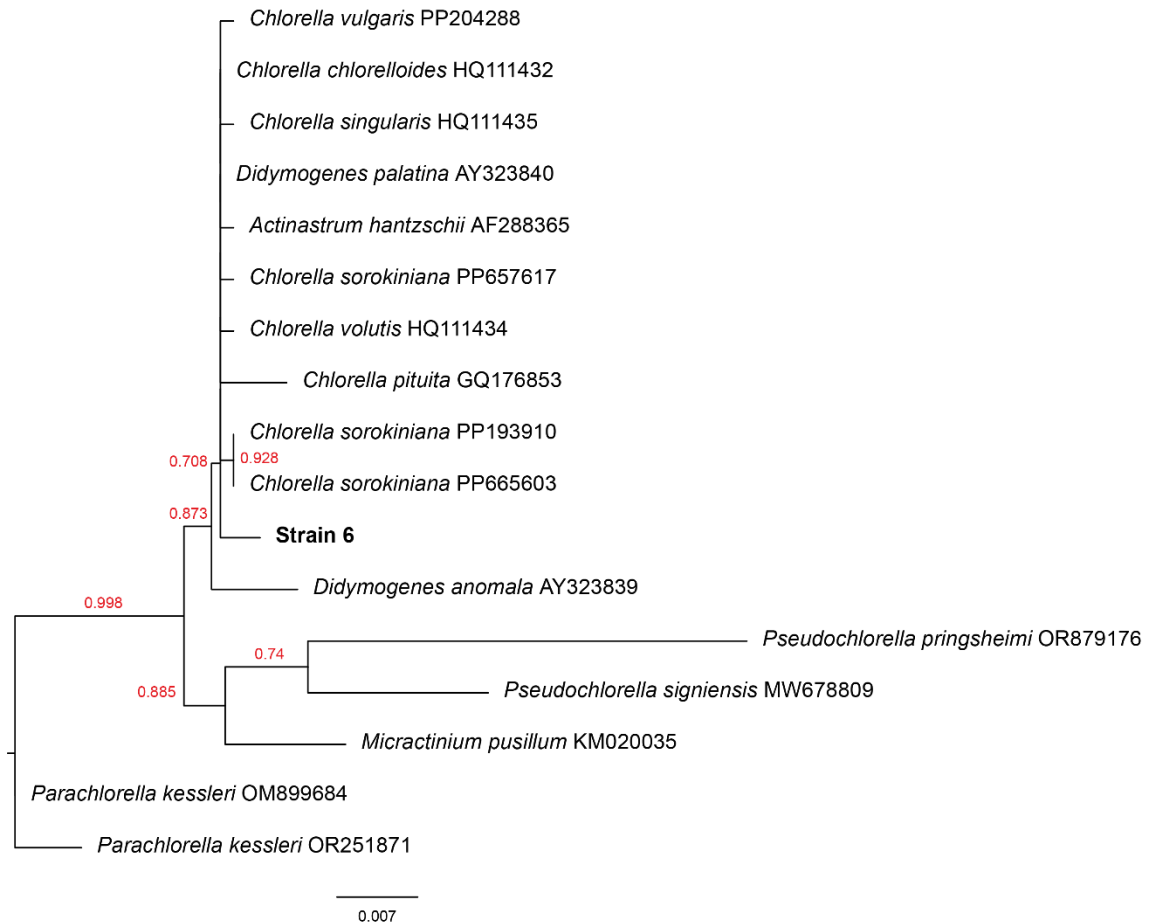


Figure 4.7- 18s gene Maximum Likelihood phylogenetic tree using the approximate likelihood-ratio test (aLRT) where Strain 6 clusters together with a *Chlorella* taxon with a branch support value of 0.708. Other taxa of other genera, including the one containing the outgroup (*Pseudochlorella*), branch off this cluster with support values of 0.998

The phylogenetic tree for Strain 7 is presented by Figure 4.8. Despite clustering with *Chlorella* species, the branch containing all *Chlorella* taxa branches off *Didymogenes* branch with low support (0.66). In addition, its NCBI BLAST best hit (*Pseudochlorella pringsheimii*) and observed morphology (Clustering) do not support this result, so it is likely that the obtained sequence was not of sufficient quality to determine the genus accurately. In any case, the results overall suggest that Strain 6 belongs to the *Chlorollesaceae*.

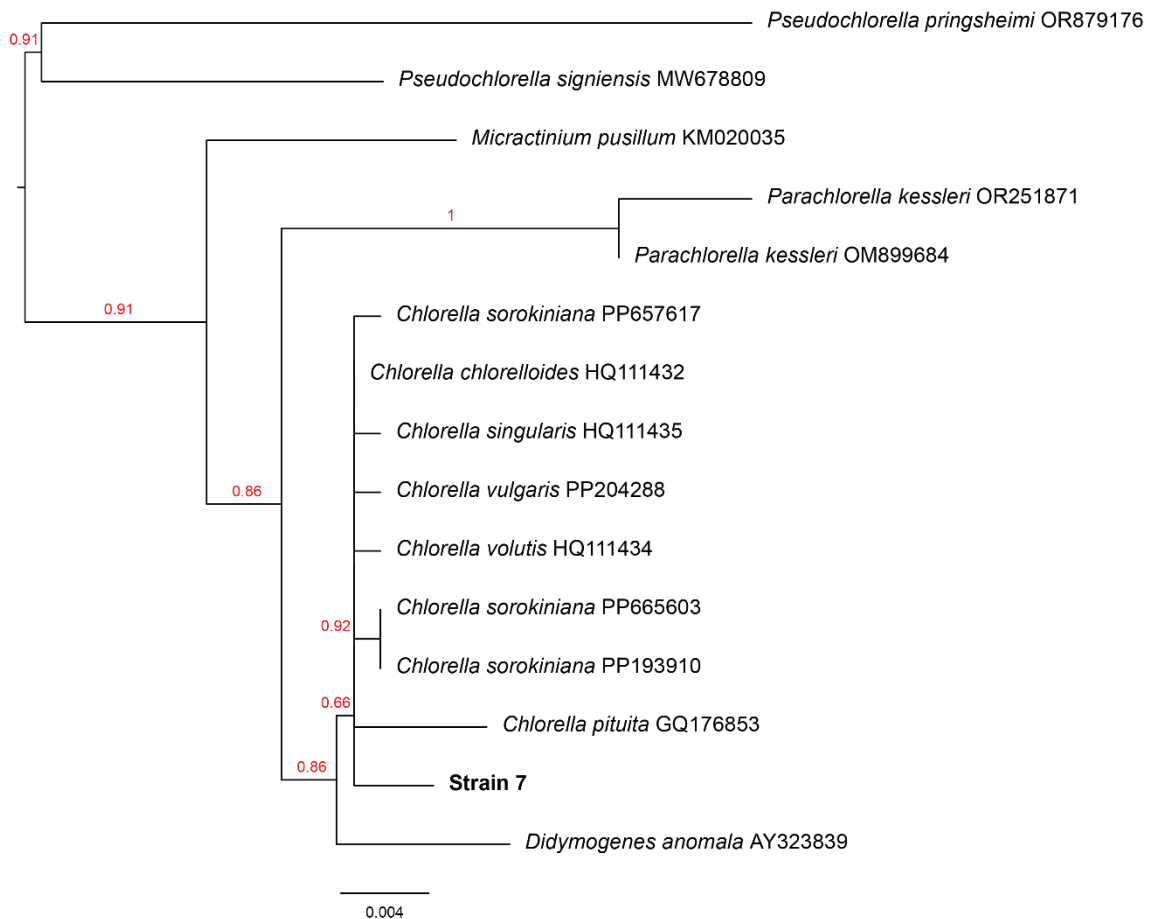


Figure 4.8- 18S Maximum Likelihood phylogenetic tree using the approximate likelihood-ratio test (aLRT) where Strain 7 clusters together with the *Chlorella* taxa with a branch support value of 0.66. Outgroup *Parachlorella* has a branch support of 0.86.

Within a related internship report developed under the supervision of professor João Varela in the MarBiotech group at CCMAR, ITS2 sequences were also analysed for Strain 6 and 7, under the names Sample 6 and Sample 7, respectively.

The ITS2 sequence of Sample 6 (Figure 4.9) clusters exclusively with a *Chlorella parva* sequence, with a moderate confidence (0.751), suggesting that, although not definitively, that strain 6 might be classified as belonging to this species. In this phylogenetic tree, taxa belonging to the closely related genus *Chloroidium* served as an outgroup branching off the *Chlorellaceae* cluster with maximum support (1.0). As *Chloroidium* belongs to the *Watanabeaceae* rather than the *Chlorellaceae*, both part of the class *Trebouxiophyceae*, this tree seems to be further supported by resolving taxa across their familial taxonomy (Li et al., 2021).

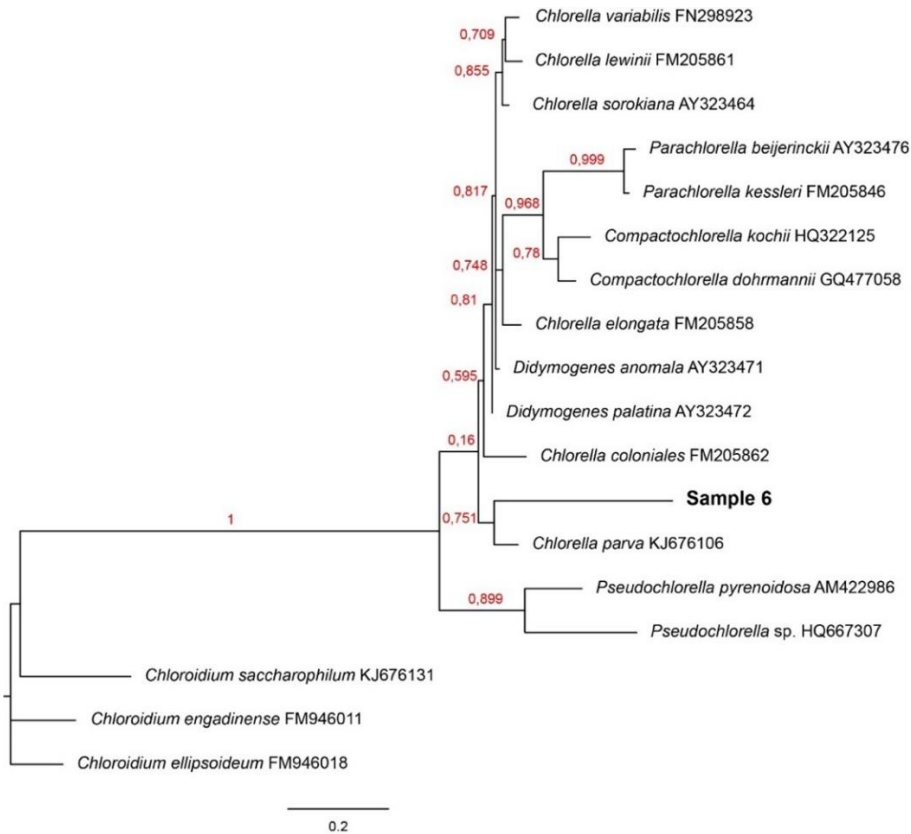


Figure 4.9- ITS2 Maximum Likelihood phylogenetic tree using the approximate likelihood-ratio test (aLRT) where Sample 6 appears in a branch near the root node with support of 1 (shown on the left, clustering together with a *Chlorella parva* sequence with a branch support value of 0.751, which appears to be a sister clade to a branch containing *Pseudochlorella* taxa and other branches containing *Chlorella*, *Parachlorella*, *Compactochlorella* and *Didymogenes* taxa.

Strain 7 (Figure 4.10) clusters with *Chlorella* sequences with moderate confidence (0.753). However, this is unlikely given its morphology and NCBI BLAST results. Sample 7 tree has the genus *Chloroidium* as an outgroup, as well as the genus *Parachlorella*.

