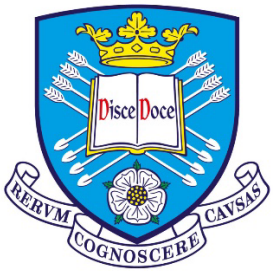

The University Of Sheffield.

An Integrated Approach for Landfill Leachate Treatment Using Plasma/UV Pre-Treatment and Indigenous Green Microalgae

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This thesis is submitted in partial fulfilment

of the requirements for the degree of

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Department of Chemical and Biological Engineering

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Declaration

I, Aya Tarif Farag, declare that this thesis is my own work and the research conducted within is the result of my own effort, unless acknowledged otherwise in the text. I am aware of the University's Guidance on the Use of Unfair Means (www.sheffield.ac.uk/new-students/unfair-means#What-is-unfair-means).

I confirm that this work has not been previously submitted for any other degree at this, or any other, university.

Acknowledgements

I would like first to thank **Allah (God)** for everything, I don't think I would have ever been able to do anything without Allah's support. My faith was the only and main thing that kept me going through all the hardships that I have been through during my PhD journey abroad and away from family. It was the only thing that kept me resilient after being hit by a car in a car accident two weeks before my first-year exam (I was left with three fractures that left me using crutches for almost 3 months), yet I entered the exam and passed. My faith also kept me going through the hard time of the lockdown during covid-19 outbreak, and it was also the main (and only) thing that kept me strong when I knew that the one person, I love the most in this world (my mother) was diagnosed with stage 4 of lung cancer (she is now recovering).

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"Mom and Dad, I hope I made you proud, I love you!"

Dedication

I am dedicating this thesis to my late grandmother "**Teta Raffaeya**" who always gave me unconditional love and support, made endless prayers for me, and fed me lots of great food!

"Studying abroad and living away from my family for almost five years made me remember you, think of you and miss you every single day grandma with your charming smile, warm heart, and beautiful soul, although you are not here with us in this world anymore, but you will always be in my heart. This thesis is for you **Teta Raffaeya**, I am sure you would have been very happy and proud just like you did when you knew I got my M.Sc. degree, I love you grandma!"

Your Granddaughter

Aya

Abstract

An increase in municipal solid waste (MSW) production is usually the inevitable result of a continuously growing population. Landfilling is the most applied method worldwide to deal with the produced MSW. However, one major drawback of landfilling is the production of a complex, toxic and hazardous type of wastewater which is landfill leachate (LL). Various methods for LL treatment exist which can mainly be divided into biological and physical-chemical methods, however integrating both approaches result in more effective treatment. In this study, soil samples (with leachate runoff) from a landfill leachate treatment site in Chesterfield, UK are collected. Indigenous microalgal strains from the samples are isolated, purified and genetically identified using five different primers which allowed the sequencing of most of the rDNA (18S, 5.8S, ITS1 & ITS2 regions). The identified sequences are submitted to the NCBI GenBank and given accession numbers, identification revealed a community of four green microalgae namely two strains of *Chlorella vulgaris*, one strain of *Chlorococcum* species and one strain of *Scotiellopsis reticulata*. The threshold concentration of LL tolerated by the four green microalgae after dilution with distilled water (v/v) is tested by growing them separately in different LL concentrations (5%, 10%, 15%, 20% and 25%). The two strains of *Chlorella vulgaris* were the only strains capable of showing a significant growth increase (p<0.05) in the challenging concentration of 20% LL (v/v) which is usually inhibitory to other algal strains. Hence, concentration 20% LL is chosen as a platform for the following screening experiment to test the most potent strain/s in growth and biotreatment of LL. One replicate of one of the strains of *Chlorella vulgaris* (C.V.M*) (CCAP 211/141) started showing an outstanding growth at the end of a 30-day experiment with a significant ammonia removal (p<0.05). To confirm these findings, the two replicates of *Chlorella vulgaris* (C.V.M* and C.V.N) are further tested in 20% LL (supplemented with phosphate) in a 30-day experiment. A dramatic increase in the growth of C.V.M* by 19-fold over its peer C.V.N with a 75% ammonia removal (starting from concentration 290.73 mg/L) are observed. This percentage is further improved when implementing plasma technology (with fluidic oscillator incorporated) as a pre-treatment step. The plasma

pre-treatment induced LL colour change after three hours (from dark-brown to yellow) and reduced ammonia-N concentration by 1.9-fold. Strain C.V.M* showed the highest growth in 20% LL pre-treated by plasma compared to the untreated LL, with a final biomass yield of 0.38 g/L, a total increase in the total ammonia-N removal (79%) and a significant decrease (p<0.05) in the pH value from 8.6 to 6.67 at the end of the experiment. Whole genome sequencing for both strains revealed differences in genotypes of 15169 single nucleotide polymorphism (SNP) loci and 2046 insertion-deletion (indel) loci. This in turn might indicate the possibility of a developed mutation or sexual reproduction that might have increased the ability of strain C.V.M* (CCAP 211/141) to tolerate harsher conditions and higher ammonia-N concentrations in 20% diluted LL. This might pave the way for a possible powerful candidate in LL treatment using a highly tolerant algal strain which when coupled with plasma pre-treatment might provide a possibly effective, eco-friendly, and sustainable LL treatment approach.

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Abreviations List

µl microlitre

µm micrometre

µmols photons m^{-2} s^{-1} micromole per square meter per second

ammonia-N ammonia-nitrogen

AOPs advanced oxidation processes

As arsenic

BLAST Basic Local Alignment Search Tool

BOD biochemical oxygen demand

C. vulgaris Chlorella vulgaris

C.v.1.1/C.V.M* denotes *Chlorella vulgaris* (the possible mutant strain)

C.v.1.2/C.V.N denotes *Chlorella vulgaris* (the wildtype/normal strain)

Chloro *Chlorococcum* sp.

COD chemical oxygen demand

Cr chromium

Cu copper

d^{-1} per day

DOM dissolved organic matter

g gram

g/L gram/litre

Gp. group (of all green microalgal strains together)

HMs heavy metals

Indel insertion-deletion

ITS1 internal transcribed spacer 1

ITS2 internal transcribed spacer 2

Kb kilobase

KHz Kilohertz

KV kilovolt

L/m litre/minute

LL landfill leachate

MEGA Molecular Evolutionary Genetic Analysis

mg/L milligram/litre

MSW municipal solid waste

NCBI national centre for biotechnology information

nF nanofarad

ng nanogram

NH_3 ammonia

nm nanometre

OD optical density

P phosphorous

Pb lead

PCR polymerase chain reaction

pmol picomole/s

PVA poly vinyl alcohol

rDNA ribosomal Deoxyribonucleic acid

rpm revolution per minute

RSD relative standard deviation

S. reticulata/S.r Scotiellopsis reticulata

SNP single nucleotide polymorphism

SSU small subunit

TOC total organic carbon

UV ultraviolet

v/v volume/volume

VFA volatile fatty acids

Wt. weight

w/v weight/volume

x *g* relative centrifugal force expressed in units of times gravity

XOCs Xenobiotic organic compounds

CHAPTER ONE

Introduction

1.1. Background

Less than 1% of the water on earth is directly available for human use (Cuellar-Bermudez *et al.*, 2017). Moreover around 70% of the world's freshwater is currently used in agriculture and this percentage may even reach 95% in some countries (Sato *et al.*, 2013). Therefore, water pollution with various wastes, together with a growing population, becomes a major threat. Unfortunately, more than 80% of the produced wastewater in the world, in general, and more than 95% of the generated wastewater in some of the least developed countries, in particular, is released to the environment without any treatment. After being discharged to different water bodies, this wastewater could either be diluted, transported downstream or infiltrates into aquifers, thus both the quality and availability of freshwater supplies could be severely affected. Wastewater discharged into rivers and lakes, eventually end up in the oceans posing a major threat to the marine environment with all its negative consequences (UNESCO, 2017).

Wastewaters could be defined as disposable liquids/water-carried wastes resulting from different practices e.g., domestic, urbanization, agricultural as well as industrial practices. Types of wastewaters include agricultural wastewater, anaerobically-digested wastewater, industrial wastewater and municipal wastewater (Gonçalves, Pires and Simões, 2017). Amongst different types of wastewaters, landfill leachate stands out as a major threat to the environment (Gotvajn, Tišler and Zagorc-Končan, 2009) with its rivers and groundwater (Kumari, Ghosh and Thakur, 2016) as well as soils and different living organisms including humans (Khanzada and Övez, 2017).

It is believed that urbanisation and increased generation of municipal solid wastes (MSW) are concomitant on a global level. The majority of the produced MSW worldwide (almost 95%) is landfilled, as landfilling is considered an affordable, widely applicable and environment friendly (if engineered landfills are implemented) technology compared to other technologies such as compositing and incineration, however landfilling still pose the threat of landfill leachate production. Landfill leachate is a highly polluted liquid with different proportions of various undesirable/toxic compounds, it is produced as a result of decomposition of different wastes in the landfill together with percolating rainwater as well as the water content already inherent in the landfill wastes. Landfill leachate represents an environmental burden, it is estimated that one tonne of solid wastes generates 0.2 m^3 of landfill leachate during the decomposition process. Landfills are also reported to continue producing leachate for more than 50 years after their closure. Although engineered landfills are usually provided by liners and leachate collecting systems, however leachate treatment still represent a necessity before being discharged into the environment, this represents a greater burden and a major problem in low- and middle-income countries (less developed countries) where open dumps and/or non-engineered landfills represent the most common practice (Renou *et al.*, 2008; Kurniawan *et al.*, 2010; Luo *et al.*, 2020; Wijekoon *et al.*, 2022). Therefore, treatment of landfill leachate (LL) before being discharged into natural water bodies in an effective and sustainable way is an extremely important prerequisite (Wiszniowski *et al.*, 2006; Renou *et al.*, 2008; Kumari, Ghosh and Thakur, 2016).

Different treatment approaches/technologies for landfill leachate (LL) are reported. Methods for LL treatments could be classified into four main categories: conventional methods, biological methods, physical-chemical methods, and integrated biological-physical-chemical methods. Integrated approaches for landfill leachate treatments are attracting a growing attention as it has been demonstrated that no single method solely was capable of treating LL in an effective and cost-effective way enough to meet the discharge standards set by different authorities (Dogaris, Ammar and Philippidis, 2020; Teng *et al.*, 2021). Common biological processes involving aerobic and anaerobic

approaches although being simple, reliable, and usually cost-effective, nonetheless they have some major drawbacks such as high production of sludge (in aerobic treatments) as well as insufficient removal of some of the most significant contaminants in LL such as ammonia-N (in anaerobic treatments with the exception of Anammox) and this might be attributed to the lack of NH_3-N degradation in the anaerobic system (Kurniawan *et al.*, 2010; Luo *et al.*, 2020), which in turn increase the urgency to search for alternative efficient biological treatments methods. In this context, LL treatment using microalgae represents a promising, relatively novel, eco-friendly, and efficient (with varying degrees) to the existing aerobic and anaerobic biological treatments. Microalgae were reported to grow in LL on both lab-scale and larger-scale studies with varying, yet promising, removal efficiencies of different contaminants in leachate (Dogaris, Ammar and Philippidis, 2020; Nawaz *et al.*, 2020).

Regarding physical-chemical treatments of LL, although being reported for its efficiency in treatment of LL (especially old/stabilised LL), however its relatively high cost and/or environmental impact risks still represent a major concern (Dogaris, Ammar and Philippidis, 2020; Luo *et al.*, 2020; Wijekoon *et al.*, 2022). Few studies have reported the efficiency of utilising Plasma/UV technologies as physical methods in LL treatment, although scaling up is still a challenge in this aspect, however being clean eco-friendly methods greatly encourage exploring these technologies as complementing steps to the microalgal treatments (Wu *et al.*, 2004; Shu *et al.*, 2006; Zhao *et al.*, 2011; Singh *et al.*, 2021).

Hence, several studies highlighted the efficiency of combining biological methods and physical-chemical methods so the scope of this study focused on this integration approach, however there was no reports in the literatures (as far as the authors know) on integrating plasma /UV as a pre-treatment step with microalgal treatment for LL, this novel integrated approach is believed to benefit from coupling efficient LL treatment with production of valuable algal biomass thus represents an addition to the sustainability, efficiency and possibly cost-effectiveness of LL treatment for the possibility of wider application in the future.

1.2. Hypothesis of the study

I hypothesise that, motivated by the considerations just broached, integrating a biological method with a physical-chemical one in LL treatment will provide a more efficient treatment approach. Biological treatment of LL using microalgae represents a promising technology thus I further hypothesise that selecting and screening indigenous strains which is naturally capable of growing in LL might provide potent candidates in the field of landfill leachate treatment. In the same context, implementing a clean technology such as plasma or UV as a complementing pre-treatment step to the microalgal treatment might further enhance algal growth by breaking down some of the organic compounds, possibly chromogenic, thus enables higher algal growth which in turn support higher removal efficiencies for different nutrients/pollutants in the leachate in a sustainable eco-friendly and possibly cost-effective way.

1.3. Aims and contributions of the study

This study aims for three main objectives:

1. Selection of indigenous microalgal strains naturally growing in landfill leachate treatment sites.
2. Screening of the isolated indigenous microalgal strains for their ability to grow in LL (20% v/v) and perform bioremediation with special focus on ammonia-N removal with the selection of the best performing alga/algae.
3. Implementing plasma/UV technologies as a pre-treatment step prior to the microalgal treatment to determine the efficiency of the integrated system for an effective LL treatment.

1.4. Thesis Structure

This thesis is divided into seven chapters:

- **Chapter 1**: is the introduction chapter which includes a brief background about the main problem addressed in the thesis, which is landfill leachate treatment, as well as the hypothesis and aims of the current study.

- **Chapter 2**: is the review of literature which describes the problem of landfill leachate in detail, starting with identifying the concept of landfill leachate as well as its characteristics and different treatment methods with special focus on the biological treatment using microalgae and the physical treatment using plasma/UV. It outlines some of the important previous studies conducted in the same context. It also addresses the gap of integrating both microalgae and plasma/UV as a novel approach in landfill leachate treatment, in addition to a brief description of the four upcoming experimental chapters (3-6).

- **Chapter 3**: is the first experimental chapter which explores the algal and cyanobacterial biodiversity in a soil sample with leachate runoff from a landfill leachate treatment site in Chesterfield, UK. It also describes the process of isolation, purification, and molecular identification of four green microalgal strains and two cyanobacterial strains from the abovementioned treatment site as indigenous microbial inhabitants in this environment.

- **Chapter 4**: is the second experimental chapter and it investigates the growth pattern of the isolated green microalgae (after excluding the cyanobacterial strains) in different landfill leachate concentrations to determine the maximum leachate concentration threshold tolerated by any of the isolated strains to be used as a platform concentration in further experiments.

- **Chapter 5**: is the third experimental chapter involving a screening experiment to examine the growth and bioremediation capability of the different

microalgal strains (individually and together) in the chosen landfill leachate concentration (20% v/v) throughout a 30-day experimental period whilst monitoring the microalgal growth, pH and COD levels, ammonia-N uptake, and heavy metals uptake continuously every five days.

- **Chapter 6:** is the fourth and final experimental chapter and it focuses on implementing plasma/UV as a pre-treatment step for the 20% LL (v/v) before applying the algal treatment (of the highest performing algal strain) whilst monitoring the algal growth and nutrients/pollutants uptake from the leachate continuously, simultaneously with testing the effect of untreated leachate vs. the plasma/UV pre-treated leachate for comparison purposes. A complete genome sequencing for the highly growing algal strain *Chlorella vulgaris* (C.V.M*) is also conducted.