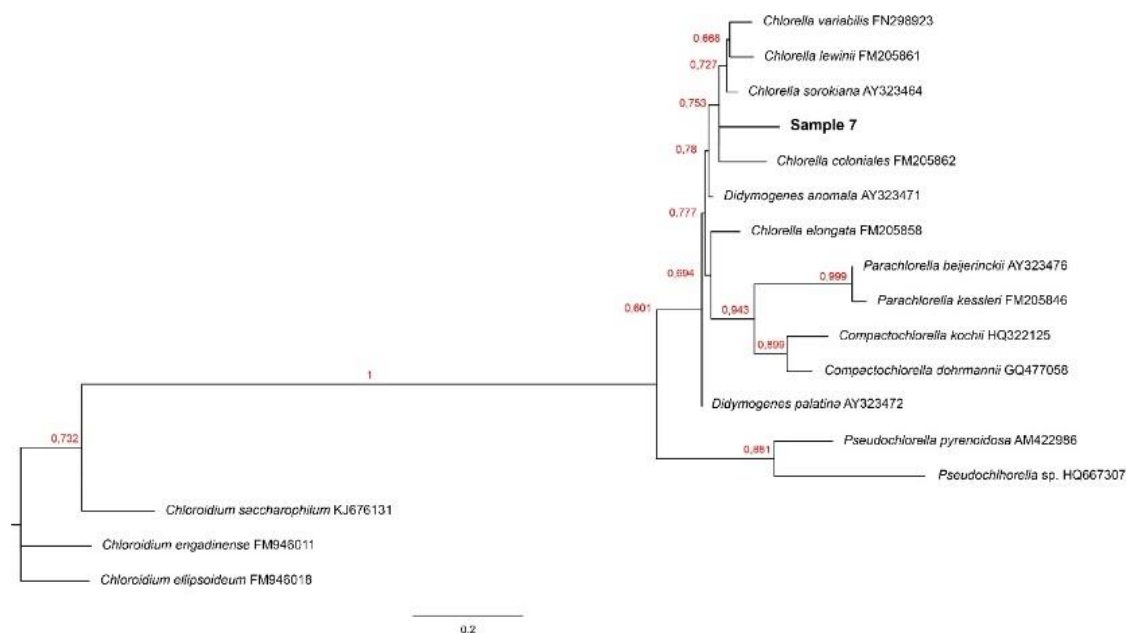


Figure 4.10- ITS2 Maximum Likelihood phylogenetic tree using the approximate likelihood-ratio test (aLRT) where Sample 7 clusters together with a *Chlorella coloniales* sequence and other *Chlorella* taxa with a branch support value of 0.753. Other taxa of other genera, including the one containing the outgroup (*Chloroidium*), branch off this cluster with support values of 0.881 and 1, respectively.

The isolated strains were identified based on morphology, NCBI BLAST similarities and respective phylogenetic trees, and given new abbreviations, as shown in Table 4.1

Table 4.1- Strain Species and new abbreviation

| Strain | Species | Abbreviation | Assignment confidence | Criteria ¹ |
|--------|----------------------------|--------------|-----------------------|-----------------------|
| 1 | <i>Desmodesmus</i> sp. | DES1 | High | P M |
| 2 | <i>Stichococcus</i> sp. | STI1 | Medium | M |
| 3 | <i>Desmodesmus</i> sp. | DES2 | High | P M |
| 4 | <i>Stichococcus</i> sp. | STI2 | Medium | M |
| 5 | <i>Desmodesmus</i> sp. | DES3 | High | P M |
| 6 | <i>Chlorella</i> sp. | CHL | High | P M |
| 7 | <i>Pseudochlorella</i> sp. | PSE | Low | M, B |

1 – P = phylogenetic inference; M = morphology; B-BLAST best hits.

The identified species match up with what can be expected to be isolated from freshwater effluent, as other microalgae obtained from wastewater (WW) samples were identified as algae belonging to the genus *Chlorella* such as *Chlorella pyrenoides* in hydroponics WW (Lu, 2017), *Chlorella vulgaris* in swine WW (Wen, 2017) and *Chlorella* sp. in dairy WW (Choi et al., 2018). *Desmodesmus* sp. has been isolated from municipal WW (Wu et al., 2013) and anaerobic digestion WW (Ji et al., 2014), whereas *Stichococcus bacillaris* has been identified in hospital WW (Ricky & Shanthakumar, 2023) and *Tetradismus* sp. has also

been found in WW (de Andrade et al., 2023). This shows the resilience of these four genera of microalgae.

Interestingly, the identification of microalgae belonging to the same genus enabled their use in trials in which the same genus was a point of comparison to the results of our lab-scale trials with these strains.

4.2 Lab-scale trials

With the strains selected and identified, lab-scale trials were used to determine the two species with best growth performance, nitrate removal ability, determine the most effective water treatment and ability to outcompete other microalgae.

4.2.1 Growth performance

To analyse the growth performance of the seven selected strains, a comparison trial was performed where all strains were grown in control growth medium (freshwater and NB+ supplementation) and in drainwater (Figure 4.11 and 4.12). When comparing the growth between the control and drainwater media, there were no significant differences in either of the tested strains.

When comparing strains, in trial A (Fig. 4.11A), the strain with a significantly higher growth performance was *Chlorella* sp. (CHL) when compared with DES1, DES2 and STI1, with a maximum OD of 1.04 ± 1.4 . In trial B (Fig. 4.11B), *Pseudochlorella* sp. (PSE) showed a significantly higher growth ($p < 0.001$) when compared to STI2 and ($p < 0.05$) DES3, with a maximum OD of 1.08 ± 0.04 . This was expected, as strains belonging to the *Chlorella* clade have shown higher growth rates than other taxa in previous reports (Das et al., 2018; Saranya & Shanthakumar, 2020; Palafox-Sola et al., 2023). The *Stichococcus* strains (STI1 and STI2) showed the slowest growth rate during these trials, which could be the result of *Stichococcus*, unlike the others, belonging to the Prasiolales, and thusly having far different optimal conditions.

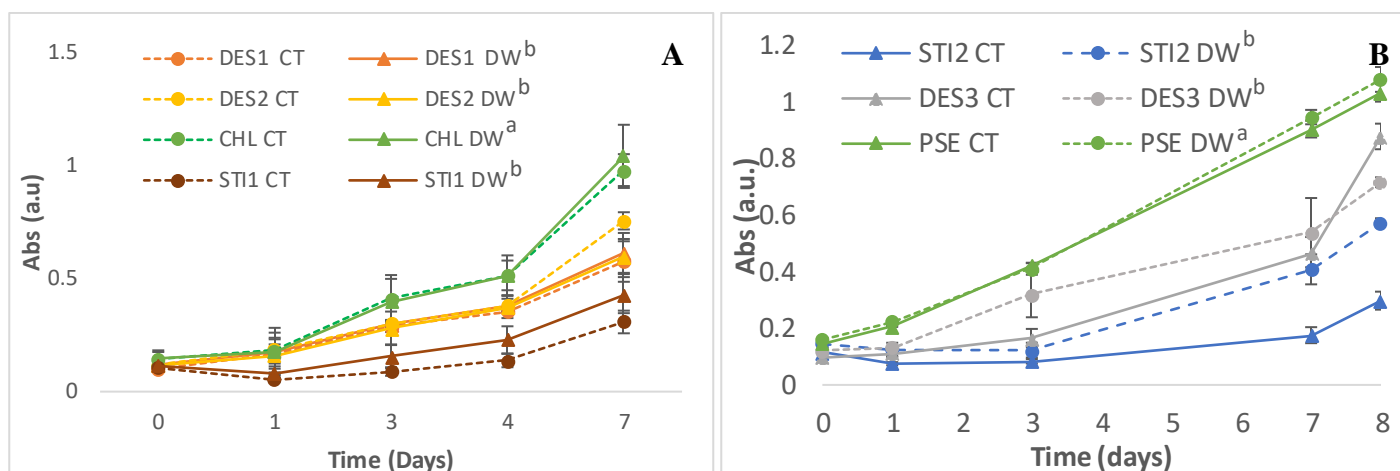


Figure 4.11- A) Growth performance of *Desmodesmus* sp. (DES1), *Stichococcus* sp. (STI1), *Desmodesmus* sp. (DES2) and *Chlorella* sp. (CHL) under control (freshwater and NB+) and drainwater conditions. Values are presented as mean±standard deviation (n=3). Absorbance (Abs) in arbitrary units. B) Growth performance of *Stichococcus* sp. (STI2), *Desmodesmus* (DES2) and *Pseudochlorella* (PSE) under control (freshwater and NB+) and drainwater conditions. Values are presented as mean±standard deviation (n=3). Absorbance (Abs) in arbitrary unit. Different letters indicate significant differences ($p < 0.05$; 2-way ANOVA) between strains grown in drainwater.

In terms of nutrient removal, all strains growing in drainwater showed a much higher nitrate removal (Table 4.2), when compared with the strains grown under control conditions, i.e., a standard growth medium for microalgae. Statistical analysis of the nitrate content showed that the difference between *Chlorella* grown in drainwater (given in the table as CHL drainwater (DW) and its control (CT), i.e., the same strain grown in standard growth medium, was statistically significant. The same conclusion can be drawn for statistical differences in nitrate levels between CHL, STI and DES1 microalgae. There was also a statistically significant difference between PSE grown in DW and its respective control (PSE CT). Statistical differences between PSE and the strains DES1, DES2 and STI1 were also found. More specifically, all strains except for one *Stichococcus* strain (STI1) showed a nitrate removal above 90%, a result that is consistent with those of several bioremediation studies showing similar percentages of nitrate removal, especially for *Chlorella* strains, although it has been reported that *Chlorella kessleri*, and *C. sorokiniana* were able to remove 98.6 (Lee & Lee, 2002) and about 100% (Arif et al., 2020) of all nitrate present in the growth medium, respectively. Nitrate removal was usually carried out in urban wastewater, but similar values were obtained with *Chlorella vulgaris* removing 98.5% of nitrates from hydroponic wastewater (Yousif et al., 2022).

Table 4.2- Nitrate concentration of each strain for each treatment (n=3). CT - Control Water; DW - Drainwater. Values are presented as mean±standard deviation. Different letters indicate significant differences (p<0.05; Kruskal-Wallis test and Dunn's post hoc test.) between strains and mediums.

| Initial | Nitrates (mM) | Nitrate removal (%) |
|-----------------|---------------------------|----------------------------|
| Control (CT) | 4.75 ± 0.23 | |
| Drainwater (DW) | 3.32 ± 0.23 | |
| Final | | |
| DES1 CT | 2.51 ± 0.21 ^{bd} | 47.15 |
| DES1 DW | 0.38 ± 0.04 ^b | 88.55 |
| STI1 CT | 2.46 ± 0.48 ^b | 48.21 |
| STI1 DW | 0.40 ± 0.28 | 87.92 |
| DES2 CT | 2.38 ± 0.76 ^d | 49.89 |
| DES2 DW | 0.18 ± 0.33 | 94.61 |
| STI2 CT | 2.21 ± 0.53 | 53.47 |
| STI2 DW | 0 ± 0.08 | 100.00 |
| DES3 CT | 1.23 ± 0.19 | 74.11 |
| DES3 DW | 0.08 ± 0.55 | 97.56 |
| CHL CT | 3.74 ± 0.11 ^b | 21.26 |
| CHL DW | 0.04 ± 0.34 ^a | 98.79 |
| PSE CT | 4.09 ± 1.73 ^d | 13.89 |
| PSE DW | 0 ± 0.17 ^c | 100.00 |

As a result of this trial, based on both growth performance and nitrate removal capabilities, the strains *Chlorella* sp. (CHL) and *Pseudochlorella* sp. (PSE) were selected as the top performing strains, to be used in further assays.

4.2.2 Water treatment

Distinct water treatment strategies were evaluated to assess the impact on growth performance of the two selected strains (Figure 4.13) to see which water pre-treatment prior to cultivation would improve the growth of the isolated strains the most.

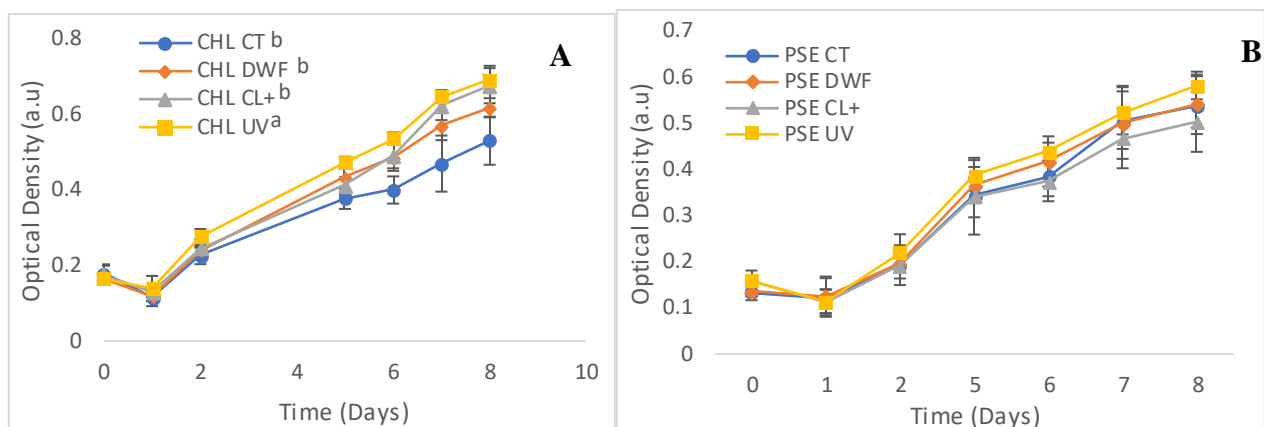


Figure 4.13- Growth performance of *Chlorella* sp. (CHL) (A) and *Pseudochlorella* (PSE) (B) when cultivated in wastewaters (WW) with or without a pretreatment step, namely: Untreated WW (CT), Filtered WW (DWF), Chlorine pretreatment WW (CL+), and ultraviolet radiation treated WW (UV). Values are presented as mean±standard deviation. Absorbance in arbitrary units (a.u). Letters indicate significant differences ($p < 0.05$; 2-way ANOVA.) between pretreatments

When comparing the tested pretreatments, ultraviolet radiation (UV) led to the highest microalgal growth performance comparing with those of other conditions tested for CHL cultures ($p < 0.05$; 2-way ANOVA). Even though this trend was also seen for PSE, the differences between pretreatments were not statistically significant ($p > 0.05$; 2-way ANOVA). CHL and PSE strains reached an OD of 0.69 ± 0.04 and 0.58 ± 0.03 , respectively. Regarding the treatment leading to the lowest growth performance, under control conditions (drainwater with no treatment) CHL only reached an OD of 0.53 ± 0.06 , which was expected given that no pretreatment of the drainwater was done to improve the growth rate or N removal. For PSE, the use of chlorine was slightly less effective treatment with an OD of 0.50 ± 0.07 .

The effectiveness of UV-treated wastewater on growth rate is consistent with studies finding that *Chlorella vulgaris* had faster growth in UV-treated WW compared to untreated WW, finding a dose-dependent response on growth rate with exposure time and chlorine concentration, potentially indicating that results may have differed with a different UV exposure time or chlorine concentration (Qin et al., 2014).

In addition, the use of UV radiation and filtration as an effluent pre-treatment for algae growth, with the purpose of removing bacteria and suspended solids from the effluent has already been studied on *Chlorella* sp. It was found that filtration improved growth and UV-B specifically proved ineffective due to the need for using higher doses, which would not be the case with UV-C (Cho et al., 2011). Some variation in results could be caused

by the number of bacteria and suspended solids present in the effluent to begin with. It is likely that combining UV and filtration pre-treatments would prove more effective than individually in not only promoting microalgal growth, but also minimizing bacterial load and the presence of suspended solids.

In terms of nutrient consumption (Table 4.3), UV-treatment led to the highest rate of nutrient removal for both strains, the difference being statistically significant. Upon growth of PSE, filtered drainwater had the highest nitrate content, whereas, with CHL, the control growth media showed the highest nitrate concentration. This discrepancy could be explained by microalgae being able to grow in both filtered and unfiltered wastewater (Calderini et al., 2021). It is also possible that the control drainwater contained bacteria that would consume nitrates.

PSE demonstrated lower nitrate concentrations relative to CHL, showing a higher removal rate, which is consistent with other trials comparing these two strains, where *Pseudochlorella pringsheimii* proved about as effective as *Chlorella vulgaris* in nitrogen removal from wastewater effluent as both were to completely remove this nutrient (Saranya & Shanthakumar, 2020).

For nitrate removal statistical analysis showed data was non homogenous so a non-parametric test was done. Krustal-Wills found statistical significance to UV's nitrate removal (p-value<0.05) relative to the other pre-treatments for both strains.

Table 4.3- Nitrate concentration for each treatment of each strain after the growth assay. C - Control (untreated drainwater) or drainwater pretreated by filtration (F), chlorine (CL+) or ultraviolet radiation (UV). Values are presented as mean±standard deviation. Nitrate Removal %, indicates what percentage of the initial nitrates were removed from the medium by the end of the trial. Different letters indicate significant differences between treatments of the same strain (p<0.05; Krustal Wallis & Dunn test) between pre-treatments.

| | Initial | Nitrates (mM) | | Nitrate removal (%) |
|------------|---------|---------------|-------------------|---------------------|
| Drainwater | 4.71 | ± | 1.09 | |
| | Final | | | |
| CHL C | 2.98 | ± | 1.63 ^b | 36.73 |
| CHL F | 1.89 | ± | 0.96 ^b | 59.87 |
| CHL CL+ | 2.89 | ± | 0.89 ^b | 38.64 |
| CHL UV | 1.75 | ± | 0.23 ^a | 62.84 |
| PSE C | 1.45 | ± | 0.63 ^d | 69.21 |
| PSE F | 3.95 | ± | 0.38 ^d | 16.13 |
| PSE CL+ | 1.66 | ± | 0.54 ^d | 64.76 |
| PSE UV | 0.01 | ± | 0.02 ^c | 99.79 |

4.2.3 Competition assays

A co-growth competition trial was done to assess the growth rate of the strains when in competition with *Tetrademus obliquus* SNS0120, a strain known for its ability to treat wastewaters (Kong et al., 2021) removing nitrates (Ma et al., 2020). The results are shown in Figure 4.14.

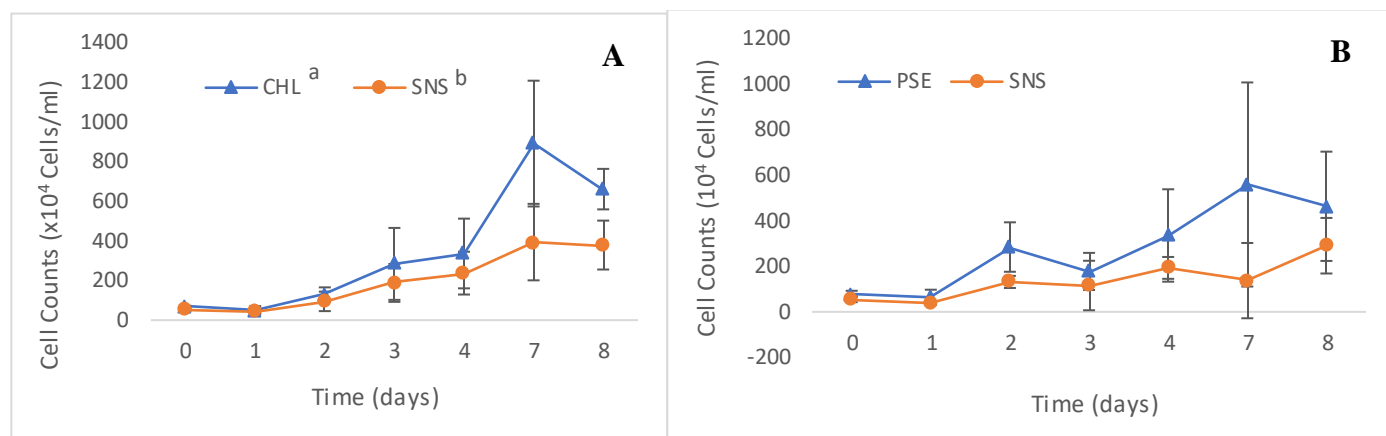


Figure 4.14- Growth performance for co-growth of *Tetrademus obliquus* with CHL (A) and SNS with PSE. (B) at 20°C. Values are presented as mean \pm standard deviation. Cell counts are presented as cells $\times 10^4$ per millilitre. Different letters indicate significant differences ($p < 0.05$; t-test) between strains.

In both cases, the cellular concentration (CC) by the end of the competition trial was higher in the selected strains when comparing with *T. obliquus*: CHL reached a CC of 8.9×10^6 cells/mL at its peak, while *T. obliquus* reached only 3.9×10^6 cells/mL. In a parallel competition trial, PSE reached a CC of 5.6×10^6 cells/mL, while *T. obliquus* reached again a lower cell concentration, 2.9×10^6 cells/mL. However, CHL had a significantly higher cellular concentration when compared to SNS ($p < 0.05$), but there were no significant differences when PSE and *T. obliquus* were co-cultivated. These results were somewhat surprising given that in another competition trial *T. obliquus* proved more effective than *C. vulgaris*, showing a relative growth rate of 1.12 and 1.16 g/L/day, in comparison to *C. vulgaris*' growth rate of 0.84 and 1.08 g/L/day for the same conditions (Escapa et al., 2017). However, in the present case, the competition trial was performed in the drainwater from which CHL and PSE were isolated from. Therefore, these strains might have already been acclimated to that environment, thusly this assay is an example of the efficacy of bioprospection of locally isolated strains.

4.3. Production of biomass in 1-L bubble columns

To assess biomass composition of the selected strains, a growth assay was performed in 1-L bubble columns (Figure 4.15). The PSE strain showed a significantly higher growth performance ($p < 0.05$), with an OD of 1.06, by the end of the trial comparing with CHL with an OD of 0.91. This result is congruent with a study finding that *Pseudochlorella pringsheimii* produced more biomass than *C. vulgaris* in WW bioremediation conditions, reaching 3.51 g/L compared to 2.84 g/L, respectively (Saranya & Shanthakumar, 2019).

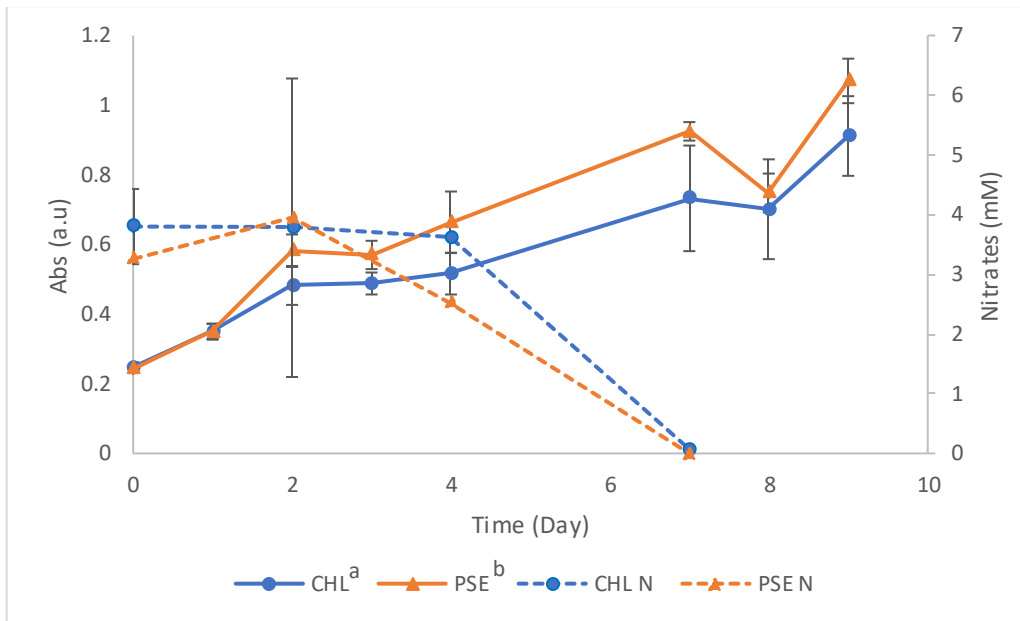


Figure 4.15-Growth performance and nitrate removal of *Chlorella* sp. (CHL) and *Pseudochlorella* sp. (PSE) at 20°C in 1-L bubble columns. Values are presented as mean \pm standard deviation. Absorbance in Arbitrary units (a.u). CHL N and PSE N- Nitrate content of respective strains on each day. Letters indicate significant differences ($p < 0.05$; T-test) between strains.

The PSE strain was on average faster at removing nitrate from the drainwater, but both strains were able to completely remove nitrate from the medium by the end of the assay, at the same time. Similar results were seen in other *Chlorella* nitrate removal trials with 100% removal (Arif et al., 2020) and nearly 100% removal: 98.6 and 98.5%, respectively (Lee, 2002; Yousif, 2022). In addition, comparison assays between *C. vulgaris* and *P. pringsheimii* showed 100% removal of ammoniacal nitrogen by both strains (Saranya & Shanthakumar, 2019). Since that same study showed higher biomass production by *Pseudochlorella pringsheimii*, it is likely that it also had a faster rate of nitrogen removal, consistent with PSE faster removal relative to CHL.

4.3.1 Biochemical composition

4.3.1.1 Proximate composition

The proximate composition in terms of lipids and proteins can be seen in Table 4.4. Lipid content varied between 16.9 and 17.9% in the two strains, while proteins ranged from 14.1 and 15%, representing a similar content of total lipids and proteins, with no significant differences ($p>0.05$) between strains.