- **Chapter 7:** is the conclusion chapter where some concluding remarks are drawn in addition to shedding some light on some possible future work

CHAPTER TWO

Review of literature

A general outline for the structure of the literature review is illustrated in Figure 2-1

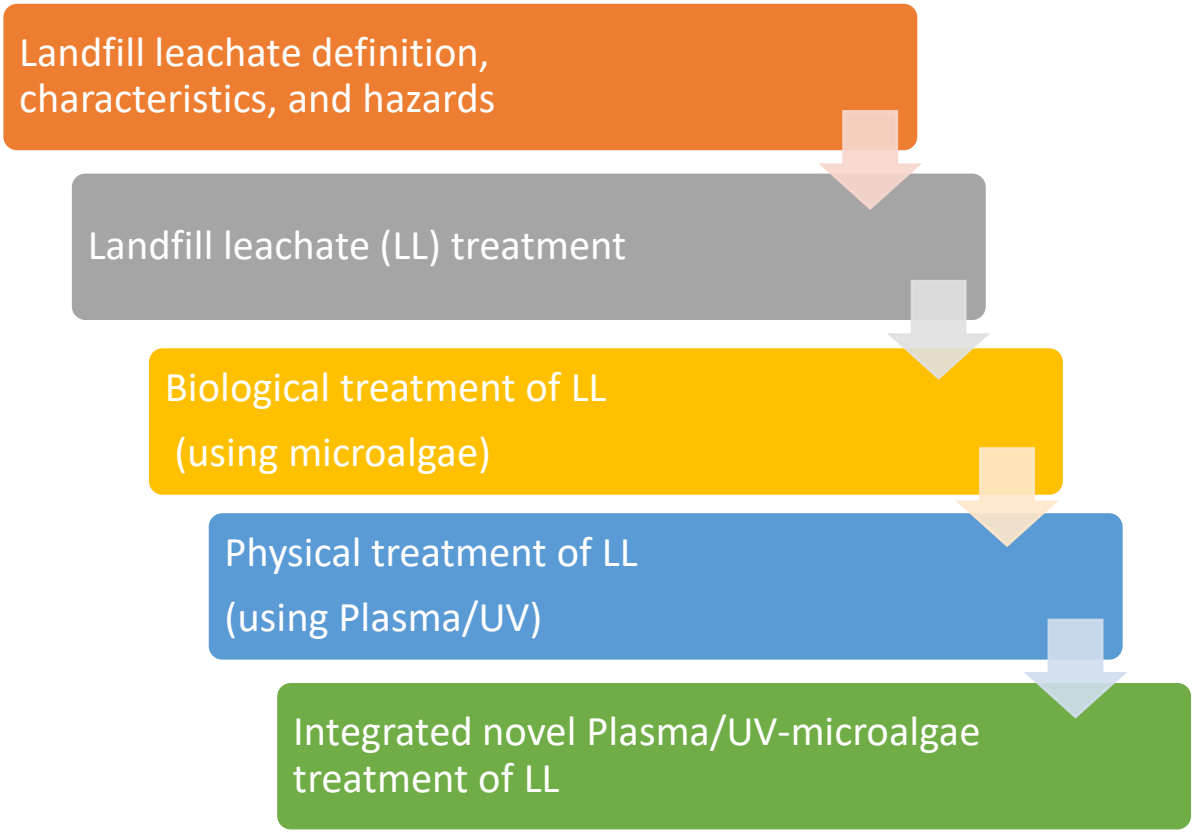

Figure 2-1 A diagram representing the workflow of the literature review in the present study.

2.1. Landfill leachate definition, characteristics, and hazards

2.1.1. Landfill leachate definition

Urbanization, industrial and commercial growth have both pros and cons. One of the major cons related to the continuing commercial and industrial growth is the rapid increase in both the municipal and industrial solid waste production. The generation of municipal solid waste (MSW) is increasing on both an overall term and even per

capita. One important example is the data comparing the waste production in the years 1994 and 1997 in Rio de Janeiro, Brazil, where the waste production were 6200 tonnes/day and 8042 tonnes/day, respectively, although the growth of population during this period was zero (Renou *et al.*, 2008). The main problem is if these huge amounts of generated wastes were not managed scientifically thus both the living organisms and the ecosystem will be subjected to deleterious effects (Kumari, Ghosh and Thakur, 2016). Although there are different means for the disposal of solid wastes e.g., landfilling, incineration, and compositing, but landfilling is the most widely accepted method for both economic and environmental reasons. Landfilling is considered the cheapest method with minimal environmental hazards as it allows wastes to decompose under controlled conditions until it reaches an inert stable state (Renou *et al.*, 2008). Almost 95% of the worldwide produced municipal solid waste (MSW) is landfilled (Khanzada and Övez, 2017). However, one of the major challenges related to landfilling of the municipal solid wastes is the generation of landfill leachate. Landfill leachate may be defined as "*the aqueous effluent generated as a result of rainwater percolation through wastes, biochemical processes in waste's cells and the inherent water content of wastes themselves*" (Renou *et al.*, 2008).

In other words, landfill leachate is generated as a result of several causes: the inherent moisture content in the wastes itself as well as the natural humidity, biological degradation of organic matter present in the landfill wastes, different biochemical, chemical and physical changes taking place in these wastes in addition to the percolation of rainwater through the landfill, all these causes result in the generation of landfill leachate which is a heavily polluted dark-coloured liquid, usually having a strong smell, and is formed beneath the landfill due to infiltration processes (Kumari, Ghosh and Thakur, 2016; Peng, 2017; Zareen T. Khanzada and Övez, 2017). Landfill leachate could infiltrate/contaminate ground water and/or surface water by mobilizing through landfills with improper lining or no lining and lacking a proper leachate collecting system (Parvin and Tareq, 2021) as illustrated in Figure 2-2.

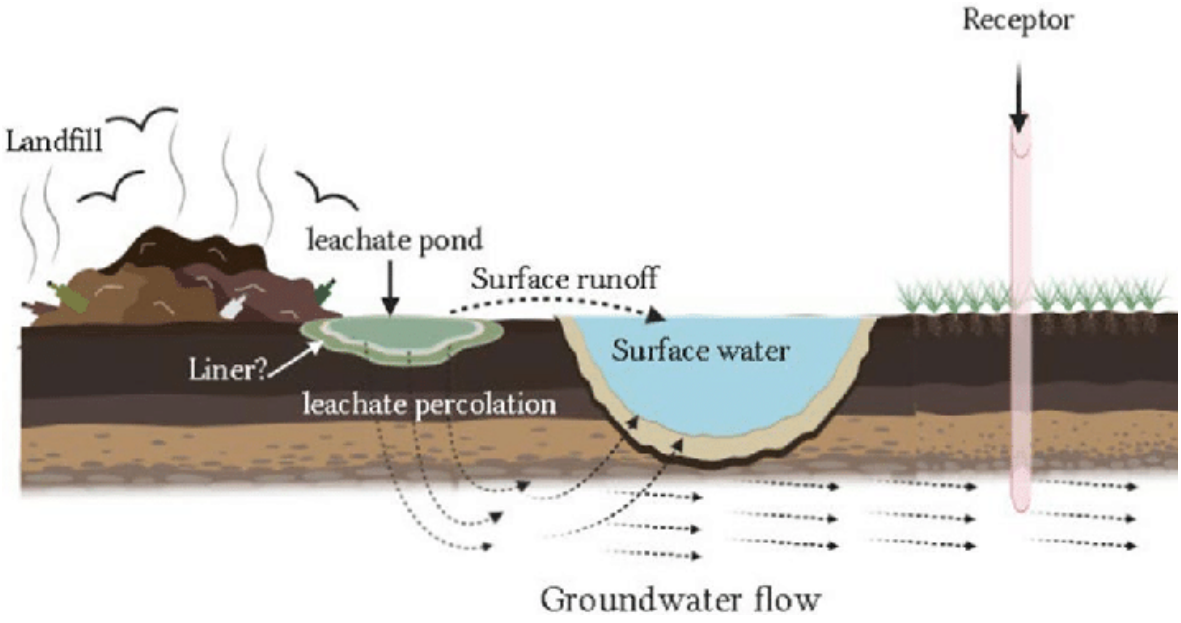

Figure 2-2 A diagram showing landfill leachate percolating groundwater and surface water through landfills with no/improper liners posing severe hazards to the surrounding environment and living organisms (Parvin and Tareq, 2021).

2.1.2. Landfill leachate characteristics

Although leachate composition may vary considerably according to several factors including the landfilling technology, age of waste which determine its decomposition state as well as the composition of wastes itself, but generally landfill leachate contains some major contaminants that may be categorized into four main groups: -

1- Dissolved organic material which may involve volatile fatty acids as well as fulvic-like and humic-like compounds.
2- Inorganic macrocomponents e.g., calcium, magnesium, sodium, potassium, ammonium, iron, manganese, sulphate, chloride, and hydrogen carbonate.
3- Heavy metals e.g., cadmium, zinc, chromium, copper, lead, and nickel.
4- Xenobiotic organic compounds (XOCs) and these are usually found in relatively small concentrations, and they include aromatic hydrocarbons, phenols, chlorinated aliphatics, pesticides and plastizers.

Other compounds: some compounds of secondary importance that may be found in very small concentrations include borate, sulphides, arsenate, selenite, barium, lithium, mercury, and cobalt (Kjeldsen *et al.*, 2002).

An important note that is worth shedding some light on is, that within the same landfill itself the leachate composition may vary, and this is attributed to the complex series of biological and chemical reactions that take place when wastes are buried in a landfill. The buried refuse undergoes several decomposition stages. These stages may be grouped into four main phases. These phases include: -

- An initial aerobic phase: in this phase (which takes only few days), the oxygen which is already present in the buried refuse is consumed resulting in the production of carbon dioxide and an increase in the waste temperature. Since the waste is covered so the consumed oxygen is not compensated thus an anaerobic environment is formed which in turn enhances the fermentation processes and this will form the core of the next phase.
- An anaerobic acid phase: this process takes place around neutral pH, in this process the anaerobic environment described earlier enhances certain groups of bacteria to break down cellulose and hemicellulose which constitute 45-60% of the dry MSW weight. There are three main groups of bacteria that are involved in these processes, and these are:

a) Hydrolytic and fermentative bacteria which hydrolyse polymers to monosaccharides and then ferment them to acids and alcohols.

Polymers → monosaccharides → carboxylic acids and alcohols.

b) Acetogenic bacteria, these bacteria convert the resulting acids and alcohols to acetates, hydrogen, and carbon dioxide.

carboxylic acids and alcohols → acetates, H_2 and CO_2 .

c) Methanogens bacteria, which convert acetates, hydrogen and carbon dioxide to methane and carbon dioxide.

acetates, H_2 and CO_2 → methane and CO_2 .

This phase is characterised by the accumulation of acids which in turn causes a decrease in pH which may increase the solubility of several compounds, also the

highest BOD (biochemical oxygen demand) and COD (chemical oxygen demand) could be detected at this phase.

- An initial methanogenic phase: at this phase a considerable amount of methane is produced, pH is becoming more neutralized supporting more growth for methanogenic bacteria which in turn convert the acids that have been accumulating during the acid phase to methane and carbon dioxide (collectively known as biogas) which causes the methane production rate to increase. The COD and BOD concentrations begin to decrease and the pH begins to increase as more acids are consumed by the methanogenic bacteria.
- The stable methanogenic phase: where the rate of methane production reaches its maximum and then starts to decrease as the amount of soluble acids decrease but, some recalcitrant compounds may still exist such as humic and fulvic acids.

These phases were documented as a result of field and laboratory data, other following decomposition phases were also hypothesized including the conversion of waste cell from anaerobic to aerobic but there were not enough data to support such hypothesis (Kjeldsen *et al.*, 2002; Dogaris, Ammar and Philippidis, 2020).

In the same context, the abovementioned phases take place over time as the landfill age increases, thus it could be concluded that the chemical composition of the landfill (based on the biochemical reactions and the decomposition phase that take place in the landfill wastes) vary according to the landfill age, which in turn influence the characteristics/parameters of the landfill leachate produced in each stage/phase of the landfill age (Table 2-1) (Renou *et al.*, 2008; Peng, 2017; Nawaz *et al.*, 2020; Wijekoon *et al.*, 2022). Therefore, landfill leachate may be classified (based on the landfill age) into three categories:

1- Young landfill leachate (<5 years)

It could also be considered as the "acetogenic" leachate, which is usually characterised by high concentrations of organic compounds which in turn reflects having relatively higher measurements of some parameters e.g., COD, BOD/COD as well as higher biodegradability, however it is characterised by relatively low ammonia-N concentrations and low pH due to the continuous acids production.

2- Intermediate landfill leachate (5-10 years):

The LL at this stage is characterised by having almost neutral pH (6.5-7.5) with lower levels of COD and BOD/COD.

3- Old/stabilised landfill leachate (>10 years):

This leachate is also called "methanogenic" LL and it is characterised by having higher pH values due to acid consumptions as well as higher concentrations of NH_3-N, but lower COD and BOD/COD levels (Peng, 2017; Nawaz et al., 2020; Wijekoon et al., 2022).

Table 2-1 Leachate characteristics/parameters based on change in landfill age (Peng, 2017).

Type of Leachate	Young	Intermediate	Stabilized
Age (years)	<5	5-10	>10
pH	<6.5	6.5-7.5	>7.5
Biodegradability	Important	Medium	Low
Kjeldahl nitrogen (g/L)	0.1-0.2	-	-
Ammonia-N (mg/L)	<400	-	>400
TOC/COD	<0.3	0.3-0.5	>0.5
Heavy metals (mg/L)	Low-medium	Low	Low
BOD/COD	0.5-1.0	0.1-0.5	<0.1
COD (mg/L)	>10,000	4,000-10,000	<4000

2.1.3. Landfill leachate hazards

Landfill leachate treatment has been attracting researchers recently for various environmental and economic reasons. Figure 2-3 clearly shows the increasing interest in the field of landfill leachate treatment by showing the growing number of published papers in the past few decades from 1973 to 2003 (Renou *et al.*, 2008).

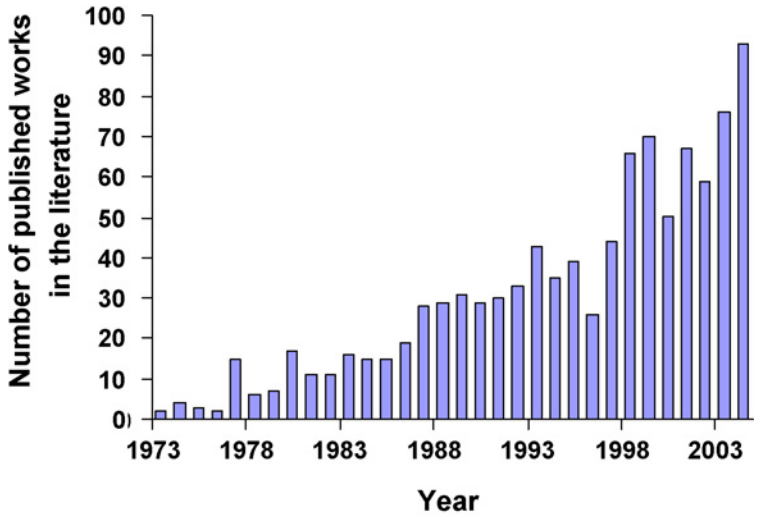

Figure 2-3 The growth in number of published work concerning landfill leachate treatment from 1973 to 2003 (Renou *et al.*, 2008).