Table 4.4-Percentage of Lipid content and Protein content of biomass collected from the 1L-Bubble column Assay. Letters denote statistical significance ($p>0.05$;t-test)

| (%) | Total Lipids | | Protein | |
|---|--------------|--------|---------|--------|
| <i>Chlorella</i> sp. (CHL) | 17.94 | ± 3.29 | 14.10 | ± 2.18 |
| <i>Pseudochlorella</i> sp. (PSE) | 16.87 | ± 3.02 | 15.01 | ± 3.77 |

The lipid content of CHL was slightly lower when compared to other reports for *C. vulgaris*, which varied between 20 and 32% (Aguoru, 2015), with the same occurring in PSE, with lower lipid content compared to *P. pringsheimii*, ranging between 18.16 and 21.89%, depending upon salinity of the growth medium (Bhatnagar et al., 2024). It is worth noting that lipid content and accumulation can be induced depending on factors such as nutrient and cultivation conditions, including stress, microalgae–bacteria interactions, use of phytohormones, EDTA and chemical additives, and improved light availability using LED-based illumination (Singh et al., 2016), salinity or the addition of gamma-aminobutyric acid (GABA) (Bhatnagar et al., 2024).

Protein content of both strains appeared to be lower when compared to those of previous reports, where *C. vulgaris* had a reported protein content between 43-58% (Irene et al, 2020), and *P. pringsheimii* whose protein contents ranged from 22.27 to 33.14 % (Bhatnagar et al., 2024). This lower protein content of *Chlorella* can be improved if WW with higher nitrogen contents were used (Xie, 2017). Another possibility would be to use greater irradiances in order to boost the protein content in this microalga (Seyfabadi et al., 2011).

4.3.1.2 Fatty Acids

Fatty acid content of both strains is shown in Table 4.5. The percentages of saturated fatty acids (SFA) were 17.85% and 36.62% for CHL and PSE respectively, which were statistically different ($p<0.05$). For monounsaturated fatty acids, the contents for CHL and

PSE were 23.75 and 20.12% respectively, which were not significantly different ($p>0.05$). For polyunsaturated fatty acids, the same cultures displayed contents of 47.07% and 32.69% respectively, which were statistically different ($p<0.05$).

The primary source of SFA was 16:0 (palmitic acid), while the primary source of PUFA was 18:2 and 18:3 n -3 (linoleic and alpha-linolenic acids respectively). The present fatty acids profile was somewhat similar to previously obtained profiles for *Chlorella vulgaris*, concerning alpha-linolenic (45.8%), palmitic (C16:0, 37.1%) (Hong et al., 2016), palmitoleic (C16:1; 2.64%) and stearic (C18:0; 1.17%) acids (Yusof, 2011) as well as having a high concentration of PUFA (47.7%) (Pratoomyot, 2005).

Table 4.5- *Chlorella sp* and *Pseudochlorella sp.* fatty Acid profile with saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acid given in percentage of total fatty acids. Cx:y notation, where x is number of carbon atoms in the chain and y is the number of double bonds. Different letters denote statistical differences between strains ($p=>0.05$; t-test)

| Fatty acids (%) | <i>Chlorella sp.</i> | <i>Pseudochlorella sp.</i> |
|-----------------|---------------------------------|---------------------------------|
| C14:0 | 0.19 ± 0.05 | 0.16 ± 0.07 |
| C16:0 | 15.39 ± 3.14 | 24.4 ± 7.32 |
| C18:0 | 2.27 ± 0.79 | 2.27 ± 0.79 |
| C23:0 | 0 ± 0 | 9.79 ± ±16.86 |
| Σ SFA | 17.85 ± 3.98^b | 36.62 ± 8.18^a |
| C16:1 | 2.01 ± 0.81 | 1.13 ± 0.71 |
| C18:1 | 20.87 ± 2.29 | 18.34 ± 6.3 |
| C18:1 | 0.75 ± 0.28 | 0.65 ± 0.14 |
| C20:1 | 0.12 ± 0.06 | 0 ± 0 |
| Σ MUFA | 23.75 ± 3.44 | 20.12 ± 7.15 |
| c16:2 | 0 ± 0 | 1.98 ± 0.34 |
| c16:3 | 0 ± 0 | 9.19 ± 2.07 |
| C18:2 | 23.68 ± 6.65 | 21.52 ± 6.84 |
| C18:3 n -3 | 23.39 ± 4.71 | 0 ± 0 |
| Σ PUFA | 47.07 ± 11.36 | 32.69 ± 9.25 |

The fatty acid profile of the PSE is also presented in Table 4.5. The percentage of SFA for PSE was 37.70%, whereas for MUFA and PUFA was 20.12% and 42.18%, respectively. The ratio of unsaturated to saturated fats was 1.65:1, much lower than for *Chlorella*. Much like CHL, the main saturated fatty acid was 16:0 (palmitic acid), the main MUFA was 18:1 (methyl-*cis*-oleate) and the main PUFA was C18:2 (linoleic acid). This is similar to the fatty acid profile of *Pseudochlorella pringsheimii* with a SFA content

of 33.98%, with the main SFA, MUFA and PUFA being 16:0 (palmitic acid), C18:1 (oleic acid) and C18:2 (linoleic acid), respectively (Abd El Baky & El Baroty, 2023). The difference between the two species was not statistically significant ($p>0.05$),

Variation in fatty acid profile is to be expected since the fatty acid content has been shown to depend upon light regimes (Seyfabadi, 2011), temperature (Mutaf, 2019) and carbon dioxide concentration (Yusof et al, 2011). PUFA, such as linoleic and linolenic acids are essential for human nutrition (Mutaf et al, 2019), and *n*-3 PUFA are commonly used in the feed industries (Remize et al, 2021) and these microalgae strains contain both.

With the protein content, lipid content and fatty acid profiles of the strains determined, it is now possible to market these specific strains for bioremediation with the additional knowledge that the resulting biomass can have additional applications, should it be employed in aquafeed, biofertilizer (Chatterjee et al., 2017) or biofuel (Giordano & Wang, 2018).

Conclusion

This study provides further evidence on the efficacy of locally isolated microalgae on bioremediation. The results obtained demonstrated that, of the strains isolated from hydroponic effluent, *Chlorella* sp. and *Pseudochlorella* sp. had the highest growth rate and nitrate removal capabilities and proved to be more effective than pre-selected microalgae strains that were not locally isolated. Several pretreatments for the effluent were tested, with cells grown in drainwater pretreated with UV radiation showing a slight increase in growth performance of the strains, compared to the other pretreatments. The competition assay demonstrated that the two selected strains performed better compared to a known bioremediating strain, with the difference being statistically significant for one of them, a result consistent with the hypothesis that locally bioprospected strains can be more effective. A scale-up assay was also performed, showing consistent results with the smaller scale assays and allowing the harvested biomass to be analysed, which enabled to determine their macronutrient profile and assess their potential for industrial applications.

This thesis has contributed to the body of evidence in favour of the efficacy of locally isolated microalgae in the treatment of hydroponic effluent and gave further insight into the microalgae that can be harvested from hydroponic effluents.

5 References

- Abd El Baky, H., & El Baroty, G. (2023). Cultivation of *Pseudochlorella pringsheimii* for biodiesel production in a scalable indoor photobioreactor: case studies from Egypt. *Journal of Genetic Engineering and Biotechnology*, 21(1), 25. <https://doi.org/10.1186/s43141-022-00450-0>
- Abdelfattah, A., Ali, S. S., Ramadan, H., El-Aswar, E. I., Eltawab, R., Ho, S.-H., Elsamahy, T., Li, S., El-Sheekh, M. M., Schagerl, M., Kornaros, M., & Sun, J. (2023). Microalgae-based wastewater treatment: Mechanisms, challenges, recent advances, and future prospects. *Environmental Science and Ecotechnology*, 13, 100205. <https://doi.org/10.1016/j.ese.2022.100205>
- Abdel-Raouf, N., Al-Homaidan, A. A., & Ibraheem, I. B. M. (2012). Microalgae and wastewater treatment. *Saudi Journal of Biological Sciences*, 19(3), 257–275. <https://doi.org/10.1016/j.sjbs.2012.04.005>
- Aguoru, C. U. , and P. O. Okibe. (2015). Content and composition of lipid produced by *Chlorella vulgaris* for biodiesel production. *Dvances in Life Science and Technology* 36, 36, 96–10.
- Aketo, T., Hoshikawa, Y., Nojima, D., Yabu, Y., Maeda, Y., Yoshino, T., Takano, H., & Tanaka, T. (2020). Selection and characterization of microalgae with potential for nutrient removal from municipal wastewater and simultaneous lipid production. *Journal of Bioscience and Bioengineering*, 129(5), 565–572. <https://doi.org/10.1016/j.jbiosc.2019.12.004>
- Alazaiza, M. Y. D., Albahnasawi, A., Ahmad, Z., Bashir, M. J. K., Al-Wahaibi, T., Abujazar, M. S. S., Abu Amr, S. S., & Nassani, D. E. (2022). Potential use of algae for the bioremediation of different types of wastewater and contaminants: Production of bioproducts and biofuel for green circular economy. *Journal of Environmental Management*, 324, 116415. <https://doi.org/10.1016/j.jenvman.2022.116415>

- Ankenbrand, M. J., Keller, A., Wolf, M., Schultz, J., & Förster, F. (2015). ITS2 Database V: Twice as Much: Table 1. *Molecular Biology and Evolution*, *32*(11), 3030–3032. <https://doi.org/10.1093/molbev/msv174>
- Arif, M., Wang, L., Salama, E.-S., Hussain, M. S., Li, X., Jalalah, M., Al-Assiri, M. S., Harraz, F. A., Ji, M.-K., & Liu, P. (2020). Microalgae Isolation for Nutrient Removal Assessment and Biodiesel Production. *BioEnergy Research*, *13*(4), 1247–1259. <https://doi.org/10.1007/s12155-020-10136-5>
- Arumugam, T. , G. S. and M. U. M. (2021). “Soilless farming of vegetable crops: An overview.” . " *Pharma Innov.*, *10*(1), 773–785.
- Beuckels, A., Smolders, E., & Muylaert, K. (2015). Nitrogen availability influences phosphorus removal in microalgae-based wastewater treatment. *Water Research*, *77*, 98–106. <https://doi.org/10.1016/j.watres.2015.03.018>
- Bhatnagar, P., Gururani, P., Rawat, J., Kumar Jaiswal, K., Gautam, P., Nanda, M., Chauhan, P. K., Vlaskin, M. S., & Kumar, V. (2024). Influence of GABA (Gamma-aminobutyric acid) supplementation on biomass, pigments, lipid and protein content of *Pseudochlorella pringsheimii* under salinity stress. *Current Research in Biotechnology*, *7*, 100223. <https://doi.org/10.1016/j.crbiot.2024.100223>
- Bligh, E. G., & Dyer, W. J. (1959). A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION. *Canadian Journal of Biochemistry and Physiology*, *37*(8), 911–917. <https://doi.org/10.1139/o59-099>
- Bohutskyi, P., Liu, K., Nasr, L. K., Byers, N., Rosenberg, J. N., Oyler, G. A., Betenbaugh, M. J., & Bouwer, E. J. (2015). Bioprospecting of microalgae for integrated biomass production and phytoremediation of unsterilized wastewater and anaerobic digestion centrate. *Applied Microbiology and Biotechnology*, *99*(14), 6139–6154. <https://doi.org/10.1007/s00253-015-6603-4>
- Boretti, A., & Rosa, L. (2019). Reassessing the projections of the World Water Development Report. *Npj Clean Water*, *2*(1), 15. <https://doi.org/10.1038/s41545-019-0039-9>
- Boubakri, A., Hafiane, A., & Al Tahar Bouguecha, S. (2015). Nitrate removal from aqueous solution by direct contact membrane distillation using two different

- commercial membranes. *Desalination and Water Treatment*, 56(10), 2723–2730. <https://doi.org/10.1080/19443994.2014.981408>
- Breil, P., Pons, M., Armani, G., Amer, R., Pienaar, H., Oberholster, P., & Namour, P. (2022). Natural-Based Solutions for Bioremediation in Water Environment. In *Sustainable Solutions for Environmental Pollution* (pp. 1–93). Wiley. <https://doi.org/10.1002/9781119827665.ch1>
- Brownlie, W. J., Sutton, M. A., Cordell, D., Reay, D. S., Heal, K. V., Withers, P. J. A., Vanderbeck, I., & Spears, B. M. (2023). Phosphorus price spikes: A wake-up call for phosphorus resilience. *Frontiers in Sustainable Food Systems*, 7. <https://doi.org/10.3389/fsufs.2023.1088776>
- C. R. Camp. (1998). SUBSURFACE DRIP IRRIGATION: A REVIEW. *Transactions of the ASAE*, 41(5), 1353–1367. <https://doi.org/10.13031/2013.17309>
- Calderini, M. L., Stevčić, Č., Taipale, S., & Pulkkinen, K. (2021). Filtration of Nordic recirculating aquaculture system wastewater: Effects on microalgal growth, nutrient removal, and nutritional value. *Algal Research*, 60, 102486. <https://doi.org/10.1016/j.algal.2021.102486>
- Carpenter, S. R. (2005). Eutrophication of aquatic ecosystems: Bistability and soil phosphorus. *Proceedings of the National Academy of Sciences*, 102(29), 10002–10005. <https://doi.org/10.1073/pnas.0503959102>
- Castresana, J. (2000). Selection of Conserved Blocks from Multiple Alignments for Their Use in Phylogenetic Analysis. *Molecular Biology and Evolution*, 17(4), 540–552. <https://doi.org/10.1093/oxfordjournals.molbev.a026334>
- Chatterjee, A., Singh, S., Agrawal, C., Yadav, S., Rai, R., & Rai, L. C. (2017). Role of Algae as a Biofertilizer. In *Algal Green Chemistry* (pp. 189–200). Elsevier. <https://doi.org/10.1016/B978-0-444-63784-0.00010-2>
- Chatzissavvidis, C. and I. T. (2014). Role of algae in agriculture. *Seaweeds*, 1–37.
- Chevenet, F., Brun, C., Bañuls, A.-L., Jacq, B., & Christen, R. (2006). TreeDyn: towards dynamic graphics and annotations for analyses of trees. *BMC Bioinformatics*, 7(1), 439. <https://doi.org/10.1186/1471-2105-7-439>

- Cho, S., Luong, T. T., Lee, D., Oh, Y.-K., & Lee, T. (2011). Reuse of effluent water from a municipal wastewater treatment plant in microalgae cultivation for biofuel production. *Bioresource Technology*, *102*(18), 8639–8645. <https://doi.org/10.1016/j.biortech.2011.03.037>
- Choi, Y.-K., Jang, H. M., & Kan, E. (2018). Microalgal Biomass and Lipid Production on Dairy Effluent Using a Novel Microalga, *Chlorella* sp. Isolated from Dairy Wastewater. *Biotechnology and Bioprocess Engineering*, *23*(3), 333–340. <https://doi.org/10.1007/s12257-018-0094-y>
- Darienko, T., Gustavs, L., & Pröschold, T. (2016). Species concept and nomenclatural changes within the genera *Elliptochloris* and *Pseudochlorella* (Trebouxiophyceae) based on an integrative approach. *Journal of Phycology*, *52*(6), 1125–1145. <https://doi.org/10.1111/jpy.12481>
- Das, C., Ramaiah, N., Pereira, E., & Naseera, K. (2018). Efficient bioremediation of tannery wastewater by monostrains and consortium of marine *Chlorella* sp. and *Phormidium* sp. *International Journal of Phytoremediation*, *20*(3), 284–292. <https://doi.org/10.1080/15226514.2017.1374338>
- de Andrade, F. P., De Farias Silva, C. E., dos Santos, J., Ribeiro, T. R. M., Medeiros, J. A., do Nascimento, M. A. A., Santos, G. K. S., dos Santos Carneiro, W., Almeida, R. M. R. G., de Oliveira, A. M. M., Feijó, F. M., da Silva Costa, M. M., de Andrade Lima, G. S., Ribeiro-Júnior, K. A. L., & Tonholo, J. (2023). Dairy wastewater treatment by *Tetradismus* sp. in open system: molecular identification and the effect of light intensity and organic load in the process. *Energy, Ecology and Environment*, *8*(4), 356–369. <https://doi.org/10.1007/s40974-023-00278-5>
- Delrue, F., Ribeiro de Jesus Cerqueira, M., Compadre, A., Alvarez, P., Fleury, G., Escoffier, C., & Sassi, J.-F. (2021). Hydroponic Farm Wastewater Treatment Using an Indigenous Consortium. *Processes*, *9*(3), 519. <https://doi.org/10.3390/pr9030519>
- Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.-F., Guindon, S., Lefort, V., Lescot, M., Claverie, J.-M., & Gascuel, O. (2008). Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Research*, *36*(Web Server), W465–W469. <https://doi.org/10.1093/nar/gkn180>

- Dong, Y., Lekbach, Y., Li, Z., Xu, D., El Abed, S., Ibsouda Koraichi, S., & Wang, F. (2020). Microbiologically influenced corrosion of 304L stainless steel caused by an alga associated bacterium *Halomonas titanicae*. *Journal of Materials Science & Technology*, 37, 200–206. <https://doi.org/10.1016/j.jmst.2019.06.023>
- Dragone, G. et al. (2010). Third generation biofuels from microalgae. *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology* 2, 1355–1356.
- Ekevwe, A. E. , et al. (2018). "Review of organic pollutants in wastewater along the Course of River Gwagwarwa and River Rafin Malam in Kano State-Nigeria. *Journal of Biotechnology and Bioengineering*, 2(2), 36–39.
- El-Bawab, F. (2020). Phylum Protozoa. In *Invertebrate Embryology and Reproduction* (pp. 68–102). Elsevier. <https://doi.org/10.1016/B978-0-12-814114-4.00003-5>
- Escapa, C., Coimbra, R. N., Paniagua, S., García, A. I., & Otero, M. (2017). Comparison of the culture and harvesting of *Chlorella vulgaris* and *Tetrademus obliquus* for the removal of pharmaceuticals from water. *Journal of Applied Phycology*, 29(3), 1179–1193. <https://doi.org/10.1007/s10811-016-1010-5>
- Eurostat, Agricultural production 2020– crops, ISSN 2443-8219, Data extracted in November 2022. (n.d.).*
- García, M. , et al. (2018). Re-utilization of drainage solution from soil less culture in protected agriculture from open to close system. *IWARESA 2018. Book of Abstracts*.
- Ghosh, A. A. M. and A. M. K. (2019). Metagenomic analysis and its applications. *Encyclopedia of Bioinformatics and Computational Biology*, 184–193.
- Giordano, M., & Wang, Q. (2018). Microalgae for Industrial Purposes. In *Biomass and Green Chemistry* (pp. 133–167). Springer International Publishing. https://doi.org/10.1007/978-3-319-66736-2_6
- González-Camejo, J., Aparicio, S., Pachés, M., Borrás, L., & Seco, A. (2022). Comprehensive assessment of the microalgae-nitrifying bacteria competition in microalgae-based wastewater treatment systems: Relevant factors, evaluation methods and control strategies. *Algal Research*, 61, 102563. <https://doi.org/10.1016/j.algal.2021.102563>

- Gouveia, L., Graça, S., Sousa, C., Ambrosano, L., Ribeiro, B., Botrel, E. P., Neto, P. C., Ferreira, A. F., & Silva, C. M. (2016). Microalgae biomass production using wastewater: Treatment and costs. *Algal Research*, *16*, 167–176. <https://doi.org/10.1016/j.algal.2016.03.010>
- Gryta, M., Tomaszewska, M., & Karakulski, K. (2006). Wastewater treatment by membrane distillation. *Desalination*, *198*(1–3), 67–73. <https://doi.org/10.1016/j.desal.2006.09.010>
- Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W., & Gascuel, O. (2010). New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. *Systematic Biology*, *59*(3), 307–321. <https://doi.org/10.1093/sysbio/syq010>
- Guiry, M. D., & Guiry, G. M. (2020, May 6). *AlgaeBase*. National University of Ireland, Galway.
- Guiry, M.D. & Guiry, G.M. 2024. *AlgaeBase*. World-wide electronic publication, National University of Ireland, Galway. (n.d.).
- Heeg, J. S., & Wolf, M. (2015). ITS2 and 18S rDNA sequence-structure phylogeny of *Chlorella* and allies (Chlorophyta, Trebouxiophyceae, Chlorellaceae). *Plant Gene*, *4*, 20–28. <https://doi.org/10.1016/j.plgene.2015.08.001>
- Hoek, C. D. G. M. and H. M. J. (1995). *Algae: an introduction to phycology*. Cambridge university press.
- Hong, J. W., Kim, O. H., Jo, S.-W., Kim, H., Jeong, M. R., Park, K. M., Lee, K. I., & Yoon, H.-S. (2016). Biochemical Composition of a Korean Domestic Microalga *Chlorella vulgaris* KNUA027. *Microbiology and Biotechnology Letters*, *44*(3), 400–407. <https://doi.org/10.4014/mbl.1512.12008>
- Iqbal, A. et al. (2023). *Sustainable Agriculture Reviews 58* (A. Iqbal, M. Iqbal, M. Alamzeb, S. Meizhen, Z. Xiling, M. Arif, X. Du, & E. Lichtfouse, Eds.; Vol. 58). Springer International Publishing. <https://doi.org/10.1007/978-3-031-16155-1>
- Ji, F., Liu, Y., Hao, R., Li, G., Zhou, Y., & Dong, R. (2014). Biomass production and nutrients removal by a new microalgae strain *Desmodesmus* sp. in anaerobic

- digestion wastewater. *Bioresource Technology*, 161, 200–207.
<https://doi.org/10.1016/j.biortech.2014.03.034>
- Joshi, D., Nainabasti, A., Bhandari, R., Awasthi, P., Banjade, D., Malla, S., & Subedi, B. (2022). A review on soilless cultivation: The hope of urban agriculture. *Archives of Agriculture and Environmental Science*, 7(3), 473–481.
<https://doi.org/10.26832/24566632.2022.0703022>
- Kalana, U. L. D. I., Kalpage, C. S., & Yatigamma, S. K. (2016). Evaluation of the suitable environmental conditions for selected freshwater microalgae species with the potential for the production of biodiesel. *Ceylon Journal of Science*, 45(3), 93.
<https://doi.org/10.4038/cjs.v45i3.7405>
- Kong, W., Kong, J., Lyu, H., Ma, J., Yuan, P., Wang, Z., Shen, B., & Feng, S. (2021). Integrating municipal wastewater treatment with CO₂ fixation and fatty acid production by cultivating *Tetradismus obliquus*. *Journal of Cleaner Production*, 320, 128916. <https://doi.org/10.1016/j.jclepro.2021.128916>
- Kristensen, P. et al. (2018). *European waters assessment of status and pressures 2018*. 85.
- Kumar, A., Ergas, S., Yuan, X., Sahu, A., Zhang, Q., Dewulf, J., Malcata, F. X., & van Langenhove, H. (2010). Enhanced CO₂ fixation and biofuel production via microalgae: recent developments and future directions. *Trends in Biotechnology*, 28(7), 371–380. <https://doi.org/10.1016/j.tibtech.2010.04.004>
- Larsdotter, K., la Cour Jansen, J., & Dalhammar, G. (2010). Phosphorus removal from wastewater by microalgae in Sweden – a year-round perspective. *Environmental Technology*, 31(2), 117–123. <https://doi.org/10.1080/09593330903382815>
- Lee, J. Y., Rahman, A., Behrens, J., Brennan, C., Ham, B., Kim, H. S., Nho, C. W., Yun, S.-T., Azam, H., & Kwon, M. J. (2018). Nutrient removal from hydroponic wastewater by a microbial consortium and a culture of *Paracercomonas saepentans*. *New Biotechnology*, 41, 15–24. <https://doi.org/10.1016/j.nbt.2017.11.003>
- Lee, K.-Y. and C.-G. Lee. (2002). Nitrogen removal from wastewaters by microalgae without consuming organic carbon sources. *Journal of Microbiology and Biotechnology*, 12(6), 979–985.