Landfill leachate toxicity to the environment is well documented (Kjeldsen *et al.*, 2002; Jones, Williamson and Owen, 2006; Öman and Junestedt, 2008; Renou *et al.*, 2008). The release of leachate to waterbodies in the environment without treatment has serious deleterious effects including partial oxygen depletion from the waterbodies it is released into, which in turn causes serious changes in the bottom fauna and flora as well as ammonia toxicity (Kjeldsen *et al.*, 2002). Besides, several bioassay tests indicated that untreated leachate may induce cytotoxicity, genotoxicity, carcinogenicity or estrogenicity as a result to the synergistic, additive, or antagonistic effects of the contaminants present in it (Kumari, Ghosh and Thakur, 2016). Moreover, several hazardous compounds are found in untreated leachate, many of which are not yet identified (Öman and Junestedt, 2008). It became a necessity, because of the previously mentioned reasons and because of the fact that landfill sites continue producing leachate for hundreds of years even after closure, to approach sustainable eco-friendly and economic methods for landfill leachate treatment before discharging it into the environment (Jones, Williamson and Owen, 2006).

Some of the important measures before releasing landfill leachate into natural waters is the removal of organic materials based on COD and BOD as well as ammonium (Renou *et al.*, 2008). One of the major causes of toxicity in landfill leachate is ammonia (Kjeldsen *et al.*, 2002). Although, generally the pollutants in LL exhibit decrease in their

concentrations over time, nevertheless, according to several studies, ammonia is considered as the most significant component of LL on the long term as the ammonia produced as a result of decomposition of organic wastes remains at high levels at the methanogenic phase. Furthermore, due to lack of other ammonia degradation mechanisms, leaching was deemed as the possible mechanism for ammonia reduction in the methanogenic phase (Dogaris, Ammar and Philippidis, 2020; Wijekoon *et al.*, 2022). Ammonia is known to be more toxic in its unionised free form (NH_3) rather than in its ionised form (NH_4), and the ammonia ionisation is a pH dependent process whereas the unionised form of ammonia is the dominant form under conditions of high pH which happens to be the prevalent condition at the stable methanogenic phase of LL, thus ammonia is considered as the most dangerous/toxic LL component with possible long-term environmental hazardous effects to different water resources (Dogaris, Ammar and Philippidis, 2020).

However, heavy metals present in untreated landfill leachate represents a major problem as well. If heavy metals are present in aquatic environments in limits exceeding the permissible ones, they will cause direct toxicity to humans and other life forms. Nonetheless, the hazardous effects of heavy metals are still possible even when they exist in dilute undetectable quantities, and this is attributed to their recalcitrance and persistence which may cause them to show toxic effects as a result to some natural processes such as biomagnification. Heavy metals are considered as one of the major inorganic contaminants in the environment because of the previously mentioned reasons and because of their mobility in the aquatic ecosystems as well as their toxicity to higher life forms (Suresh Kumar *et al.*, 2015). Heavy metals may also exist in landfill leachate in considerable amounts representing an environmental issue if the leachate contaminates the surface or ground water and a treatment issue when attempting to collect and treat leachate before discharging it into the environment (Baun and Christensen, 2004).

Dissolved organic matter (DOM) is another major component in LL which account for the dark brown colour of LL, DOM affect the transportation and bioavailability of heavy metals via complexes formation, and they could also interact with other

pollutants in the Leachate. Concentrations of DOM in LL could be estimated via different parameters measurements such as COD, BOD, TOC, VFA as well as individual compounds e.g., methane (Wijekoon *et al.*, 2022).

Landfill leachate (due to the presence of high levels of the abovementioned pollutants) poses a major threat to the environment and must be effectively treated before being safely discharged into the environment, otherwise soils, ground water, surface water, different living organisms and eventually humans would be severely affected as illustrated in Figure 2-4 (Wijekoon *et al.*, 2022).

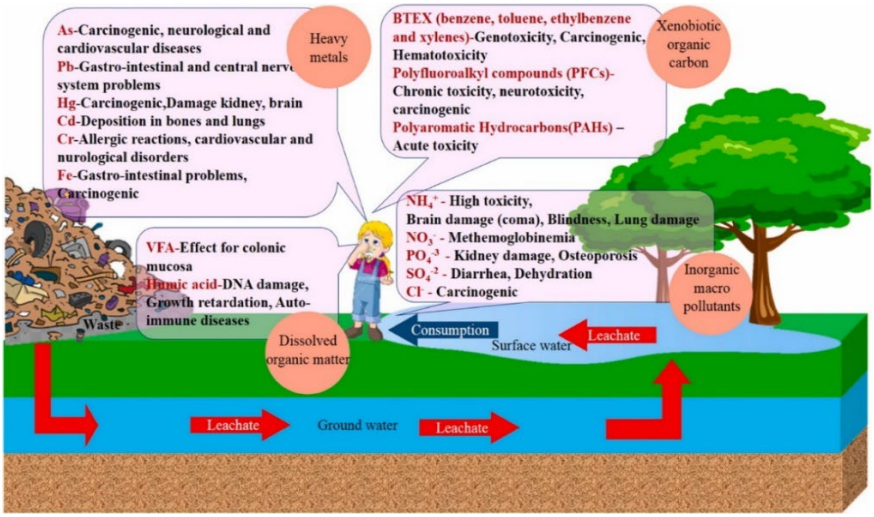

Figure 2-4 Different deleterious effects of LL components on ecosystems and humans for both short and long terms (Wijekoon et al., 2022).

2.2. Landfill leachate (LL) treatment

Treatment of landfill leachate before discharging it into the environment is a necessity (Wiszniowski *et al.*, 2006) due to the abovementioned reasons. A vast majority of technologies concerning landfill leachate treatment are applied. Renou *et al.* (2008) classified the landfill leachate treatment methods into conventional methods and new methods whilst Wiszniowski *et al.* (2006) classified them into biological methods and chemical-physical methods. However, this classification in addition to leachate transfer and integrated approaches which involve combining more than one method of physical-chemical and/or biological treatments of LL is widely reported in the literature (Dogaris, Ammar and Philippidis, 2020; Luo *et al.*, 2020; Nawaz *et al.*, 2020;

Wijekoon *et al.*, 2022). In the present study, the former classification will be applied and outlined, also both advantages and disadvantages of the different methods will be mentioned.

Different approaches for LL treatment are outlined in Figure 2-5, briefly LL treatment methods can be classified into:

2.2.1. Leachate transfer

Leachate transfer involves either treatment of LL in conjunction with domestic sewage or recycling the leachate back to the landfill (Figure 2-5). Advantages of these methods involve low operational cost, the presence of nitrogen in LL and phosphorus in sewage enhance the biological treatment process, also leachate recycling increases the moisture content in the landfill waste which in turn provide more nutrients for the microorganisms responsible for waste decomposition. On the other hand, major drawbacks of these methods involve the inhibitory effects possibly caused by toxicants in LL to the decomposing microorganisms (Dogaris, Ammar and Philippidis, 2020; Nawaz *et al.*, 2020).

2.2.2. Biological treatments

Biological treatments are divided into aerobic treatment and anaerobic treatment, in aerobic treatments microorganisms degrade organic compounds to carbon dioxide and sludge whilst in anaerobic treatment microorganisms degrade organic compounds to carbon dioxide and methane (biogas) (Luo *et al.*, 2020). Microorganisms in biological treatments are either grown in suspended mode or attached mode (Dogaris, Ammar and Philippidis, 2020). Both aerobic and anaerobic treatment methods are listed in Figure 2-5. Main advantages of biological treatments include being efficient especially in treatment of LL with high organic content, relatively inexpensive (in most cases), reliable and simple. However, the main drawbacks of the biological treatment methods generally involve the hampering effect caused by toxic compounds in leachate or by high concentrations of refractory substances which might exert an inhibitory effect on the biological activity thus affect the treatment efficiency. Biological treatments are deemed efficient in treatment of young LL with

high percentage of organic matter (Dogaris, Ammar and Philippidis, 2020; Luo *et al.*, 2020; Wijekoon *et al.*, 2022).

2.2.3. Physical-chemical treatments

Physical-chemical treatment methods involve several different methods such as coagulation-flocculation, chemical precipitation, adsorption, membrane filtration, air stripping, chemical oxidation/advanced oxidation processes (AOPs), Bio electrochemical treatments (Figure 2-5) and membrane technologies e.g., microfiltration, nanofiltration, ultrafiltration as well as reverse osmosis.

Physical-chemical treatments have the advantage of being efficient in removal of non-biodegradables (e.g., humic and fulvic acids) as well as undesirable compounds such as heavy metals. They are usually considered effective for old leachate and as a pre-treatment step before biological treatment or a final polishing step before discharging the LL to the environment (Dogaris, Ammar and Philippidis, 2020; Luo *et al.*, 2020). Main drawbacks of physical-chemical methods involve relatively high cost, high sludge production in some methods e.g., coagulation-flocculation and possibility of air pollution in case of certain methods e.g., air stripping (Wijekoon *et al.*, 2022).

2.2.4. Integrated approaches of physical-chemical and biological treatments

Each of the methods implemented in the treatment process of landfill leachate has both advantages and disadvantages and no method solely was capable of achieving an effective treatment that can meet the limits for release into surface waters (Wiszniowski *et al.*, 2006; Gotvajn, Tišler and Zagorc-Končan, 2009). Integration of two or more methods (Figure 2-5) could increase the efficiency of both/all by combining the advantages of the implemented methods and overcoming their respective defects thus achieving more efficient LL treatment with lower cost (Teng *et al.*, 2021). This is, in fact, widely supported by several studies in the literature (Renou *et al.*, 2008; Dogaris, Ammar and Philippidis, 2020; Luo *et al.*, 2020; Teng *et al.*, 2021; Wijekoon *et al.*, 2022). Integrated systems for LL treatments may involve combination of two or more physical-chemical treatments, combination of two or more biological treatments as well as combination of a physical-chemical and a biological treatment methods (Luo *et al.*, 2020). However, the recommendation of a sustainable treatment technology/s

depend on several criteria e.g., efficiency of the treatment method, its cost, and its environmental impact.

The present study will mainly focus on the combination of plasma/UV technologies as a physical treatment with using green microalgae as the biological treatment (Figure 2-5) to achieve an efficient, sustainable, environmentally friendly, and possibly an applicable cost-effective method for the treatment of landfill leachate.

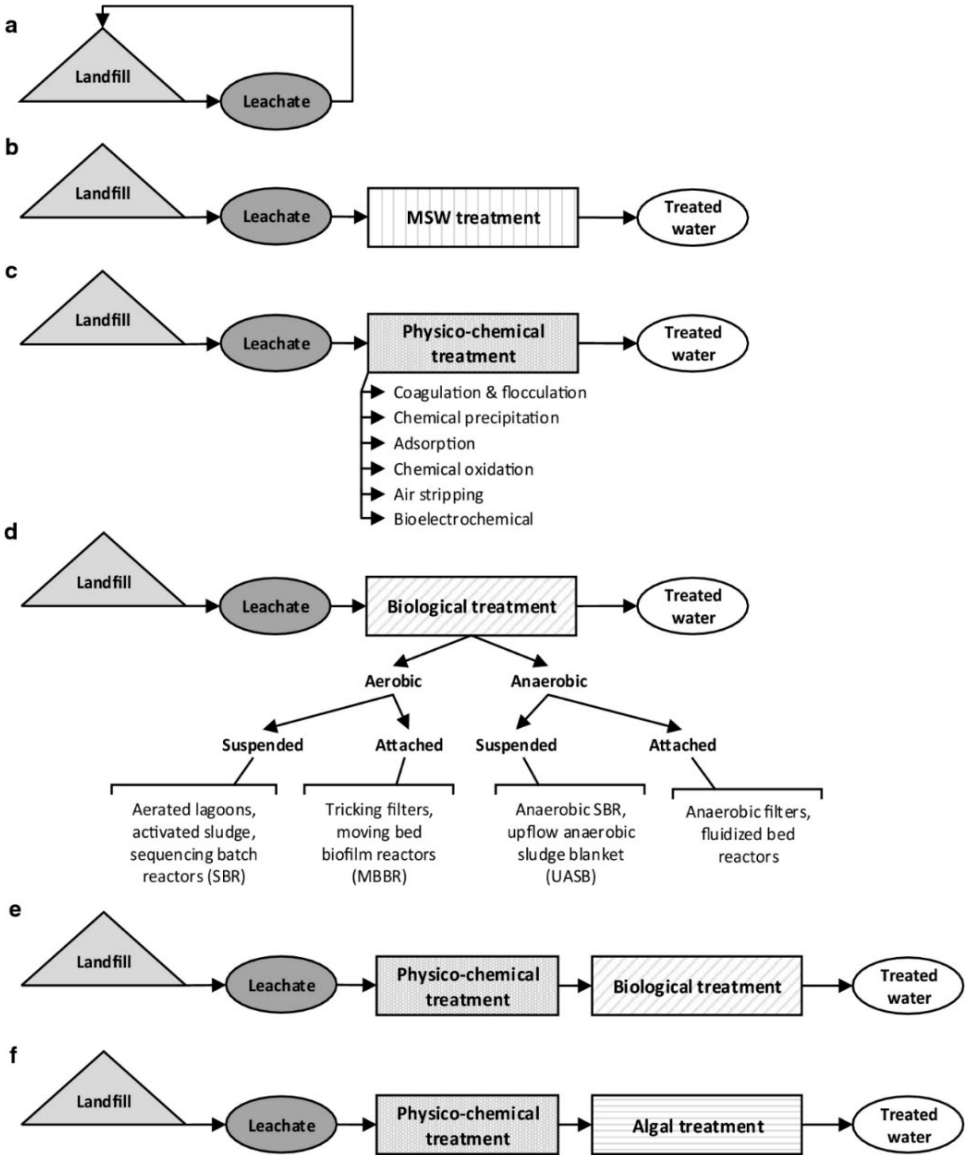

Figure 2-5 Overview of the main landfill leachate treatment methods: a) recirculation; b) mixing with municipal solid waste (MSW); c) physico-chemical treatments; d) biological treatments; e) combination of physico-chemical and biological treatments; f) combination of physico-chemical and algal treatments (Dogaris, Ammar and Philippidis, 2020).

2.3. Biological treatment of LL

The limitations of biological treatments is well documented (Renou *et al.*, 2008; Kurniawan *et al.*, 2010; Dogaris, Ammar and Philippidis, 2020; Luo *et al.*, 2020; Teng *et al.*, 2021; Wijekoon *et al.*, 2022). Thus the urge for exploring novel biological LL treatment approaches (that could overcome these limitations) is increasing, especially as the discharge standards for LL in most countries is getting harder (Renou *et al.*, 2008; Dogaris, Ammar and Philippidis, 2020; Nawaz *et al.*, 2020). In this context, landfill leachate treatment using microalgae may provide a novel alternative that is worth exploiting its potentials which will be further discussed below.