- Lee, K.-Y. ; L. C.-G. (2002). Nitrogen Removal from Wastewaters by Microalgae Without Consuming Organic Carbon Sources. *Journal of Microbiology and Biotechnology*, 12(6), 979–989.
- Leliaert, F., Smith, D. R., Moreau, H., Herron, M. D., Verbruggen, H., Delwiche, C. F., & De Clerck, O. (2012). Phylogeny and Molecular Evolution of the Green Algae. *Critical Reviews in Plant Sciences*, 31(1), 1–46. <https://doi.org/10.1080/07352689.2011.615705>
- Lemieux, C., Otis, C., & Turmel, M. (2014). Chloroplast phylogenomic analysis resolves deep-level relationships within the green algal class Trebouxiophyceae. *BMC Evolutionary Biology*, 14(1), 211. <https://doi.org/10.1186/s12862-014-0211-2>
- Lemieux, C., Vincent, A. T., Labarre, A., Otis, C., & Turmel, M. (2015). Chloroplast phylogenomic analysis of chlorophyte green algae identifies a novel lineage sister to the Sphaeropleales (Chlorophyceae). *BMC Evolutionary Biology*, 15(1), 264. <https://doi.org/10.1186/s12862-015-0544-5>
- Lepage, G., & Roy, C. C. (1984). Improved recovery of fatty acid through direct transesterification without prior extraction or purification. *Journal of Lipid Research*, 25(12), 1391–1396. [https://doi.org/10.1016/S0022-2275\(20\)34457-6](https://doi.org/10.1016/S0022-2275(20)34457-6)
- Li, S., Tan, H., Liu, B., Zhu, H., Hu, Z., & Liu, G. (2021). Watanabeales ord. nov. and twelve novel species of Trebouxiophyceae (Chlorophyta). *Journal of Phycology*, 57(4), 1167–1186. <https://doi.org/10.1111/jpy.13165>
- Lu, H. et al. (2017). Isolation, identification of Chlorella pyrenoides from aquaculture wastewater and its purification of wastewater. *Transactions of the Chinese Society of Agricultural Engineering* , 33(4), 273–277.
- LYNCH, J. M., & MOFFAT, A. J. (2005). Bioremediation - prospects for the future application of innovative applied biological research. *Annals of Applied Biology*, 146(2), 217–221. <https://doi.org/10.1111/j.1744-7348.2005.040115.x>
- Ma, S., Yu, Y., Cui, H., Li, J., & Feng, Y. (2020). Utilization of domestic wastewater as a water source of Tetrademus obliquus PF3 for the biological removal of nitric oxide. *Environmental Pollution*, 262, 114243. <https://doi.org/10.1016/j.envpol.2020.114243>

- Marin, B., & Melkonian, M. (2010). Molecular Phylogeny and Classification of the Mamiellophyceae class. nov. (Chlorophyta) based on Sequence Comparisons of the Nuclear- and Plastid-encoded rRNA Operons. *Protist*, *161*(2), 304–336. <https://doi.org/10.1016/j.protis.2009.10.002>
- Martínez-Dalmau, J., Berbel, J., & Ordóñez-Fernández, R. (2021). Nitrogen Fertilization. A Review of the Risks Associated with the Inefficiency of Its Use and Policy Responses. *Sustainability*, *13*(10), 5625. <https://doi.org/10.3390/su13105625>
- Melo, J. M., Telles, T. S., Ribeiro, M. R., de Carvalho Junior, O., & Andrade, D. S. (2022). *Chlorella sorokiniana* as bioremediator of wastewater: Nutrient removal, biomass production, and potential profit. *Bioresource Technology Reports*, *17*, 100933. <https://doi.org/10.1016/j.biteb.2021.100933>
- Mostert, J. F., & Frylinck, L. (2003). WHEY AND WHEY POWDERS | Applications of Dialysis. In *Encyclopedia of Food Sciences and Nutrition* (pp. 6167–6170). Elsevier. <https://doi.org/10.1016/B0-12-227055-X/01291-8>
- Muggia, L., Leavitt, S., & Barreno, E. (2018). The hidden diversity of lichenised Trebouxiophyceae (Chlorophyta). *Phycologia*, *57*(5), 503–524. <https://doi.org/10.2216/17-134.1>
- Pacheco, D., Rocha, A. C., Pereira, L., & Verdelhos, T. (2020). Microalgae Water Bioremediation: Trends and Hot Topics. *Applied Sciences*, *10*(5), 1886. <https://doi.org/10.3390/app10051886>
- Palafox-Sola, M. F., Yebra-Montes, C., Orozco-Nunnelly, D. A., Carrillo-Nieves, D., González-López, M. E., & Gradilla-Hernández, M. S. (2023). Modeling growth kinetics and community interactions in microalgal cultures for bioremediation of anaerobically digested swine wastewater. *Algal Research*, *70*, 102981. <https://doi.org/10.1016/j.algal.2023.102981>
- Pandey, A., Srivastava, S., & Kumar, S. (2019). Isolation, screening and comprehensive characterization of candidate microalgae for biofuel feedstock production and dairy effluent treatment: A sustainable approach. *Bioresource Technology*, *293*, 121998. <https://doi.org/10.1016/j.biortech.2019.121998>

- PARVIN, M., ZANNAT, M. N., & HABIB, M. A. B. (2007). Two Important Techniques for Isolation of Microalgae. *Asian Fisheries Science*, 20(1). <https://doi.org/10.33997/j.afs.2007.20.1.010>
- Pereira, H., Barreira, L., Figueiredo, F., Custódio, L., Vizetto-Duarte, C., Polo, C., Rešek, E., Engelen, A., & Varela, J. (2012). Polyunsaturated Fatty Acids of Marine Macroalgae: Potential for Nutritional and Pharmaceutical Applications. *Marine Drugs*, 10(9), 1920–1935. <https://doi.org/10.3390/md10091920>
- Pereira, H., Barreira, L., Mozes, A., Florindo, C., Polo, C., Duarte, C. V, Custódio, L., & Varela, J. (2011). Microplate-based high throughput screening procedure for the isolation of lipid-rich marine microalgae. *Biotechnology for Biofuels*, 4(1), 61. <https://doi.org/10.1186/1754-6834-4-61>
- Pereira, H., Gangadhar, K. N., Schulze, P. S. C., Santos, T., de Sousa, C. B., Schueler, L. M., Custódio, L., Malcata, F. X., Gouveia, L., Varela, J. C. S., & Barreira, L. (2016). Isolation of a euryhaline microalgal strain, *Tetraselmis* sp. CTP4, as a robust feedstock for biodiesel production. *Scientific Reports*, 6(1), 35663. <https://doi.org/10.1038/srep35663>
- Pereira, H., Schulze, P. S. C., Schüler, L. M., Santos, T., Barreira, L., & Varela, J. (2018). Fluorescence activated cell-sorting principles and applications in microalgal biotechnology. *Algal Research*, 30, 113–120. <https://doi.org/10.1016/j.algal.2017.12.013>
- Pham, T.-L., & Bui, M. H. (2020). Removal of Nutrients from Fertilizer Plant Wastewater Using *Scenedesmus* sp.: Formation of Bioflocculation and Enhancement of Removal Efficiency. *Journal of Chemistry*, 2020, 1–9. <https://doi.org/10.1155/2020/8094272>
- Phang, S. M., Miah, M. S., Yeoh, B. G., & Hashim, M. A. (2000). Spirulina cultivation in digested sago starch factory wastewater microalgae. *Journal of Applied Phycology*, 12(3/5), 395–400. <https://doi.org/10.1023/A:1008157731731>
- Pratoomyot, J. P. S. and T. N. (2005). Fatty acids composition of 10 microalgal species. *Songklanakarinn J. Sci. Technol* , 27(6), 1179–1187.
- Qin, L., Shu, Q., Wang, Z., Shang, C., Zhu, S., Xu, J., Li, R., Zhu, L., & Yuan, Z. (2014). Cultivation of *Chlorella vulgaris* in Dairy Wastewater Pretreated by UV Irradiation

- and Sodium Hypochlorite. *Applied Biochemistry and Biotechnology*, 172(2), 1121–1130. <https://doi.org/10.1007/s12010-013-0576-5>
- Rashmi, I., Karthika, K. S., Roy, T., Shinoji, K. C., Kumawat, A., Kala, S., & Pal, R. (2022). Soil Erosion and Sediments: A Source of Contamination and Impact on Agriculture Productivity. In *Agrochemicals in Soil and Environment* (pp. 313–345). Springer Nature Singapore. https://doi.org/10.1007/978-981-16-9310-6_14
- Rekha Kathal, V. C. , et al. (2016). Pollution Status of Yamuna River, India—A national concern. *International Research Journal of Environment Sciences*, 5, 1–6.
- Renuka, N., Guldhe, A., Prasanna, R., Singh, P., & Bux, F. (2018). Microalgae as multi-functional options in modern agriculture: current trends, prospects and challenges. *Biotechnology Advances*, 36(4), 1255–1273. <https://doi.org/10.1016/j.biotechadv.2018.04.004>
- Ricky, R., & Shanthakumar, S. (2023). Removal of ciprofloxacin from aqueous media by *Chlorella pyrenoidosa*, *Scenedesmus obliquus*, and isolated *Stichococcus bacillaris*: A comparative study on toxicity, removal mechanism and biochemical composition. *Journal of Environmental Chemical Engineering*, 11(3), 109990. <https://doi.org/10.1016/j.jece.2023.109990>
- Ruane, J., Sonnino, A., & Agostini, A. (2010). Bioenergy and the potential contribution of agricultural biotechnologies in developing countries. *Biomass and Bioenergy*, 34(10), 1427–1439. <https://doi.org/10.1016/j.biombioe.2010.04.011>
- Safi, C., Zebib, B., Merah, O., Pontalier, P.-Y., & Vaca-Garcia, C. (2014). Morphology, composition, production, processing and applications of *Chlorella vulgaris*: A review. *Renewable and Sustainable Energy Reviews*, 35, 265–278. <https://doi.org/10.1016/j.rser.2014.04.007>
- Salazar, J., Santana-Sánchez, A., Näkkilä, J., Sirin, S., & Allahverdiyeva, Y. (2023). Complete N and P removal from hydroponic greenhouse wastewater by *Tetradismus obliquus*: A strategy for algal bioremediation and cultivation in Nordic countries. *Algal Research*, 70, 102988. <https://doi.org/10.1016/j.algal.2023.102988>
- Salazar, J., Valev, D., Näkkilä, J., Tyystjärvi, E., Sirin, S., & Allahverdiyeva, Y. (2021). Nutrient removal from hydroponic effluent by Nordic microalgae: From screening

- to a greenhouse photobioreactor operation. *Algal Research*, 55, 102247. <https://doi.org/10.1016/j.algal.2021.102247>
- Sample, D. J., & Liu, J. (2014). Optimizing rainwater harvesting systems for the dual purposes of water supply and runoff capture. *Journal of Cleaner Production*, 75, 174–194. <https://doi.org/10.1016/j.jclepro.2014.03.075>
- Sanchez Rizza, L., Sanz Smachetti, M. E., Do Nascimento, M., Salerno, G. L., & Curatti, L. (2017). Bioprospecting for native microalgae as an alternative source of sugars for the production of bioethanol. *Algal Research*, 22, 140–147. <https://doi.org/10.1016/j.algal.2016.12.021>
- Saranya, D., & Shanthakumar, S. (2019). Green microalgae for combined sewage and tannery effluent treatment: Performance and lipid accumulation potential. *Journal of Environmental Management*, 241, 167–178. <https://doi.org/10.1016/j.jenvman.2019.04.031>
- Saranya, D., & Shanthakumar, S. (2020). Effect of culture conditions on biomass yield of acclimatized microalgae in ozone pre-treated tannery effluent: A simultaneous exploration of bioremediation and lipid accumulation potential. *Journal of Environmental Management*, 273, 111129. <https://doi.org/10.1016/j.jenvman.2020.111129>
- Saxena, P., & Bassi, A. (2013). Removal of nutrients from hydroponic greenhouse effluent by alkali precipitation and algae cultivation method. *Journal of Chemical Technology & Biotechnology*, 88(5), 858–863. <https://doi.org/10.1002/jctb.3912>
- Schulze, P. S. C., Carvalho, C. F. M., Pereira, H., Gangadhar, K. N., Schüler, L. M., Santos, T. F., Varela, J. C. S., & Barreira, L. (2017). Urban wastewater treatment by *Tetraselmis* sp. CTP4 (Chlorophyta). *Bioresource Technology*, 223, 175–183. <https://doi.org/10.1016/j.biortech.2016.10.027>
- Seyfabadi, J., Ramezanpour, Z., & Amini Khoeyi, Z. (2011). Protein, fatty acid, and pigment content of *Chlorella vulgaris* under different light regimes. *Journal of Applied Phycology*, 23(4), 721–726. <https://doi.org/10.1007/s10811-010-9569-8>
- Shrimali, M., & Singh, K. P. (2001). New methods of nitrate removal from water. *Environmental Pollution*, 112(3), 351–359. [https://doi.org/10.1016/S0269-7491\(00\)00147-0](https://doi.org/10.1016/S0269-7491(00)00147-0)

- Siebert, S., Burke, J., Faures, J. M., Frenken, K., Hoogeveen, J., Döll, P., & Portmann, F. T. (2010). Groundwater use for irrigation – a global inventory. *Hydrology and Earth System Sciences*, *14*(10), 1863–1880. <https://doi.org/10.5194/hess-14-1863-2010>
- Silva, A., Delerue-Matos, C., Figueiredo, S., & Freitas, O. (2019). The Use of Algae and Fungi for Removal of Pharmaceuticals by Bioremediation and Biosorption Processes: A Review. *Water*, *11*(8), 1555. <https://doi.org/10.3390/w11081555>
- Singh, J., Yadav, P., Pal, A. K., & Mishra, V. (2020). *Water Pollutants: Origin and Status* (pp. 5–20). https://doi.org/10.1007/978-981-15-0671-0_2
- Singh, P., Kumari, S., Guldhe, A., Misra, R., Rawat, I., & Bux, F. (2016). Trends and novel strategies for enhancing lipid accumulation and quality in microalgae. *Renewable and Sustainable Energy Reviews*, *55*, 1–16. <https://doi.org/10.1016/j.rser.2015.11.001>
- Sreekala, K. G. , et al. (2019). Microalgal Pigments as Natural Color: Scope and Applications. *Phytochemicals from Medicinal Plants. Apple Academic Press*, 257–278.
- Tamil Selvan, S., Velramar, B., Ramamurthy, D., Balasundaram, S., & Sivamani, K. (2020). Pilot scale wastewater treatment, CO₂ sequestration and lipid production using microalga, *Neochloris aquatica* RDS02. *International Journal of Phytoremediation*, *22*(14), 1462–1479. <https://doi.org/10.1080/15226514.2020.1782828>
- Tan, J. Sen, Lee, S. Y., Chew, K. W., Lam, M. K., Lim, J. W., Ho, S.-H., & Show, P. L. (2020). A review on microalgae cultivation and harvesting, and their biomass extraction processing using ionic liquids. *Bioengineered*, *11*(1), 116–129. <https://doi.org/10.1080/21655979.2020.1711626>
- Templeton, D. W., & Laurens, L. M. L. (2015). Nitrogen-to-protein conversion factors revisited for applications of microalgal biomass conversion to food, feed and fuel. *Algal Research*, *11*, 359–367. <https://doi.org/10.1016/j.algal.2015.07.013>
- Tossavainen, M., Lahti, K., Edelmann, M., Eskola, R., Lampi, A.-M., Piironen, V., Korvonen, P., Ojala, A., & Romantschuk, M. (2019). Integrated utilization of microalgae cultured in aquaculture wastewater: wastewater treatment and

- production of valuable fatty acids and tocopherols. *Journal of Applied Phycology*, 31(3), 1753–1763. <https://doi.org/10.1007/s10811-018-1689-6>
- Vaccari, D. A. (2009). Phosphorus: a looming crisis. *Scientific American*, 300(6), 54–59.
- Varela, J. C., Pereira, H., Vila, M., & León, R. (2015). Production of carotenoids by microalgae: achievements and challenges. *Photosynthesis Research*, 125(3), 423–436. <https://doi.org/10.1007/s11120-015-0149-2>
- Vidali, M. (2001). Bioremediation. An overview. *Pure and Applied Chemistry*, 73(7), 1163–1172. <https://doi.org/10.1351/pac200173071163>
- Visconti, S., Astolfi, M. L., Battistoni, A., & Ammendola, S. (2022). Impairment of the Zn/Cd detoxification systems affects the ability of Salmonella to colonize Arabidopsis thaliana. *Frontiers in Microbiology*, 13. <https://doi.org/10.3389/fmicb.2022.975725>
- Visviki, I., & Santikul, D. (2000). The pH Tolerance of Chlamydomonas applanata (Volvocales, Chlorophyta). *Archives of Environmental Contamination and Toxicology*, 38(2), 147–151. <https://doi.org/10.1007/s002449910018>
- Wang, L., Min, M., Li, Y., Chen, P., Chen, Y., Liu, Y., Wang, Y., & Ruan, R. (2010). Cultivation of Green Algae Chlorella sp. in Different Wastewaters from Municipal Wastewater Treatment Plant. *Applied Biochemistry and Biotechnology*, 162(4), 1174–1186. <https://doi.org/10.1007/s12010-009-8866-7>
- Warsinger, D. M., Tow, E. W., Nayar, K. G., Maswadeh, L. A., & Lienhard V, J. H. (2016). Energy efficiency of batch and semi-batch (CCRO) reverse osmosis desalination. *Water Research*, 106, 272–282. <https://doi.org/10.1016/j.watres.2016.09.029>
- Wu, Y., Guan, K., Wang, Z., Xu, B., & Zhao, F. (2013). Isolation, Identification and Characterization of an Electrogenic Microalgae Strain. *PLoS ONE*, 8(9), e73442. <https://doi.org/10.1371/journal.pone.0073442>
- Yasmin Anum Mohd Yusof. (2011). Fatty acids composition of microalgae Chlorella vulgaris can be modulated by varying carbon dioxide concentration in outdoor culture. *African Journal of Biotechnology*, 10(62). <https://doi.org/10.5897/AJB11.1602>

- Yousif, Y. I. D., Mohamed, E. Sh., & El-Gendy, A. S. (2022). Using chlorella vulgaris for nutrient removal from hydroponic wastewater: experimental investigation and economic assessment. *Water Science and Technology*, 85(11), 3240–3258. <https://doi.org/10.2166/wst.2022.157>
- Zhao, G., Wang, X., Hong, Y., Liu, X., Wang, Q., Zhai, Q., & Zhang, H. (2022). Attached cultivation of microalgae on rational carriers for swine wastewater treatment and biomass harvesting. *Bioresource Technology*, 351, 127014. <https://doi.org/10.1016/j.biortech.2022.127014>
- Zhou, W., Li, Y., Min, M., Hu, B., Chen, P., & Ruan, R. (2011). Local bioprospecting for high-lipid producing microalgal strains to be grown on concentrated municipal wastewater for biofuel production. *Bioresource Technology*, 102(13), 6909–6919. <https://doi.org/10.1016/j.biortech.2011.04.038>

6 Annex

Extracted Sequences

>Strain1_DES1_18S

GAGCTTAGGCTTGTCTTCAAAGATTAGCCATGCATGTCTAAGTATAAACTGCT
TATACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATAGTTTATTTGGTGGT
ACCTTCTTACTCGGAATAACCGTAAGAAAATTAGAGCTAATACGTGCGTAAAT
CCCGACTTCTGGAAGGGACGTATATATTAGATAAAAGGCCGACCGGGCTCTG
CCCGACCCGCGGTGAATCATGATATCTTCACGAAGCGCATGGCCTTGTGCCG
GCGCTGTTCCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATAGAGG
CCTACCATGGTGGTAACGGGTGACGGAGGATTAGGGTTCGATTCCGGAGAGG
GAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATT
ACCCAATCCTGATACGGGGAGGTAGTGACAATAAATAACAATACCGGGCATT
CATGTCTGGTAATTGGAATGAGTACAATCTAAATCCCTTAACGAGGATCCATT
GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTA
TATTTAAGTTGTTGCAGTTAAAAGCTCGTAGTTGGATTCGGGTGGGTCTCA
GCGGTCCGCCTATGGTGAGTACTGCTGTGGCCTTCCTTACTGTGCGGGGACCTG
CTTCTGGGCTTCATTGTCCGGGACAGGGATTCGGCATGGTTACTTTGAGTAAA
TTAGAGTGTTCAAAGCAGGCTTACGCCGTGAATACTTTAGCATGGAATAACAT
GATAGGACTCTGCCCTATTCTGTTGGCCTGTAGGAGTGGAGTAATGATTAAGA
GGAACAGTCGGGGGCATTCGTATTTTCATTGTCAGAGGTGAAATTCTTGATTT
ATGAAAGACGAACTACTGCGAAGCATTGTCAGGATGTTTTTCATTAATCAGA
ACGAAGTGGGGGCTCGAGACGATTAGATAACGTCGTAGTCTCACATAACGAT
GCGACTAGATGGCCGACGGTCTGCATGACTCGTCAGCACCTGAGAGCATCAG
CTTAGCTCAGGCGAGATTGTGATATGCTCATCAGCAGTGTCTGATATAATGCC

>Strain2_STII_18S

AATGCGCCAGATGTATTCTCCTGGCAGACTGGCGGAGTGTCTGCATCCAGTAT
AACTGCTTTATACTGTGAAACTGCGAATGGCTCATTAGAATCAGTTATAGTTTA
TTTGATGGTACCTACTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGTGC
GCAAATCCCGACTTCCGGAAGGGACGTATTTATTAGATAAAAGGCCGACCGG
GCTTGCCCGACTCGCGGTGAATCATGATAACTTCACGAATCGCATGGCCTTGC
GCCGGCGATGTTTCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATAG
AGGCCTACCATGGTGGTAACGGGTGACGGGGGATTAGGGTTCGATTCCGGAG

AGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCA
AATTACCCAATCCTGACACAGGGAGGTAGTGACAATAAATAACAATACCGGG
CTATTTTAGTCTGGTAATTGGAATGAGTACAATCTAAATCCCTAACGAGGATC
AATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAG
CGTATATTTAAGTTGCTGCAGTTAAAAAGCTCGTAGTTGGATTCGGATGGGT
ACCGTCGGTCCGCCTAACGGTGGTACTGGCGGCGCCCATCTTGCTGTCGGG
GACGGGCTCCTGGGCTTCACTGTCTGGGACTCGGAGTCGACGAGGTTACTTT
GAGTAAATTAGAGTGTTCAAAGCAGGCCTACGCTCTGAATACATTAGCATGG
AATAACACGATAGGGACTCTGGCCTATCTTGTTGGTCTGTAGGACCGGGAGTA
ATGATTAAGAGGGACAGTCGGGGGCATTCGTTATTTTCATTGTCAGAGGTGGA
AATTCTTGATTTATTGAAGGACGAACTACTGCGAAGCATTGCGCAAGGGATT
GTTTTTCATTAATCAAGGACGAAAGTTGGGGGCTCGAGACGATTAGCATAAC
CGTTCCTTAGTCTTCAACCATTAACGATGGCCGACTAGGATTGGTGATTGTT
CTTCTTGATGACTCCCACCAGCCACCTATTGAGAAAGTCCAAAGCTATCGG
TTCCAGTGGAGATATTGATGTGTTCCGCCACAGACT

>Strain3_DES2_18S

CTAGGCTTCGGGGGGGCGGATGAGGTGTCGGCATTAGCCATGCATGTCTAA
GTATAAACTGCTTATACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATAGT
TTATTTGGTGGTACCTTCTTACTCGGAATAACCGTAAGAAAATTAGAGCTAAT
ACGTGCGTAAATCCCGACTTCTGGAAGGGACGTATATATTAGATAAAAGGCCG
ACCGGGCTCTGCCCGACCCGCGGTGAATCATGATATCTTCACGAAGCGCATG
GCCTTGTGCCGGCGCTGTTCCATTCAAATTTCTGCCCTATCAACTTTCGATGG
TAGGATAGAGGCCTTCCTTGGTGGTAACGGGTGACGGAGGATTAGGGTTCGA
TTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAG
GCGCGCAAATTACCCAATCCTGATACGGGGAGGTAGTGACAATAAATAACAA
TACCGGGCATTTCATGTCTGGTAATTGGAATGAGTACAATCTAAATCCCTAAC
GAGGATCCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCAGCT
CCAATAGCGTATATTTAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTCG
GGTGGGTCTCAGCGGTCCGCCTATGGTGAGTACTGCTGTGGCCTTCCTTACTG
TCGGGGACCTGCTTCTGGGCTTCATTGTCCGGGACAGGGATTCGGCATGGTT
ACTTTGAGTAAATTAGAGTGTTCAAAGCAGGCTTACGCCGTGAATACTTTAGCA
TGGAATAACATGATAGGACTCTGCCCTATTCTGTTGGGCCTGTAGGAGTGGAG
TAATGATTAAGAGGAACAGTCGGGGGCATTCGTTATTTTCATTGTCAGAGGTGA

AATTCTTGATTTATGAAGACGAACTTACTGCGAAAGCATTGCAAGGATGTTTC
ATTATTCAAGAACGAAAGTGGGGCCTCGAGACGATAGATACGTCGTAGTCTC
AACATAACGATGCGACTAGGAATGCCGAACGTTTTGGCATGACTTCGTACG
CACTGAGCATCAGTTGGTTCGGGGATAGTAATAGTGG