2.3.1. Biological treatment of LL using microalgae

Algae are a very diverse and large group of microscopic and macroscopic eukaryotic organisms that carry on oxygenic photosynthesis. Algae are known to be ubiquitous in nature as they inhabit a wide variety of terrestrial and aquatic (marine and fresh water) habitats which made them extensively studied by researchers in different fields (Wang and Chen, 2009). Another reason for studying different algal species was their fascinating capability of being useful in different fields such as food and health supplements production, aquaculture support, pharmaceuticals and biofuel production as well as waste water treatment (Cuellar-Bermudez *et al.*, 2017) which will be the scope of this study. The study of microalgae for wastewater treatment purposes has been going for a while (Paskuliakova *et al.*, 2018), hence comes the expression phycoremediation. Phycoremediation has been reported since 1957 (Cuellar-Bermudez *et al.*, 2017) and can be defined as the use of algae to take up and/or biotransform the organic and inorganic pollutants in wastewaters during their growth in it (Paskuliakova, Tonry and Touzet, 2016). Generally, wastewater can represent a source of available free nutrients (especially nitrogen and phosphorus) which can be utilized by algae for growth thus coupling the process of wastewater treatment with the process of biomass production in addition to the possibility of recovery of the desired compounds from the wastewater e.g., heavy metals (Cuellar-Bermudez *et al.*, 2017). Although there are various studies on the possibility of treating wastewater using microalgal biomass but there is not as much number of studies regarding the

biotreatment of landfill leachate using microalgae which may be possibly attributed to the complex nature of landfill leachate and its possible toxicity to living organisms (Paskuliakova, Tonry and Touzet, 2016). Nevertheless, microalgae have shown some promising results in this regard which make them possible candidates in effective leachate treatment.

Bioremediation of LL using microalgae is attracting an increasing attention although being a relatively recent field with inadequate coverage in the literature compared to other leachate treatment technologies in general. The main reasons behind this is mainly because LL treatment technologies using microalgae have a couple of attractive merits as they could valorise low-value wastewater and leachate by production of valuable algal biomass utilising only nutrients that already exist in the leachate and/or wastewater, this is concurrent with the advantage of LL bioremediation/treatment which in turn may add to the sustainability and/or cost-effectiveness of the whole process by producing valuable algal biomass (by the end of the treatment) which could further be used in the production of biofuel, bioactive compounds and/or resources recovery (Dogaris, Ammar and Philippidis, 2020; Nawaz *et al.*, 2020).

Landfill leachate is usually very complex in its composition with variable constituents in varying proportions depending on landfill age amongst other factors, however it usually contains significant amounts of ammonia nitrogen and organic matter as well as other components (Gotvajn, Tišler and Zagorc-Končan, 2009). Ammonia is one of the most common constituents of LL, which results from the process of biological degradation of amino acids and other nitrogen-containing organic matter found in the leachate (Cheung, Chu and Wong, 1993). Although ammonium is considered as a preferred nitrogen source for algae to uptake and assimilate, nonetheless high ammonia concentrations may have very toxic effects on different living organisms as well as the environment (Hellebust and Ahmad, 1989; Wijekoon *et al.*, 2022). Toxicity/inhibitory effects of high levels of ammonia-N present in LL (> 110 mg/L) to microalgal growth is widely reported in the literature (Cheung, Chu and Wong, 1993; Zhao *et al.*, 2014; Hernández-García *et al.*, 2019; Zheng *et al.*, 2019).

On the other hand, concentrations of ammonia-N tolerated by microalgae vary from one species to another (Zheng *et al.*, 2019) as shown in Table 2-2.

Table 2-2 Ammonia-N levels tolerated by different green microalage.

Microalgal strain	Ammonia-N tolerance (mg L^{-1})	Reference
Chlorella pyrenoidosa	134 mg L^{-1}	Lin *et al.*, 2007
Chlamydonomas snowiae (LK)	134 mg L^{-1}	Lin *et al.*, 2007
Chlorella pyrenoidosa (LK)	405 mg L^{-1}	Lin *et al.*, 2007
Scenedesmus obliquus	≥167 mg L^{-1}	Hernández-García *et al.*, 2019
Desmodesmus spp.	≥167 mg L^{-1}	Hernández-García *et al.*, 2019
Chlorella vulgaris FACHB-30	110 mg L^{-1}	Zheng *et al.*, 2019

The presence of ammonia in leachate in the form of ammonium (NH_4^+)/free ammonia (NH_3) is a temperature and pH dependent process. Ammonia in its unionised free form (NH_3) is reported to be more toxic to different living organisms especially in high concentrations, its acute toxicity and sometimes lethal effect to fish, duckweed, algae, and other microorganisms is well reported in the literature (Cheung, Chu and Wong, 1993; Clément and Merlin, 1995; Francis-Floyd *et al.*, 2009; Wijekoon *et al.*, 2022). However, the relatively high pH value (>8) characterising the stabilised/old/methanogenic LL favours the formation of the unionised/free form of ammonia (more toxic form) especially at high temperatures thus contributes to the increasing toxicity of ammonia-N in LL (Cheung, Chu and Wong, 1993). Despite being a highly toxic component of LL (especially in high concentrations), high removal

efficiencies were exhibited by different tested microalgae in diluted LL and were reported in several studies in the literature (Cheung, Chu and Wong, 1993; Zhao *et al.*, 2014; Hernández-García *et al.*, 2019; Zheng *et al.*, 2019).

Heavy metals (HMs) represent an important component of landfill leachate. Removal of heavy metals can be challenging as they represent a persistent hazard in the environment which cannot be chemically nor biologically degraded, they can only undergo transformation, circulation and eventually accumulation throughout the food chain representing a serious threat to human beings and other living organisms. The metal uptake capacity of certain algal strains may exceed those of activated carbon, natural zeolite, and synthetic ion-exchange resin plus many other advantages regarding the speed, time and energy saving, ease of handling, low cost, large surface to volume ratio, being both user and eco-friendly as well as providing the possibility of easy recovery of the desired metals. Microalgae can remove heavy metals from solutions using two main processes:

- The passive removal process: this is a non-metabolic, rapid, and reversible step. This step takes place at the cell surface and can be done by both living and non-living cells. In this step, heavy metals are adsorbed to the different functional groups at the surface of the cell by various electrostatic interactions.
- The active removal process: this is a metabolic-dependent, slow, and usually irreversible step. It can be done only by living cells and takes place within the cell. This process involves the transportation of metal ions across the cell membrane barrier and then accumulation inside the cell after binding to intracellular organelles and/or compounds (Suresh Kumar *et al.*, 2015).

Dissolved organic matter (DOM) present in landfill leachate includes amino acids, volatile fatty acids, hydrophilic acids, fulvic-like and humic-like compounds. The functional groups in the DOM could possibly interact with other contaminants in the leachate. They might form complexes with heavy metals present in the leachate which might further influence their transportation, stability, and bioavailability. They also account for the leachate dark brown colour. DOM can be characterised using several bulk parameters e.g., COD, BOD, BOD/COD ratio, TOC, VFA and other individual

compounds, i.e., methane (Teng *et al.*, 2021; Wijekoon *et al.*, 2022). Adequate amounts of COD were reportedly removed from LL by different tested algal strains (Lin *et al.*, 2007; El Ouaer *et al.*, 2017). Algae are also known by their efficiency in phosphorous removal as phosphorous is essential for many vital activities in the cell and is also required by algal cells as a building block (Lin *et al.*, 2007).

However, as mentioned previously, LL biotreatment using algae (phycoremediation) is considered relatively novel. In a review article conducted by Nawaz *et al.* (2020), they reported finding 22 research articles on LL treatment using microalgae since 2007 till the date of review article publication. Herein we will review some of the studies and findings involving bioremediation of LL using microalgae/microalgae-bacterial consortia.

Lin *et al.* (2007) reported the removal of significant amounts of ortho-P, ammoniacal-N and COD by the tested microalgal strains from the leachate, where the removal rate was higher in case of lower LL concentrations (10% and 30%) than in case of higher LL concentrations (50%, 80% and 100%) for the same strain. Lin *et al.* (2007) also compared the phytotoxicity of algal-treated leachate to algal-free leachate and found that the phytotoxicity decreased in the 10%, 30% and 50% concentrations of leachate treated with algae, and that the germination rates of *Brassica Chinensis* seeds were significantly higher in these treated concentrations than in the same concentrations of algal-free leachate. In an experiment performed by Mustafa, Phang and Chu (2012), a consortium of five microalgal species namely *Chlorella vulgaris*, *Scenedesmus quadricauda*, *Euglena gracilis*, *Ankistrodesmus convolutus* and *Chlorococcum oviforme* was inoculated in a high-rate algal pond (HRAP) with a daily removal of 400 ml (1%) of the pond and replacement with equal amount of pre-treated LL (this percentage increased to 2% on day 197 and then to 4% on day 309 of the experiment), the experiment continued for 351 days in open conditions. A significant decrease in COD (91%), ammonia-N (99.9%) and orthophosphate (86%) was reported, algal biomass production ranging from 2-5.54 g/L (dry weight) was concomitant. Richards and Mullins (2013) tested the HMs removal ability of a mixture culture of four marine microalgae seeded in a photobioreactor containing a mixture of leachate and

hypersaline solution that simulates the salinity of a typical seawater. Results obtained by Richards and Mullins (2013) showed an outstanding ability of the marine algal mixture to efficiently remove 95% of the heavy metals in the leachate-hypersaline solution mixture where some metals were completely removed (lanthanum and cerium) and others were almost completely removed (Aluminium and iron). Additionally, high lipid content was reported for the two dominating algal species in this study indicating the dual successful ability of the tested marine microalgae in heavy metal treatment of leachate as well as lipid production. Edmundson and Wilkie (2013) concluded that LL can be used as a growth medium rich in nutrients for some microalgae. They found that the microalga *Scenedesmus* cf. *rubescens* could grow well in 100% LL when pH is controlled, moreover the results of *Scenedesmus* cf. *rubescens* growth in the pH controlled 100% LL were comparable to those obtained from its growth in Bold Basal Medium (BBM) in terms of mean growth rate and cell yield. Furthermore, the maximum biomass yield (g/L/day) was 0.55±0.084 and 0.58±0.036 in case of the algal growth in LL and BBM, respectively. In a study conducted by Thongpinyochai (2014), the obtained results indicated that *Chlorella vulgaris* successfully removed 65.77% total phosphorus, 53.91% of ammonia-N, 31.74% of nitrate-N, 51% of COD and 52.78% of BOD from 30% diluted landfill leachate samples in Thailand. Thongpinyochai (2014) has also reported the ability of *Chlorella vulgaris* to effectively remove 70% and 66% of Cr and Ni, respectively, from the tested landfill leachate samples in Thailand. Another study carried out by Paskuliakova, Tonry and Touzet (2016) showed the ability of an algal strain of *Chlamydomonas sp.* (isolated from raw leachate) to effectively remove 90.7% of ammonia nitrogen from a 10% raw leachate supplemented with phosphate together with producing higher algal biomass and this was possible at relatively low light and temperature conditions.

High ammonia-nitrogen and phosphorus removal rates (up to 95%) were obtained by algae-bacteria consortium treating municipal wastewater samples spiked with 10% landfill leachate spike ratio (from a Chinese landfill) with a maximum biomass yield of 1.58 g/L whereas the tested microalga (*Chlorella pyrenoidosa*) is the dominating microorganism i.e., representing 94% of the total biomass concentration (Zhao *et al.*, 2014). They also revealed the effectiveness of this consortium in treating landfill

leachate from other organic contaminants with the possibility of carbon fixation and lipid production. Another study by Costa *et al.* (2014) showed, the possibility of algae-bacteria consortia in open pond systems to treat sanitary landfill leachate in Brazil where the obtained results showed a removal of 75% of BOD and ammonium which becomes below the limit established by the Brazilian regulations for wastewater discharge. In a study performed by Kumari, Ghosh and Thakur (2016), landfill leachate samples from an Indian landfill were treated using an algal strain, a bacterial strain, a combination of both (algal-bacterial consortium). Interestingly, it was found that the algal-bacterial consortium showed the highest efficiency in removal of toxic organic compounds and heavy metals present in the tested leachate samples while individual bacterial-treated and algal-treated samples showed less ability in removing the same contaminants. Moreover, Kumari, Ghosh and Thakur (2016) reported the superiority of algal-bacterial consortium over individual treatments in mineralizing the cytotoxic compounds found in the untreated leachate to non-toxic ones and this was confirmed by various bioassay tests indicating the possible capability of algal-bacterial co-culture in effective landfill leachate treatment.

Khanzada and Övez (2017) investigated the growth and ammonium-N removal efficiency of a mixed culture of indigenous freshwater microalgae (*Chlorella vulgaris* and *Chlamydomonas reinhardtii*) in different dilutions (10%, 30%, 50%, 70%, 90% and 100%) of autoclaved and non-autoclaved treated LL (with a pH value maintained at 6.5-7.5) from Istanbul municipal landfill in Turkey. Their results revealed a maximum dry biomass yield in case of algal cultures growing in 50% leachate whilst a complete ammonium-N removal (100%) was recorded for algal cultures growing in 10% Leachate which contain almost 50 mg/L of NH_4^+-N. In another study done by Hernández-García *et al.* (2019), two microalgal species (*Desmodesmus* spp. and *Scenedesmus obliquus*) were examined for their growth and nutrients removal from wastewater containing different leachate concentrations (0%, 7%,10% and 15%). The highest biomass yield for both species *Desmodesmus* spp. and *Scenedesmus obliquus* were 1.3 ± 0.1 g/L and 1.2 ± 0.07 g/L, respectively, and they were observed at the concentration 7%. This concentration also favoured the removal of 82% and 79% of NH_4^+-N as well as 41% and 43% of ortho-phosphate content in the tested leachate-

wastewater samples for both algal species *Desmodesmus* spp. and *Scenedesmus obliquus*, respectively. On the other hand, Nordin, Samsudin and Yusof (2019) isolated two strains of indigenous microalgae from a landfill leachate treatment plant and identified them (using molecular biology tools) as *Chlorella vulgaris* UPSI-JRM01 and *Tetradesmus obliquus* UPSI-JRM02. Nordin et al (2109) cultivated the two isolated strains in synthetic nitrified LL with high $N\text{-}NO_3$ concentration (1500 mg/L) and monitored their growth and nitrate uptake every two days for the time interval of the experiment (30 days), the obtained results indicated the superiority of *Tetradesmus obliquus* UPSI-JRM02 in terms of biomass production over *Chlorella vulgaris* UPSI-JRM01 whereas the biomass productivities were 40.49 mg/L/day and 36.28 mg/L/day, respectively. However, the $N\text{-}NO_3$ removal efficiencies amongst both strains were comparable; 37% and 44% removal percentages by *Tetradesmus obliquus* UPSI-JRM02 and *Chlorella vulgaris* UPSI-JRM01 which although less than 50% but corresponds to removal of significant amounts of $N\text{-}NO_3$ (> 500 mg/L), moreover the produced biomass contained significant protein content (> 50%) which might add further commercial value by the possibility of using the produced biomass as fertilizers and/or animal feed after toxicological assessments. In the same context, results obtained from a study by El Ouaer *et al.* (2020) reported that the growth of a *Chlorella* sp. (isolated from a natural lagoon in Tunis) was promoted by 10% (v/v) leachate by two times and ten times in terms of cell density and chlorophyll content, respectively, compared to its growth in the standard culture medium BBM. El Ouaer *et al.* (2020) further reported a removal efficiency of 60%, 100% and 10% for COD, NH_4^+-N and salinity, respectively, recorded for pure cultures of *Chlorella* sp. in diluted LL. In an attempt to optimise algal microbiome with the dominating algal species being *Chlorella vulgaris*, Okurowska *et al.* (2021) enriched algal-bacterial consortium isolated from a landfill treatment site in the UK and subjected it to 24 months of adaptive laboratory evolution. Okurowska *et al.* (2021) compared the algal growth and nutrients removal efficiencies of both the original and adapted consortia and they found that a 3-fold improvement in the algal growth rate of the adapted consortium was noticeable compared to that of the original one, however the reduction in NH_4^+-N removal rates were comparable between both of them with a slightly higher

removal rate in case of adapted consortium (97.28 ± 0.3 %) at LL concentration of 20% (v/v).

2.3.2. Limitations facing LL treatment by microalgae

Although biological treatment of leachate has good reputation because it is simple, reliable, highly cost-effective, and eco-friendly (Renou *et al.*, 2008) but it still faces some challenging limitations. Examples of these limitations include:

- Scaling-up problems: several studies have proven the effectiveness of algae and/or algae-bacteria consortia in treatment of landfill leachate but many of these studies were conducted on small (laboratory) scale after treating the leachate by autoclaving, filtering or dilution to eliminate or reduce the effect of competitive microorganisms in the leachate itself, decrease its turbidity and increase light transmittance, all of which represent major scale-up problems (Cuellar-Bermudez *et al.*, 2017).
- Phosphorous supplementation requirement: another important limitation is the need to supplement algal cultures growing in LL with external source of phosphorus (P) due to the limited amount of P existing in leachate and this limits the sustainability of the process, as P itself is a non-renewable resource (Nawaz *et al.*, 2020).
- Toxic effect of raw Landfill Leachate on algae: The efficiency of LL treatments using microalgae is usually hampered by the inhibitory/toxic effect exerted by high LL concentrations on algal species which requires either pre-treatment of LL or diluting it with water which make the process of LL treatment using algae uneconomical as well as limiting its sustainability (Dogaris, Ammar and Philippidis, 2020; Nawaz *et al.*, 2020).
- Poor understanding of the algae leachate system: since this field is relatively novel so a better understanding for the mechanism of algal growth in LL would greatly enhance the development of the treatment processes (Nawaz *et al.*, 2020).

2.3.3. Suggested possibilities for overcoming abovementioned limitations

The abovementioned limitations for LL treatment using microalgae could be overcome by several approaches:

- Selection of species that could better tolerate the harsh conditions of LL thus could tolerate higher ammonia-N concentrations which in turn will mitigate the need to perform pre-treatment/dilution steps. This could be either single highly tolerating species or consortia of different species (algae and/or bacteria) which could be fulfilled by different aspects either screening different species for the most potent ones or by adaptive laboratory evolution (Nawaz *et al.*, 2020).
- Regarding the need for P supplementation, it might be a good idea to dilute LL with agricultural wastewaters (agricultural runoffs) which already contains high P concentrations (Nawaz *et al.*, 2020).
- Increasing efficiency of LL treatment using microalgae could be carried out by integrating biological treatment with another physical-chemical treatment technology (Dogaris, Ammar and Philippidis, 2020).
- Better understanding for the algal leachate interaction could be achieved through more studies directed towards developing a standard protocol for treatment processes with mathematical modules aiming for predicting as well as explaining the performance of the treatment scheme (Nawaz *et al.*, 2020).

2.4. Physical-chemical treatment of LL

Despite being acknowledged as effective in removal of several pollutants from leachate e.g., suspended solids, floating material, colloidal particles, colour, non-biodegradables as well as other undesirable pollutants e.g., heavy metals and ammonia, however, some major drawbacks involve the pollutants (e.g., sludge and ammonia) that may result in from using some of these methods raise some concerns about their impact on the environment in addition to their high costs (Renou *et al.*, 2008; Luo *et al.*, 2020). This in turn increase the need to search for more eco-friendly methods with less impact on the environment as well as higher cost-effectiveness. In this context, treating LL with plasma or UV represent a potential candidate.

2.4.1. Physical-chemical treatment of LL using Plasma/UV

Plasma technology is deemed a green technology with no generated postproduction wastes (Siswanto *et al.*, 2018). The term "plasma" refers to a gas that has been partially

or totally ionised and consists mainly of photons, ions, free electrons, and atoms (in their fundamental or excited states), however the net charge of plasma is neutral because both positive and negative charge carriers are equal in number (Misra *et al.*, 2011). Plasma could be also referred to as the fourth state of matter (Niveditha *et al.*, 2021). Plasma is divided into two main classes; non-thermal (cold) plasma and thermal plasma and this classification mainly depend on the method by which the plasma is generated as well as the relative energetic levels of electrons and heavy specie (i.e., ions and atoms) of the plasma. Non-thermal plasma could be generated by electric discharge in a gas at low pressure/atmospheric pressure or by using microwaves, while thermal plasma is generated at higher pressures and requires higher power (Misra *et al.*, 2011; Pankaj and Keener, 2017). Plasma could be generated at atmospheric pressure using different approaches such as corona discharge, dielectric barrier discharges (DBD), radio frequency plasma (RFP) as well as the gliding arc discharge (Misra *et al.*, 2011). Non-thermal (cold) plasma will be utilised in LL treatment in this study and will be further discussed in detail regarding its application in landfill leachate treatments.

The use of non-thermal (cold) plasma provides several attractive advantages e.g., lack of toxic residues, simplicity, low energy consumption and non-temperature operating conditions (Niveditha *et al.*, 2021). Research in the field of non-thermal (cold) plasma applications, revealed the possibility of using it for surface decontamination to decrease the microbial load from the surfaces of some raw and fresh products e.g., fruit, vegetables, meat, meat products, grains as well as some powders (onion powders) as well as surface modifications of packaging materials, however this approach still has some scalable issues to be addressed in order to become widely and commercially applicable (Misra *et al.*, 2011; Niveditha *et al.*, 2021). Cold plasma is also useful in significant degradation of pesticides and mycotoxins present in the agricultural produce (Pankaj and Keener, 2017) in addition to detoxification of some cyanotoxins (Pandhal *et al.*, 2018). Other applications of cold plasma include biomedical applications especially in the fields of sterilisations, wound healing, and diseases treatments (Pankaj and Keener, 2017; Ahmed *et al.*, 2018), in industry for polymers sterilisation, surface modifications and functionalisation as well as in the

field of water treatment for decontamination and degradation of pesticides and dyes (Pankaj and Keener, 2017).

However, hence cold plasma is deemed a novel technology (Pankaj and Keener, 2017) so its application in landfill leachate treatment is relatively recent. This in turn explains the inadequate number of studies available in this particular field.

Plasma was reported to be effective in ammonia-nitrogen removal with a removal efficiency reaching 83% after six hours of treatment using a dielectric barrier discharge system in atmospheric pressure (Zhao *et al.*, 2011). Zhao *et al.* (2011) also reported a gradual change in the deep grey-black colour of the leachate to deep-yellow (after one hour) then to light-yellow (after two hours) then to almost decolourised/complete transparent after six hours of plasma treatment which in turn indicates the efficiency of plasma treatment in the gradual destruction/decomposition of the chromogenic substances in the leachate. In the same context, plasma-based water treatment was reported to effectively remove PFOS (Perfluorooctane sulfonate) and PFOA (Perfluorooctanoic acid), from landfill leachate samples to below USEPA's (United States Environmental Protection Agency's) health advisory concentration levels within 10-75 minutes, with effective decolourisation achieved in 30 minutes (Singh *et al.*, 2021).

Similar to using the plasma technology in landfill leachate treatment, there were few reports on using UV in landfill leachate treatment, although usually being applied concurrently with hydrogen peroxide or ozone (Wu *et al.*, 2004; Shu *et al.*, 2006). Wu *et al.* (2004) compared the efficiency of different ozone-based advanced oxidation processes (AOPs) namely ozone, ozone/UV, and ozone/Hydrogen peroxide (H_2O_2) in the treatment of raw leachate obtained from a landfill in Taiwan (after applying coagulation with ferric chloride as pre-treatment step). Wu *et al.* (2004) concluded that the biodegradability was increased in all the treatment processes, however the ozone/UV treatment was the most effective among the other approaches in enhancing the biodegradability (indicated by BOD_5/COD ratio) as well as the decolourisation of the leachate. They also recommended the usage of this approach prior to the biological treatment process. Another study conducted by Shu *et al.* (2006) indicated that LL

treated with a maximum dosage of four UV lamps together with 232.7 mM of H_2O_2 were effective in removal of 72% and 65% of colour and COD, respectively, in 300 minutes from raw leachate, this percentage further increased to 91% and 87% of colour and COD, respectively, in only 120 minutes for 20% diluted LL.

2.4.2. Limitations facing LL treatment using plasma/UV treatments

Although LL treatment using plasma or UV has proven to effective in removal of significant amounts of different pollutants e.g., Ammonia-N, COD, as well as other harmful organic compounds from leachate, in addition to effective LL decolourisation in relatively short times (Wu *et al.*, 2004; Shu *et al.*, 2006; Zhao *et al.*, 2011; Singh *et al.*, 2021) however, large scale applications of such technologies might still face some challenges especially those concerning the cost effectiveness of the whole process.

2.4.3. Suggested possibilities for overcoming abovementioned limitation

In addition to the need for more detailed studies regarding the possibility of wide scale applications of plasma and/or UV technologies in LL treatment, another possible addition to the cost effectiveness of these processes might be possible by coupling leachate treatment with microalgal treatment thus producing valuable algal biomass as mentioned previously which might have different applications in biofuel productions, bioactive compounds productions and/or resources recovery as well as carbon dioxide sequestration. This in turn might pave the way for more sustainable, more cost-effective, as well as efficient landfill leachate treatment with the possibility of larger-scale application in the future.

2.5. Integrated novel Plasma/UV-microalgae treatment of LL

Integrating both approaches of plasma/UV with microalgae for landfill leachate treatment is worth exploring based on the reasons mentioned in detail in sections 2.3.2, 2.3.3, 2.4.2 and 2.4.3, which might be briefly summarised as follows:

- Plasma treatment of landfill leachate was reported to be effective in ammonia-N removal, leachate decolourisation as well as removal of some harmful organic compounds (Zhao *et al.*, 2011; Singh *et al.*, 2021). In the same context, LL treatment

using UV lamps coupled with H_2O_2 or ozone was also effective in removal of adequate amounts of COD with effective colour removal as well as enhancing the leachate biodegradability (Wu *et al.*, 2004; Shu *et al.*, 2006). A major drawback of such technologies is its scalability challenge, which might be a result of the relative high cost of the whole process. Integrating such processes with a biological process such as microalgae treatment might help the cost-effectiveness of the whole process by producing valuable algal biomass. However, there was almost no reports about integrating algal treatment with plasma/UV treatments which might be beneficial for both, as algae will greatly benefit from the decolourisation achieved by plasma/UV technologies as pre-treatments steps, also the plasma/UV technologies will help improve leachate quality by enhancing COD and/or ammonia-N removal as reported in the previously mentioned studies, which in turn might pave the way for better algal growth and performance in landfill leachate treatment.

- One of the main drawbacks of implementing microalgae as a LL treatment method is that it requires pre-treatment of leachate with some hazardous chemicals such as HCl to neutralise it high pH (Edmundson and Wilkie, 2013), diluting leachate to very low concentrations to mitigate its toxic effects on algal growth (usually 10%) which increase environmental footprints of the whole process (Paskuliakova, Tonry and Touzet, 2016; El Ouaer *et al.*, 2020) and/or long term adaptation for algal strains to better tolerate the harsh environment of LL, with the dilution still represent a necessity (Okurowska *et al.*, 2021). This in turn increases the urgency for selecting highly tolerant microalgal strains to be able to tolerate the harsh leachate conditions without the need to carry on the abovementioned measures.
- Integrating plasma/UV as a pre-treatment step with microalgal LL treatment (especially after choosing a potent highly tolerant strain) might therefore perform a dual function of enhancing leachate quality as mentioned earlier and alleviate the need to perform the environmentally exhausting measures mentioned above with the possibility of decreasing the cost-effectiveness of the whole process by

producing valuable algal biomass that might be used in different purposes e.g., biofuels.

Based on the abovementioned reasons, it was concluded that integrating a biological treatment approach (using microalgae) with a physical treatment approach (using plasma/UV) would provide an efficient, eco-friendly, possibly simple, and cost-effective approach for landfill leachate treatment.

As far as the author's know this is the first report on landfill leachate treatment using an integrated plasma/UV-green microalgae approach/technology.

2.6. Conclusions

This chapter gives a detailed description of the landfill leachate problem with its definition, characteristics, hazardous effects, and different treatments methods. An outline for different treatments methods was provided including both biological and physical-chemical treatments with special focus on the biological treatments using microalgae and physical treatments using plasma /UV, hence comes the importance of integrating both treatments in a novel plasma/UV- microalgae treatment approach.

The next chapters include four experimental chapters (3-6), each chapter starts with an abstract, a brief introduction followed by materials and methods section, results, discussion, and conclusion. The first experimental chapter includes samples collection from a landfill leachate treatment site in the UK. Isolation, purification and molecular-biology based identification of different algal strains in the sample (chapter 3), followed by trials to grow the isolated microalgae in different LL concentrations (5%, 10%, 15%, 20% and 25%) to test their threshold tolerance (chapter 4). Screening experiment is then carried out to explore the growth profile and the pollutants uptake ability of the different algae (chapter 5). The highly performing algal strain is then used for treatment of LL which has been pre-treated by plasma/UV and both growth as well as bioremediation efficiencies is tested followed by complete genome sequencing for the highly growing *Chlorella vulgaris* strain (C.V.M*) in chapter 6.

CHAPTER THREE

Molecular Identification

of Microalgal and Cyanobacterial Strains from a Landfill Leachate Treatment Site

3.1. Abstract

Landfill leachate is a challenging complex environment for microorganisms to thrive in as it is usually toxic, high in ammonia content and rich with different pollutants such as organic, inorganic compounds and heavy metals. Naturally occurring microorganisms in such environments are usually the most powerful candidates for bioremediation of these environments. Green algae are well known for their ability to treat wastewater, and some have been studied for landfill leachate treatment. In this study, samples of soils from a landfill leachate treatment site were collected and cultivated in BG11 medium and the strains that grew are further treated to produce pure cultures. Four microalgal strains are identified using five different primers that allowed the sequencing of most of the rDNA (18S, 5.8S, ITS1 & ITS2 regions) thus using a powerful and accurate molecular biology identification tool. The identification revealed two strains of *Chlorella vulgaris* MT137379 and *Chlorella vulgaris* MT137382, one strain of *Chlorococcum* sp. MT152906 and one strain of *Scotiellopsis reticulata* MT151679. Two strains of cyanobacteria were also identified using PCR amplification of DNA sequences from the 16S rDNA region. The cyanobacterial strains were identified as *Phormidium autumnale* MT152907 and *Phormidium autumnale* MT153248. This chapter sheds some light on the microalgal and cyanobacterial diversity in a landfill leachate environment. This in turn will provide possible candidates for a future biological treatment of landfill leachate.

3.2. Introduction

Water scarcity represents a global concern. Less than 1% of the water on earth is directly available for human use from which 70% is used in agriculture. Therefore, water pollution with various wastes, together with a growing population, becomes a major threat (Cuellar-Bermudez *et al.*, 2017). Landfill leachate in particular is a complex, challenging and costly wastewater type to treat (Paskuliakova, Tonry and Touzet, 2016). It may pollute ground water as well as surface water. Landfill leachate contains various types of pollutants which may be categorised into four groups: dissolved organic matter, inorganic macro- components, heavy metals, and xenobiotic organic compounds (Kjeldsen *et al.*, 2002). The release of leachate to water bodies in the environment without treatment has serious deleterious effects including partial oxygen depletion from the water bodies it is released into, which in turn causes serious changes in the bottom fauna and flora as well as ammonia toxicity (Kjeldsen *et al.*, 2002). Several bioassay tests indicated that untreated leachate may induce cytotoxicity, genotoxicity, carcinogenicity or estrogenicity as a result of the synergistic, additive or antagonistic effects of the contaminants present in it (Kumari, Ghosh and Thakur, 2016). Moreover, several hazardous compounds are found in untreated leachate, many of which are not yet identified (Öman and Junestedt, 2008). It became a necessity, due to these reasons and since landfill sites continue producing leachate for hundreds of years even after closure, to approach sustainable eco-friendly and economic methods for landfill leachate treatment before discharging it into the environment (Jones, Williamson and Owen, 2006). One of the most promising technologies in this regard is the application of microalgae for landfill leachate treatment (Dogaris, Ammar and Philippidis, 2020).