>4_STI2_18S

GTGAACATTGCAGGCCTCCCTGGCATTACGCCATGCATGTCTCAGTATAAAC
TGCTTTATACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATAGTTTATTTG
ATGGTACCTACTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGTGCGCA
AATCCCGACTTCCGGAAGGGACGTATTTATTAGATAAAAGGCCGACCGGGCT
TGCCCGACTCGCGGTGAATCATGATAACTTCACGAATCGCATGGCCTTGCGCC
GGCGATGTTTCATTCAAATTTCTGCCCTATCAACTTTCGATGGGTAGGGATAG
AGGCCTACCATGGGTGGGTAACGGGGTGACGGGGGAATTAGGGGTCCAATT
CCCGGAGAAGGGAGGCCTGGAAAAACGGCCTCCACATCCCAAGGAGGGG
GGGCGGGGGCCCCAAATAACCCAATCCTGGCCCCCGGGGGGGAGGGGCAA
AAAAAAAAAAACCCGGGCTATTTTTATTTCGGGGATTTGGAAAGGGGACAAA
CCCAATCCCCTTAAACAGGGATTATTTGGGGGGGAAATTTGGGGGCCCCCCC
CCCCCGGTTATTTCCCCCCCCAAAACGGATATTTTAAGTTGGGGGCGGTAAA
AAACCCTGTTTGGTTTTTGGAGAGGGGGCCCCGGGGGGCCCCACAAAGGGG
GGTTTTTCGGGGGGCCCCCTTTTTTTTTTTGGGAGAGAGCCCCGCGTGTCTCT
CTGTGTGGGGGACCCACAACCACAGCATCTTTTGTGTATAAATATAATGTGTG
TCAGCGAGCGCCCCTCCCTTGTATATATATTATAGAAAAAACACAAAAGAAG
TGCGGCGCGCTCTCTATTGTGTGTGCGTGACGCAGCGCACATAATATTATAG
GAGAGACGCGCGGCGACTCTCTTTCTCTGTGCGGAAGAGGAGTATTTGTG
TTGTATTATAACAC

>5_DES3_18S

CGGAATCCTATTGAGGTGCGGCATGAGCCATGCATGTCTAAGTATAAACTGC
TTATACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATAGTTTATTTGGTGG
TACCTTACTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGTGCGTAAATC
CCGACTTGGAAACGAAGTATATTATTTAGATAAAAGGCCGACCGAGCTTTGCTC
GACCCCGCGGGAAACTGTACTCCACGA

GCTGAAGCGAGTGTGGCGGCTGCGCTCGCCTTTTTCTTAAAAAAATTTTG
TCGCGCCCATAATCAAACCTTCGATGGTAGGATAGAGGCCTACCATGGTGGTA

ACGGGTGACGGAGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACG
GCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGATAC
GGGGAGGTAGTGACAATAAATAACAATACCGGGCATTTCATGTCTGGTAATTG
GAATGAGTACAATCTAAATCCCTTAACGAGGATCCATTGGAGGGCAAGTCTG
GTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTTAAGTTGTTGC
AGTTAAAAAGCTCGTAGTTGGATTCGGGTGGGTCTAGCGGTTCCGCCTATG
GTGAGTACTGCTTATGGCTTCTTTCTGTCTGGGACGGCTTCTGGCTTCACTTGT
CCGGGACTCGGAGTCCGACGTGGTACTTTGGTAATTTAGAGTGGTCAAGCA
GGCTACCCCGAATACTTTAACCTGGATACCAATGGACTCTGCTATCTTGTTGTC
TGTA GAACCGATATGATAGGGACGTTCCGGGGCATTCTTATTGCGAGTGA
ATTCTTGATTTTAAAAGCACTCTGGAAGCCTGCCAGAGGTTTCTATATCACA
ATGGTCTGGACATACTACCGCGATGTCACATATGCCAGTGCAGATACAAGTTC
GAC

>Strain6_CHL_18S

TAGACCCAGATATCTGCATTCTGCTGGCATGACGCCATGCATGCTGCTAAGTA
TAAACTGTTTTATACTGTGAAACTGCGAATGGCTCATTAATCAGTTATAGTTT
ATTTGATGGTACCTACTACTCGGATACCCGTAGTAAATCTAGAGCTAATACGTG
CGCAAATCCCGACTTCTGGAAGGGACGTATTTATTAGATAAAAGGCCGACCG
GGCTTGCCCGACTCGCGGTGAATCATGATAACTTCACGAATCGCATGGCCTCG
TGCCGGCGATGTTTCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATA
GAGGCCTACCATGGTGGTAACGGGTGACGGAGGATTAGGGTTCGATTCCGGA
GAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGC
AAATTACCCAATCCTGACACAGGGAGGTAGTGACAATAAATAACAATACTGG
GCCTTTTCAGGTCTGGTAATTGGAATGAGTACAATCTAAACCCCTTAACGAGG
ATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAA
TAGCGTATATTTAAGTTGCTGCAGTTAAAAGCTCGTAGTTGGATTCGGGTG
GGGCCTGCCGGTCCGCCGTTTCGGTGTGCACTGGCAGGGCCCACCTTGTTGC
CGGGGACGGGCTCCTGGGCTTCACTGTCCGGGACTCGGAGTCCGGCGCTGTTA
CTTTGAGTAAATTAGAGTGTTCAAAGCAGGCCACGCTCTGAATACATTAGCA
TGGAATAACACGATAGGACTCTGGCCTATCCTGTTGGTCTGTAGGACCGGA
GTAATGATTAAGAGGGACAGTCGGGGGCATTTCGATTTTCATTGTCAGGAGGT
GAAATTCTTGATTTATGAAGACGAACTACTGCGAAAGCATTGCGCAAGGAT
GTTTCATTAATCAAGAACGAAGGTTGGGGGGCTCGAAGACGATTAGAATACC

GTCCTAGTCTCACCCATAACCGATGGCCGACTAGGATCGCCGAATGTTTTCTC
GATGACCTCCCGCCCCGCCACCTTTATGGAGAAAATCAAGTATCGTACAGGGG
GCATAGATGAGATGCGCTGCTCGCACAGGTGGCTA

>7_PSE_18S

CCCAGGGTGGAGGTATCGCTGCAGCTGCATGACTCATGCGTGCGCCAGTATA
AACTGTTTCATACTGTGAAACTGCGAATGGCTCATTAATCAGTTATAGTTTAT
TTGATGGTACCTACTACTCGGATACCCGTAGTAAATCTAGAGCTAATACGTGC
GCAAATCCCGACTTCTGGAAGGGACGTATTTATTAGATAAAAGGCCGACCGG
GCTTGCCCGACTCGCGGTGAATCATGATAACTTCACGAATCGCATGGCCTCGT
GCCGGCGATGTTTCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATAG
AGGCCTACCATGGTGGTAACGGGTGACGGAGGATTAGGGTTCGATTCCGGAG
AGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCA
AATTACCCAATCCTGACACAGGGAGGTAGTGACAATAAATAACAATACTGGG
CCTTTTCAGGTCTGGTAATTGGAATGAGTACAATCTAAACCCCTTAACGAGGA
TCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAAT
AGCGTATATTTAAAGTTGCTGCAGTTAAAAGCTCGTAGTTGGATTTCGGGTG
GGGCCTGCCGGTCCGCCGTTTCGGTGTGCACTGGCAGGGGCCACCTTGTTGC
CGGGGACGGGCTCCTGGGCTTCACTGTCCGGGACTCGGAGTCGGCGCTGTTA
CTTTGAGTAAATTAGAGTGTTCAAAGCAGGCCTACGCTCTGAATACATTAGCA
TGAATAACACGATAGGACTCTGGCCTATCCTGTTGGTCTGTAGGACCGGAGTA
ATGATTAAGAGGGACAGTCGGGGGCATTCGTAATTCATTGTCAGAGGTGAAA
TTCTTGGATTTATGAAAGACGAACTACTGCGAAAGCCATTTGCCAAGGATGTT
TTCATTAATCAAGACCGAAAGTTTGGGGCTCGAGAACGATTAGATAACCGTCCT
AGTCCTCAACCATAAACGATGCGGACTAGGGAATCGGCGAATGTTTCTTCGA
ATGACTTCGCTCACGTATGGAATTCAGTTTGGGTTCGCGGAAGTA

>Strain6_CHL_ITS055

CAAATGTAGAGAGTCAGCGATGACTCTGGCAGACTGACGCCAGTTCCTGAG
ACTCAAGAGCGCCCTTCGTACCTCGCGTCTGAACCGGGCGGCATAGCCGGGG
CAACAACCCCCGGTTTTATCCCGCCCGCACAACCCATGCTGACCAGCGCCCA
GCGCATTCAACCAACCGTGATTCAAGCGTGTGCGTGGGCGCACGCACGCGA
GCCATTCAATCCCCTCTCACCAATTTCAAGTACTTATTA ACTCTCTCTCCAAAG

TACGTTCCATCTTTCTCTCGCGCTAGTTGTTGCTATCGATCTCCCTTCCGTCT
TTATCCATACATGGAAGTTACCACCTGCTTGGTGTTCATGCATAACAACCC
AACTCTTCCAAAGCGTCCCGTGCATGGAGCCGCTCGCGCCGCTCCCGACTTC
TCACTCTCTTTGACTGACCTTTCTTGGGGAGTTGCGGGCAGACTGACTGAGA
CAGAGATTCTCTAGATTAACATAACTCGCCAGACGCAGGATATTTCTTGCT
GGTCTTTGCCCGGCTCGCTCGCTCTTACTAACGACATCCTGCCTAGTTACTTT
CCCTCCGCTTACTGATATGCTTAAGTACAGCGAGCAGTCTTGCCTGACCTCAC
GCAGATCGTGAGGGTGGGTTCAGTTCGCGGCACGGAGAGCACCGAATTCAT
GCTGGCTCTCCATCAAGACACATCCGGCAACGGCCGGCTGCGTACGGAGTGC
GTACCTACCTAGCCATCCACCTGCATACAGACTCCATGTTTCATTCATCTCTCTT
CCATTCAACCCAGACGGAATGCCGGCAGAAGGAGGGACGCACGAACCAGCA
GGACAGCTCCGCTCCCTCCCGCTCTTCATGCAAAGGAGGCGGGCGCGAGAA
GATGTGAGCGACCTAACTCAAGACATGACCTGCGTCGAACCATGGGTGCAAT
TACTTGCGATAAATCGATCGATGATGAATTAATCATTCAATCACTTACGAATTT
CATGCGTGCCTACTCGAATCGAGAGCAGACTACAATCGTGAAAGATGACTTT
GCATGCTCGCGTTCAAGCAAGGCTAATACGCAATAGATTGCTAGAATAACGCTA
GGTGGAAAAGATGCATAGAATGGGCGTTCCATCA

>7 ITS055R (ITS)_F7_20240116_102812.ab1

CATACGGTACGAGTACACATGACGATGACTTCATGGCAGACGGAGACAGATT
CCGGAGACTCAAGAGCGCCCTTCGTACCTCGGCTCTGACCGGGCGGCATAGC
CGGGGCAACAACCCCCGGTTTTATCCCGCCCGCACAAACCCATGCTGACCAGC
GCCAGCGCATTCAACCAACCGTGAAGCAGGCGTGTGCCTGGGCGCACCCG
TGGGATCCAATCGCTTCCCTCTCAACAATTTCAAGTACTTTTTAACTCTCTTTT
CAAAGTTCTTTTCATCTTTCCCTCGCGGTACTIONTTCGCTATCGGTCTCCCGTC
CGTATTTAGCCTTAGATGGGATTTACCACCTGCTTTGGGCTGCATTCCCAAAC
AACCCGACTCTTCGAAAGCGTCCCGTGGAGCCCCAGGCTCCGGTCCCAAACG
GGGTTCTCACCTCTTTGACGCCCCTTTCCAGGGGACTTGGGGCCAGACTGG
GGCAGAGAGCGCTTCTCTAGACTACAATTCGCCAGCCGGAGGCTGGAGATTT
TCAAGTTGGGCTTTGCCCGGTTGCTCGCCGTTACTAAGGGCATCCTGGTTAG
TTTCTTTTCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTAGTCTTGCCTGA
GCTCAGGTCGAAAGTGAGGGTGGTTTCAGGGGCCGCAAGGCGACCACCGAA
TTCTGCTGGGCCTCCAACAAAGACCCCTCGGGCAACGGCAGGCTGCGTAG
GGGGTGCCTACCTACCAAGCCATCGCCCTGCAGACGGGGTCCATGTTCAAGC

CTCTACGCTTCAACCAACCCGGACGGCGTCCCGGCACAAGGGGGGACGCTC
GGAGCCGGGAGGGCCAGCTCCGCTCCCGCACGCTCTTCAATGAGAGGGGGC
GGGGGCGAGAGGTGTGAGCCGACGCTGAGGCAGACATGCCCTCGGCCGAA
GCCTTGGGCGCAATTTGGCGTTCAAGATTCGATGGTTCACGGGAATTCTGCA
ATTCACACTACGTTATCGCATTTGCTGCGTTCTTTCATCGAATAACGGAGCA
GAATATCCGTTATGAAAGTTGTCATTGGATTTGGCTTGCCTCGAGCCAAGCTT
CCGAACGCAATGGCTCATTGAGTCAAGCTGGGGGTGTAAAGGCGTTGGTCTT