Although there are various studies on the possibility of treating wastewater using microalgal biomass, a process known as phycoremediation, there are fewer studies regarding the biotreatment of landfill leachate using microalgae. This might be attributed to the complex nature of landfill leachate and its possible toxicity to living organisms (Paskuliakova, Tonry and Touzet, 2016). Nevertheless, microalgae have shown some promising results in this regard which make them possible candidates in

effective leachate treatment. However, a key step in this process is the selection of suitably tolerant microalgal strains. The capability of algae isolated from landfill leachate itself is usually better than other adaptive strains for the leachate treatment (Paskuliakova, Tonry and Touzet, 2016), but the research on indigenous microalgae inhabiting and growing in landfill leachate is limited (Edmundson and Wilkie, 2013; Cheah *et al.*, 2016).

There are very few studies (Paskuliakova, Tonry and Touzet, 2016; Nordin, Samsudin and Yusof, 2019) that have used molecular biology tools, to distinctively and unequivocally, identify the indigenous algal strains inhabiting the landfill leachate with the potential of being powerful candidates in further phycoremediation processes.

In addition to green microalgae, cyanobacteria (blue-green algae) also represent a valuable and effective source for wastewater treatment (Markou and Georgakakis, 2011) as well as nitrified landfill leachate treatment (Nordin, Yusof and Samsudin, 2017; Nawaz *et al.*, 2020) Cyanobacteria have the advantage of high biomass production when cultivated in wastewater and landfill leachate (Markou and Georgakakis, 2011; Nordin, Yusof and Samsudin, 2017; Nawaz *et al.*, 2020). Therefore, having the ability to isolate and identify more indigenous strains might allow the possibility of using them in future biotreatment of wastewater and landfill leachate, as well as biomass production which might be of several uses as biofuel, biodiesel, or feedstock.

However, there is not enough research on green algae and/or cyanobacteria inhabiting landfill leachate environments in the UK. Identifying organisms that can withstand the harsh landfill leachate environments is a crucial step towards effective treatment strategies for these types of wastewaters. In this study, four green microalgal strains and two cyanobacterial strains isolated from a landfill leachate treatment site in Chesterfield, UK were cultured and purified. Five primers (18S Lim, 18S Huss, 5.8S, ITS1 and ITS2) were used to identify the rDNA in the four green algae isolates, in addition to the 16S primer for identification of the cyanobacteria isolates. This study, therefore, provides more accurate identification of these strains and

provides evidence for a biodiverse environment of indigenous leachate-inhabiting green algae and cyanobacteria. This will allow the future possibility of using them as powerful landfill leachate and wastewater treatment candidates.

3.3. Materials and Methods

3.3.1. Sample collection

Soil samples were collected in May 2018 from Erin landfill leachate treatment site in Chesterfield, UK, owned by Viridor, a British waste management company (53°14'42.6"N 1°19'45.9"W).

3.3.2. Algae and cyanobacterial isolation

Soil samples are then divided into separate lots. Each lot is used to inoculate 250 ml Erlenmeyer flasks containing 100 ml fresh liquid BG11 medium. BG11 medium is prepared according to Stanier *et al.* (1971). The flasks are then incubated in a temperature-controlled growth room (25 ± 2^{o}C) in shaking incubators (80 rpm, Stuart orbital shaker). Cultures are grown under continuous illumination provided by daylight fluorescent lamps with a light intensity of 25 ± 5 µmols photons m^{-2} s^{-1}.

The grown liquid cultures are then used to inoculate BG11 solid medium (1.5% w/v Bacteriological Agar) by streaking the inoculum across the agar surface as described by (Andersen, 2005). The plates are then incubated under the same conditions mentioned above. This step is repeated periodically until pure cultures were obtained. The purity of obtained isolates is checked using microscopic examination.

3.3.3. Molecular biology methods

3.3.3.1. Genomic DNA Extraction:

Five to ten ml of homogenized algal cultures (OD_{595} for algal cultures used were all above 1) at their exponential phase are centrifuged for 1 minute at 1500 x *g* (Ohaus, Germany). The supernatant is discarded, and the pellet is used for further DNA extraction. DNA extraction method using DNeasy® Plant Pro kit (50) (QIAGEN, Germany) is carried out as detailed in the manufacturer's instructions.

500 µl Solution CD1 is added to the algal pellet in a 2 ml tissue disruption tube. Vortexed (FISONS Scientific Equipment, UK) briefly to mix for 5 seconds. Homogenization using a Bead Bug (Microtube Homogenizer, D1030-E) is then carried out. There is a duplicate for each sample, one is bead beaten for 5 minutes and the other for 8-9 minutes.

After the bead beating step, the Tissue Disruption Tubes (0.5 mm Silica glass beads, acid washed, Sigma-Aldrich) are then centrifuged at 12,000 x g for 2 min (Sigma, Germany). Subsequently, the supernatant is transferred to a 1.5 ml collection tube. About 350–450 µl supernatant is expected. Since the supernatant may still contain some algal particles, 200 µl Solution CD2 is added and vortexed for 5 s. A centrifugation step at 12,000 x g for 1 min at room temperature is then carried out. Avoiding the pellet, the supernatant is transferred to a 1.5 ml collection tube. Note: Expected supernatant amount at this step is 400–500 µl. 500 µl Buffer APP is added to the collection tube and vortexed for 5 s. 600 µl of the lysate is loaded onto an MB Spin Column and centrifuged at 12,000 x g for 1 min. The flow-through is then discarded and the previous step is repeated to ensure that all lysate has passed through the MB Spin Column. The MB Spin Column is then carefully placed into a clean 2 ml collection tube. 650 µl Buffer AW1 is added to the MB Spin Column, then centrifuged at 12,000 x g for 1 min. The flow-through is discarded and the MB Spin Column is placed back into the same 2 ml collection tube. 650 µl Buffer AW2 is added to the MB Spin Column, then centrifuged at 12,000 x g for 1 min. The flow-through is discarded and the MB Spin Column is placed into the same 2 ml collection tube, then centrifuged at up to 16,000 x g for 2 min. The MB Spin Column is carefully placed into a new 1.5 ml collection tube. 50–100 µl of Buffer EB is added to the centre of the white filter membrane, then centrifuged at 12,000 x g for 1 min. The MB Spin Column is then discarded. The DNA bound to the MB Spin Column membrane is resolubilized into Buffer EB. Buffer EB is 10 mM Tris (pH 8.0).

The DNA is left overnight to resolubilize in the fridge and was stored at -20 ^{o}C in the freezer after that.

3.3.3.2. Determining the DNA quality and concentration:

The concentration and quality of the extracted genomic DNA is estimated using two methods.

Firstly: using the Nanodrop (Jenway Genoa Nano, UK) where 1 µl of DNA is transferred to the Nanodrop and the absorbance is measured at wavelengths 260, 280 and 320 nm. EB (Elution Buffer) is used to blank the instrument at the same wavelengths. The purity of DNA is estimated using the ratio A_{260}/A_{280} given by a Nanodrop like spectrophotometer (Thermo Scientific, USA):

$$A_{260}/A_{280} = \frac{(A260 - A320)}{(A280 - A320)}$$

Secondly: Agarose gel electrophoresis is also used to determine genomic DNA and/or PCR products size and concentration. Agarose gel (1%) is prepared using molecular biology grade agarose (Fisher Bioreagents, USA). Gel Red (product number 41003) is added to allow visualisation of the DNA and a ladder (1 Kb gene ruler, Biolabs) is used to estimate sizes. A bioimaging system (SYNGene) and Gene Snap computer software are used to visualise and record the gels.

3.3.3.3. Polymerase chain reaction (PCR):

The genomic DNA whose quality and quantity are assessed using the previously mentioned methods is then used for further PCR reactions using different primers. Five different primers are used, each to amplify a certain region in the ribosomal DNA of the tested green microalgal genomes. For the cyanobacteria, one primer (16S) is used to amplify the 16S rDNA region. A list of the used primers is shown in the Table 3-1.

Table 3-1 Primers Sequences used in PCR reactions.

Primer	Forward Sequence (5′ – 3′)	Reverse Sequence (5′ – 3′)	References
18S Lim	GCGGTAATTCCAGCTC CAATAGC	GACCATACTCTCCCC CCGGAACC	(Lim *et al.*, 2012)
18S Huss	ACCTGGTTGATCCTGC CAGT	GATCCTTCYGCAGGTT CACCTAC	(Huss *et al.*, 1999)
5.8S	GTCAGAGGTGAAATTC TTGG	CAATGATCCTTCCGC AGGTT	(Nakayama *et al.*, 1996) (Hoshina *et al.*, 2005)
ITS1	TACCTGGTTGATCCTGC CAG	AACTAAGAACGGCCA TGCAC	(Nakayama *et al.*, 1996), (Hoshina *et al.*, 2005)
ITS2	TGGTGAAGTGTTCGGA TTGG	TCCCAAACAACCCGA CTCT	(Hoshina, Kamako and Imamura, 2004) (Hoshina *et al.*, 2005)
16S	AGAGTTTGATCCTGGC TCAG	TACGGCTACCTTGTTA CGACTT	(Stackebrandt and Goodfellow, 1991)

The PCR mixtures for all the different primers are prepared for each tested genomic DNA (as shown in Appendix 1). All the mixtures are added to 0.2 ml PCR tubes (StarLab, USA) and the tubes are transferred to a MyCycler thermocycler (Bio-Rad, USA) where the reaction can take place. A control tube with no genomic DNA is always prepared to make sure there is no contaminating DNA bands and that any resulting bands are only the products of the PCR reaction. The conditions used for running different PCR reactions are variable amongst different primers. (Conditions for running the various primers are shown in Appendix 2, 3, 4 and 5).

After the completion of PCR cycles, the PCR products are visualized using 1% agarose gel electrophoresis (as described previously). The PCR bands sizes are estimated compared to the loaded DNA ladder and are further purified using a PCR purification Kit.

3.3.3.4. PCR products purification

The PCR products are purified using QIAquik® PCR purification Kit (Qiagen, Germany) to clean up the amplified PCR products from any impurities such as primers, nucleotides, enzymes, mineral oil, salts, and other impurities. The manufacturer's instructions are followed. Five volumes of Buffer PB are added to one

volume of the PCR product to be purified and vortexed briefly. If the colour is orange to violet, 10 µl 3 M sodium acetate pH 5.0 is added to the solution and mixed briefly by vortexing. The colour of the mixture will turn yellow indicating a pH ≤7, which is the pH allowing the DNA to bind to the spin column in order to be further washed by the washing buffer in the following steps. The sample is then applied to the QIAquick spin column and centrifuged for 1 minute at 17900 g. The flow-through is then discarded and the QIAquick column is then placed back into the same tube. 750 µl of washing Buffer PE is added to the QIAquick column and centrifuged for 1 minute at 17900 g.

The flow-through is then discarded and the QIAquick column is then placed back into the same tube and is centrifuged again for another minute to remove any residual washing Buffer. The QIAquick column is then placed in a clean 1.5 ml microcentrifuge tube and 50 µl of Elution Buffer (EB) is added to the centre of the white QIAquick membrane and centrifuged for another minute.

The purified PCR products are then visualized using 1% agarose gel electrophoresis where their purity could be assessed. Quantification of the purified PCR products took place using a Nanodrop (Jenway Genoa Nano, UK).

3.3.3.5. DNA sequencing:

The purified PCR products are diluted to the required concentrations (15 µl, 5 ng μl^{-1}) and each sample is prepared in duplicate to be sequenced, one in forward direction and one in reverse direction. The primers used for PCR reactions both forward and reverse are diluted to 10 pmol μl^{-1} (volume 15 µl) and send together with the samples to Eurofins Genomics (Koln, Germany) for sequencing.

The produced sequences (for the green microalgal strains) are assembled using BioEdit Sequence Alignment Editor software (Version 7.0.5.3) (Hall, 1999). The resulting sequences are compared against other closely resembling sequences (maximal score, identity and query coverage) in the National Centre for Biotechnology Information (NCBI) database (Coordinators, 2016) using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990).

3.3.3.6. Phylogenetic analysis:

The produced DNA sequences are aligned automatically using MUSCLE alignment provided by MEGA X software (Molecular Evolutionary Genetic Analysis) version 10.1.7 (Kumar *et al.*, 2018) under default parameters. Phylogenetic trees for the isolated strains are constructed using Neighbour-joining method. The best mathematical model to compute the evolutionary distance for each tree is determined using MEGA X and is mentioned in the description box under each tree, it was either Tamura-nei or Kimura-2-parameters model. The robustness of the statistical confidence for the constructed trees is determined using bootstrap tests based on 500 replicates and values are shown next to the tree branches.

3.4. Results

3.4.1. Sample collection

To identify some local indigenous microalgae and cyanobacteria for the possibility of future use in landfill leachate treatment, soil samples were collected in May 2018 from a landfill leachate treatment site in Chesterfield, UK (Figure 3-1)

Figure 3-1 Pictures for the landfill leachate treatment site owned by Viridor, from which soil samples were collected to be used to isolate green microalgae and cyanobacteria. The soil samples were collected from the green areas shown in the pictures (A, B, C and D)

3.4.2. Algae and cyanobacterial isolation:

The obtained soil samples are cultivated as mentioned earlier in liquid BG11 media. Purification of the obtained isolates is conducted via the traditional agar streaking method until pure algal single colonies or filaments were obtained (Figure 3-2). The cultures purity is regularly checked by microscopic examination.

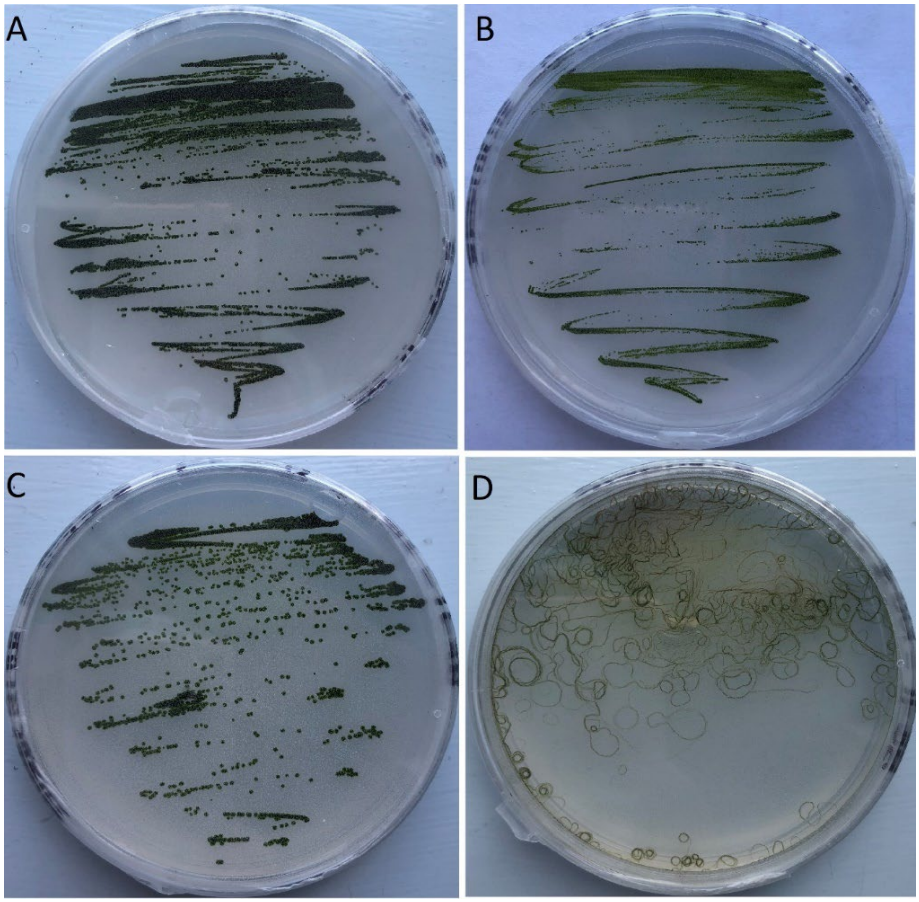

Figure 3-2 Petri dishes showing single colonies of the isolated microalgae after purification (A, B and C), as well as purified filaments (D).

3.4.3. Molecular biology methods:

3.4.3.1. Genomic DNA extraction:

The DNeasy® Plant Pro kit is used to extract Genomic DNA from the purified microalgal isolates. The efficacy of bead beating the cells for different time intervals (5-9 minutes) during the DNA extraction process is also tested. The presence of genomic DNA is detected using gel electrophoresis and the Genomic DNA bands are visualized under UV Bioimaging system (Figure 3-3).

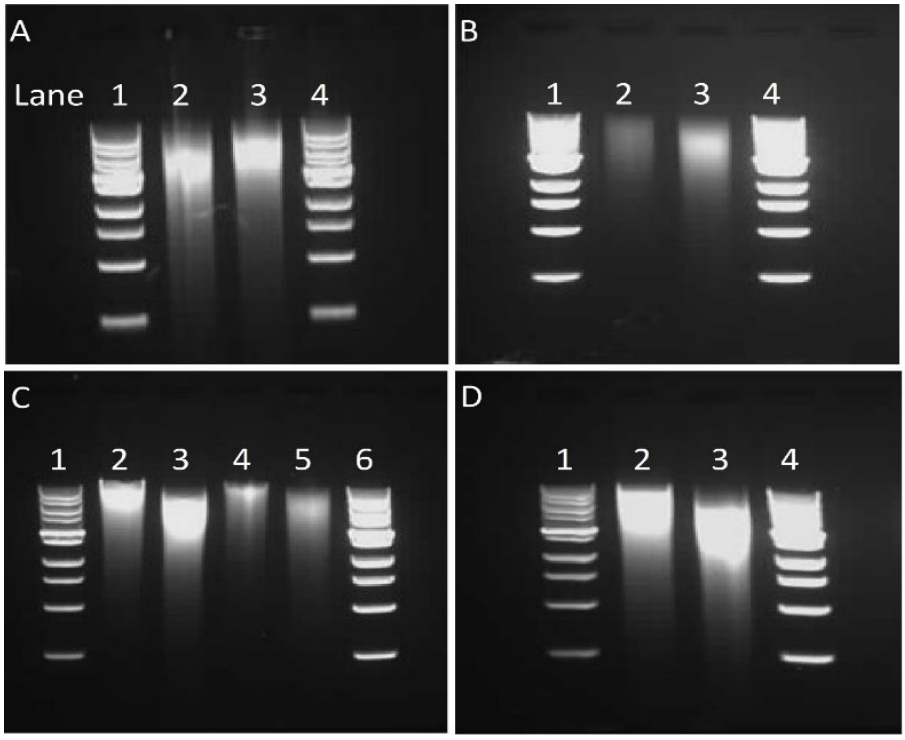

Figure 3-3 Genomic DNA extracted from the four isolated strains of green microalgae. A) Genomic DNA extracted from *Chlorella vulgaris* ATFG1. Lanes 1 and 4 contain the 1 Kb DNA ladder whilst lane 2 has the genomic DNA extract from *C. vulgaris* ATFG1 (bead beaten for 9 minutes), lane 3 includes genomic DNA from *C. vulgaris* ATFG1 (bead beaten for 5 minutes). B) Genomic DNA extracted from *Chlorococcum* sp. ATFG. Lanes 1 and 4 are the 1 Kb DNA ladder whilst lane 2 has the genomic DNA from *Chlorococcum* sp. ATFG (bead beaten for 5 minutes), lane 3 includes genomic DNA from *Chlorococcum* sp. (bead beaten for 8 minutes). C) Genomic DNA extracted from *Scotiellopsis reticulata* ATFG. Lanes 1 and 6 contain the 1 Kb DNA ladder whilst lane 2 has the genomic DNA from *S. reticulata* (bead beaten for 5 minutes), lane 3 includes genomic DNA from *S. reticulata* (bead beaten for 8 minutes). Lanes 4 and 5 are the same as 2 and 3 but with half the amounts of DNA, respectively. D) Genomic DNA extracted from *Chlorella vulgaris* ATFG2. Lanes 1 and 4 are the 1 Kb DNA ladder whilst lane 2 has the genomic DNA from *C. vulgaris* ATFG2 (bead beaten for 5 minutes), lane 3 includes genomic DNA from *C. vulgaris* ATFG2 (bead beaten for 8 minutes).

3.4.3.2. Polymerase chain reaction (PCR) and PCR products purification:

For each green algal strain five PCR reactions with five different primers are carried out to amplify most of the 18S ribosomal DNA so that we can get a better and more accurate genetic identification. After the PCR was done, purification for the PCR products is conducted. Purity of the bands is then detected using gel electrophoresis

and the results are shown in the following figures Figure 3-4, Figure 3-5, Figure 3-6, Figure 3-7.

Two primers (18S Lim & 18S Huss) are used to amplify the 18S rRNA gene of the genomic DNA for all the purified green microalgal strains by PCR. The PCR amplification resulted in bands of different sizes which is approximately 500 BP in case of 18S Lim primer and ranged from 1500-2000 BP in case of 18S Huss primer as shown in Figure 3-4 and Figure 3-5, respectively.

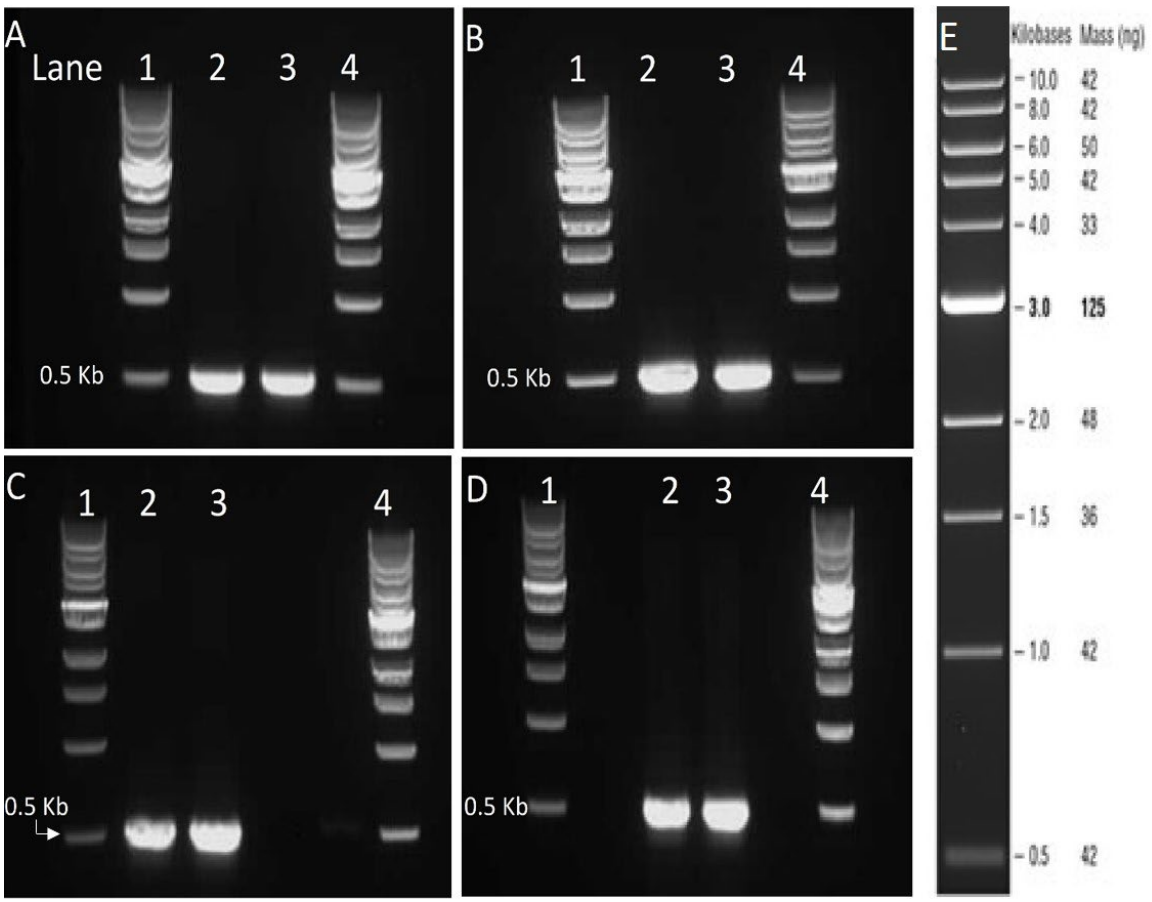

Figure 3-4 The purified 18S rDNA PCR products resulted from using 18S Lim primers for the four green algal isolates. A) Lanes 1 and 4 are the DNA Ladders whilst 2 and 3 are the purified PCR products from *Chlorella vulgaris* ATFG1. B) Lanes 1and 4 are the DNA Ladders whilst 2 and 3 are the purified PCR products from *Chlorococcum* sp. C) Lanes 1 and 4 are the DNA Ladders whilst 2 and 3 are the purified PCR products from *Scotiellopsis reticulata*. D) Lanes 1 and 4 are the DNA Ladders whilst 2 and 3 are the purified PCR products from *Chlorella vulgaris* ATFG2. E) The 1 Kb DNA ladder.

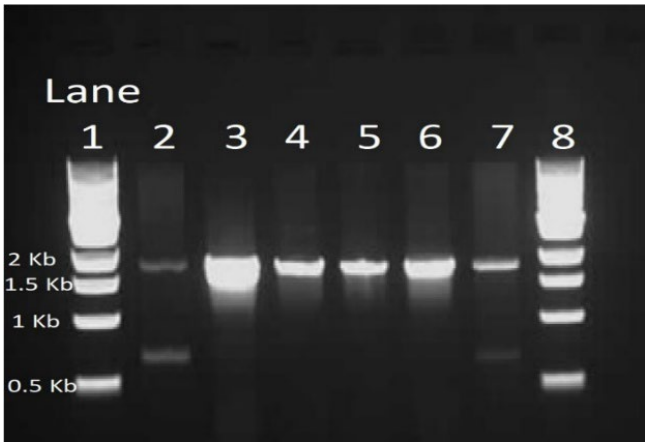

Figure 3-5 The purified 18S rDNA PCR products resulted from the 18S Huss primers amplification. Lanes 1 & 8 are the DNA ladders. Lane 2 is the PCR product from *Chlorella vulgaris* ATFG1. Lane 3 is the PCR product from *Chlorococcum* sp. Lanes 4, 5 and 6 are repetitions for the PCR product from *Scotiellopsis reticulata*. Lane 7 is the PCR product from *Chlorella vulgaris* ATFG2.

Three primers (5.8S, ITS1 and ITS2) are used to amplify the ITS region in the rDNA of the microalgal strains. The PCR amplification of 5.8S rRNA gene resulted in bands with size ranging from 500-1000 BP (Figure 3-6). The purified PCR products resulted from amplification of ITS1 and ITS2 regions of rDNA ranged from 1000 - 15000 BP in size as shown in Figure 3-7.

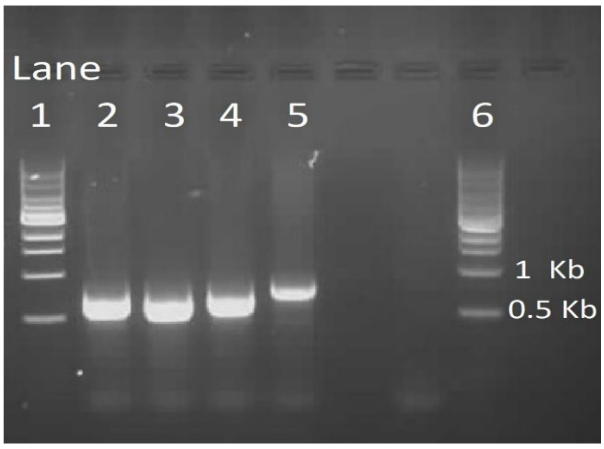

Figure 3-6 The 5.8S rRNA purified PCR products. Lanes 1 and 6 are the DNA ladders. Lane 2 is the PCR product from *Chlorella vulgaris* ATFG1. Lane 3 is the PCR product from *Chlorococcum* sp. Lanes 4 is the PCR product from *Scotiellopsis reticulata*. Lane 5 is the PCR product from *Chlorella vulgaris* ATFG2.

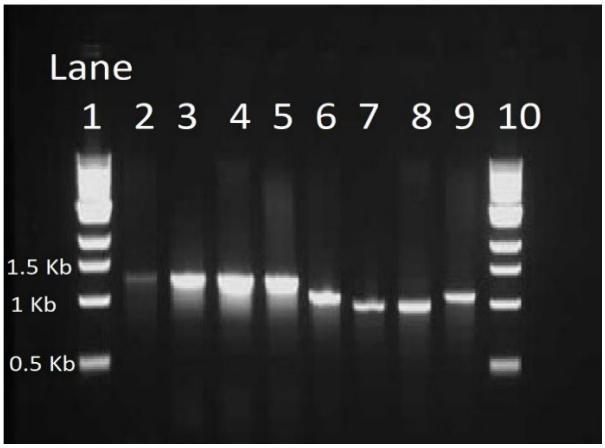

Figure 3-7 The ITS1 and ITS2 purified PCR products. Lanes 1 and 10 are the DNA ladders. Lanes 2 and 6 are the purified PCR products from *Chlorella vulgaris* ATFG1 for the ITS1 and ITS2, respectively. Lanes 3 and 7 are the purified PCR products from *Chlorococcum* sp. for the ITS1 and ITS2, respectively. Lanes 4 and 8 are the purified PCR products from *Scotiellopsis reticulata* for the ITS1 and ITS2, respectively. Lanes 5 and 9 are the purified PCR products from *Chlorella vulgaris* ATFG2 for the ITS1 and ITS2, respectively.

The 16S primer is used to amplify the 16S rDNA region for the two filamentous cyanobacterial strains by PCR, resulting in bands that were further purified and visualised after performing agarose gel electrophoresis. The purified PCR products ranged between 1000-1500 BP in size (Figure 3-8).

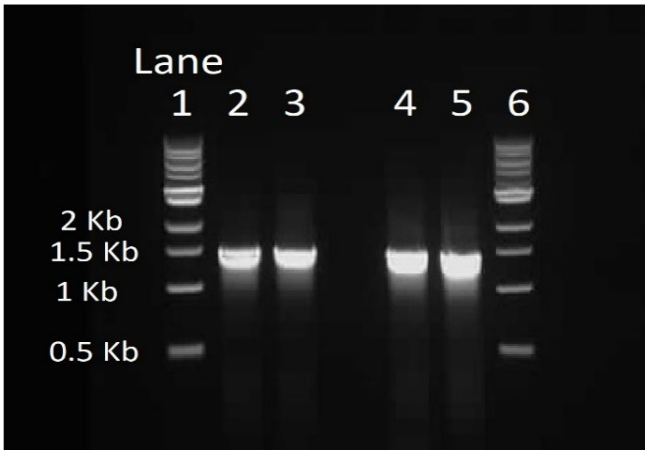

Figure 3-8 The purified 16S rDNA PCR products from the cyanobacterial strains. Lanes 1 and 6 are the DNA ladders. Lanes 2 and 3 are the purified 16S PCR products from *Phormidium autumnale* ATFG5. Lanes 4 and 5 are the purified 16S PCR products from *P. autumnale* ATFG6.

3.4.3.3. DNA sequencing:

Two primers (18S Lim and 18S Huss) in addition to another three primers (ITS1, 5.8S & ITS2) are collectively used to amplify the 18S and ITS regions of the rDNA for each green algal strain, respectively. The resulted contig sequences as well as the 16S cyanobacterial sequences were searched using NCBI BLAST after which they were submitted to the NCBI GenBank database and were given accession numbers. The identification revealed two strains of *C. vulgaris* MT137379 and *C. vulgaris* MT137382, one strain of *Chlorococcum* sp. MT152906 and one strain of *S. reticulata* MT151679. The cyanobacterial strains were identified as *P. autumnale* MT152907 and *P. autumnale* MT153248 (Figure 3-9).

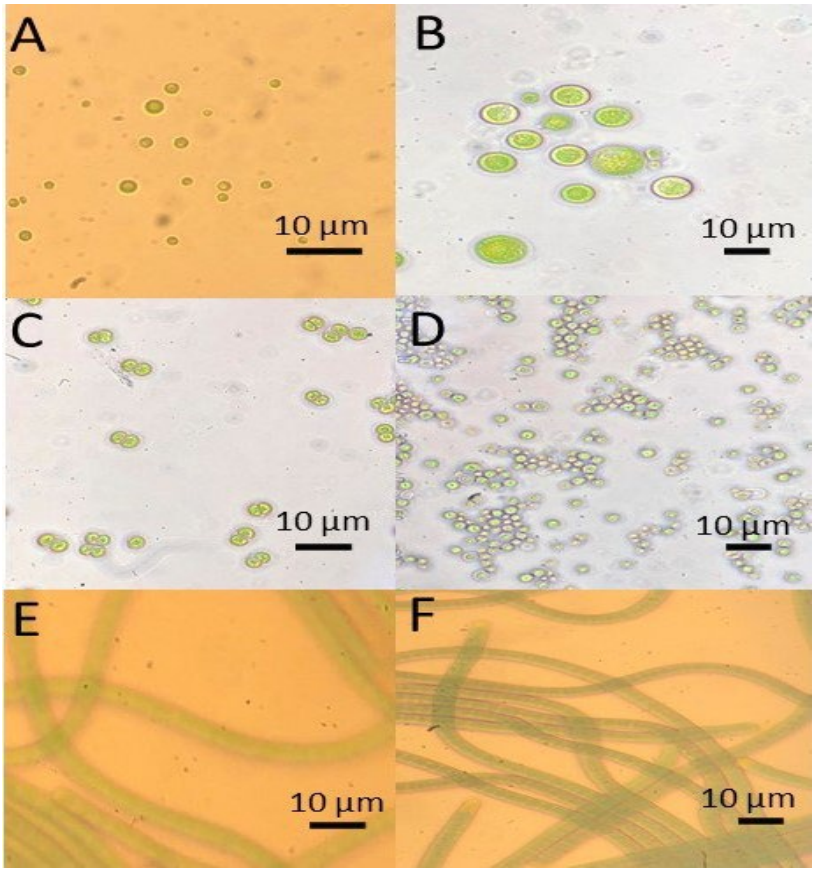

Figure 3-9 Light microscope pictures for the six strains identified in this study. A) *Chlorella vulgaris* strain ATFG1 MT137379. B) *Chlorococcum* sp. Strain ATFG MT152906. C) *Scotiellopsis reticulata strain* ATFG MT151679. D) *Chlorella vulgaris* strain ATFG2 MT137382. E) *Phormidium autumnale* strain ATFG5 MT152907. F) *Phormidium autumnale* strain ATFG6 MT153248. Magnification: 40 X.

3.4.3.4. Phylogenetic analysis:

The phylogenetic description of the six strains is based on the Mega X program as previously described in the materials and methods section. The resulting phylogenetic trees for each strain are shown in Figure 3-10, Figure 3-11, Figure 3-12 & Figure 3-13.

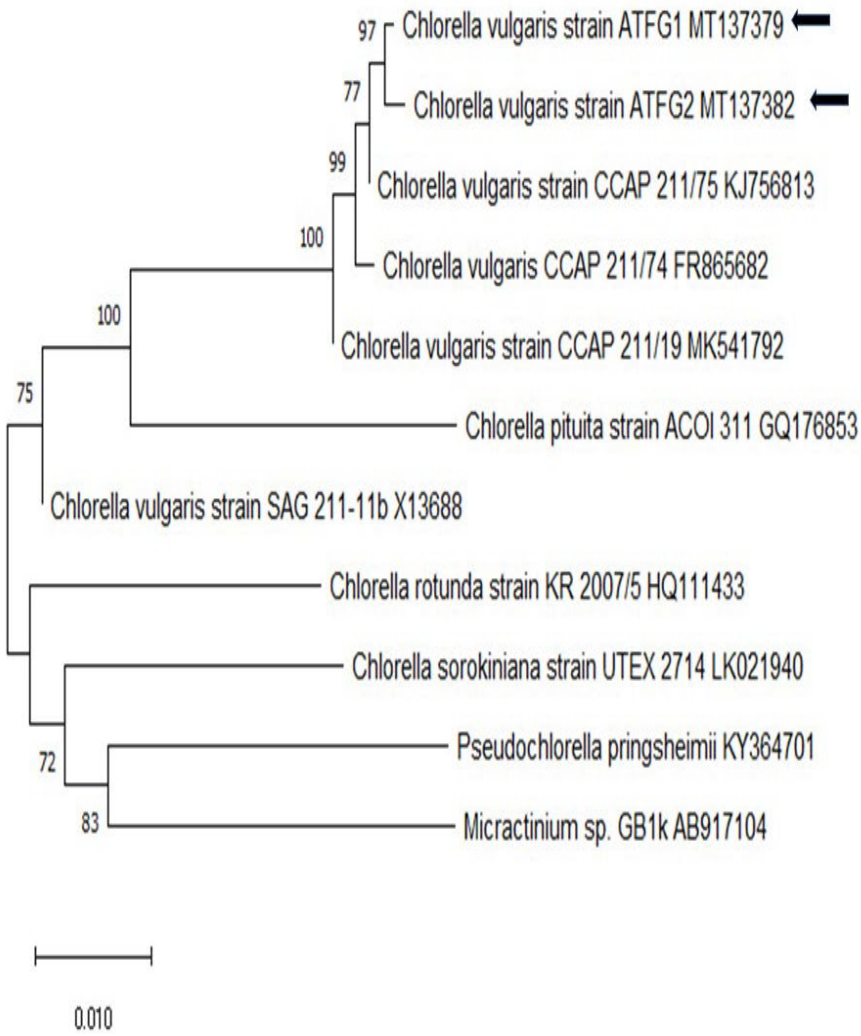

Figure 3-10 Phylogenetic tree for the aligned sequences of both *Chlorella vulgaris* ATFG1 and *Chlorella vulgaris* ATFG2, inferred using the Neighbour-Joining method. Significant bootstrap values (>50%) are based on 500 replicates and reported to the corresponding internodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura-Nei method. Arrows refer to the strains characterised in this work.

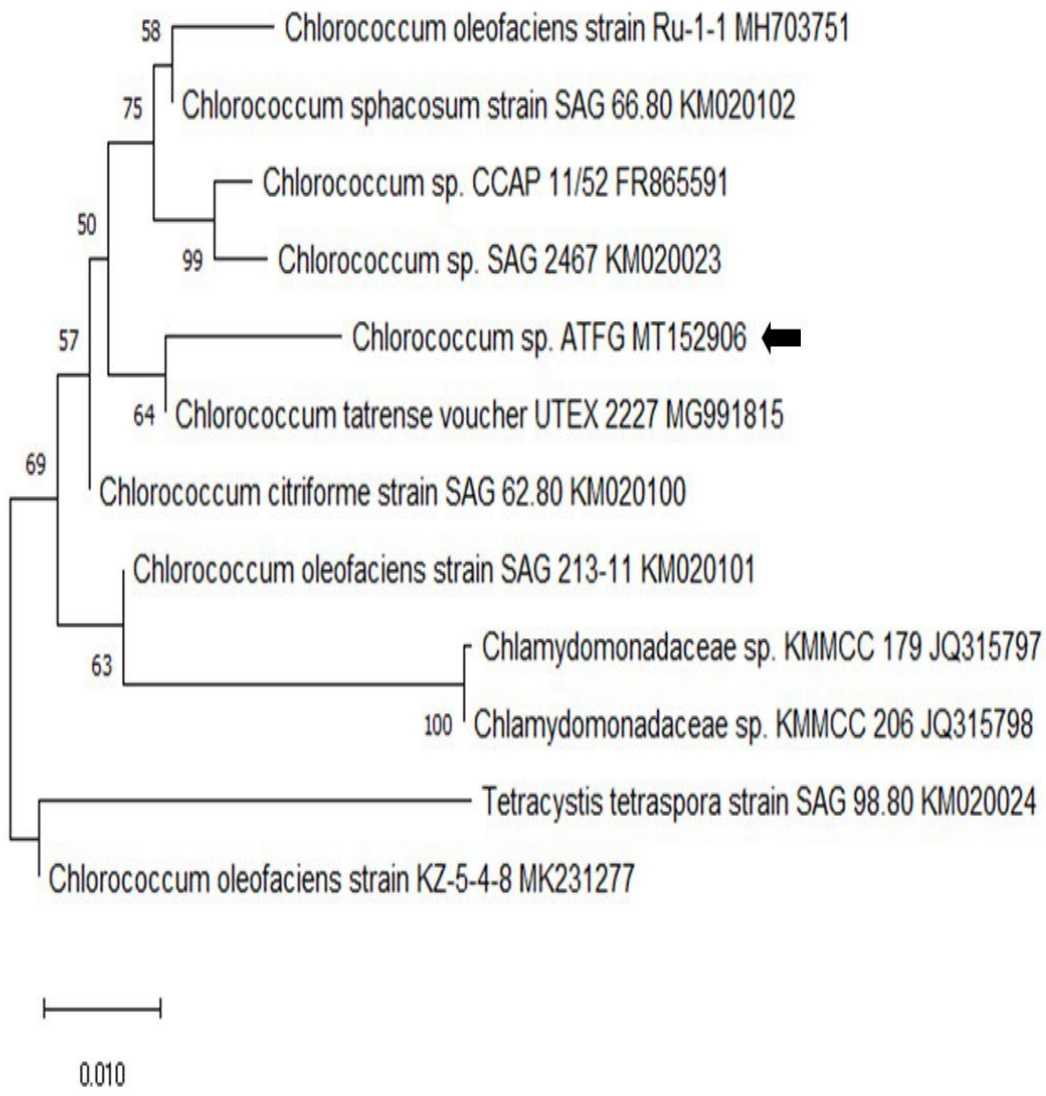

Figure 3-11 Phylogenetic tree for the aligned sequences of *Chlorococcum* sp. ATFG, inferred using the Neighbour-Joining method. Significant bootstrap values (>50%) are based on 500 replicates and reported to the corresponding internodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method. Arrow refers to the strain characterised in this work.

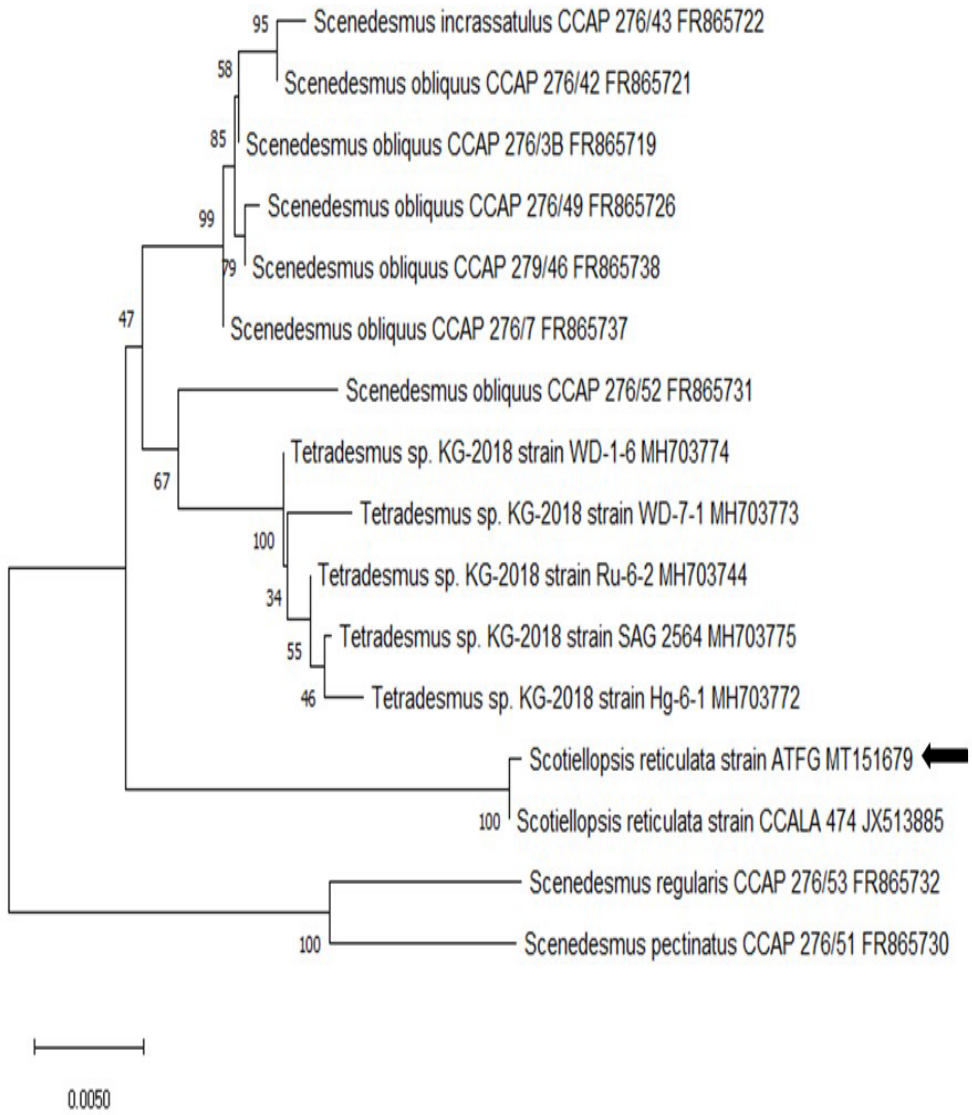

Figure 3-12 Phylogenetic tree for the aligned sequences of *Scotiellopsis reticulata* ATFG, inferred using the Neighbour-Joining method. Significant bootstrap values (>50%) are based on 500 replicates and reported to the corresponding internodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method. Arrow refers to the strain characterised in this work.

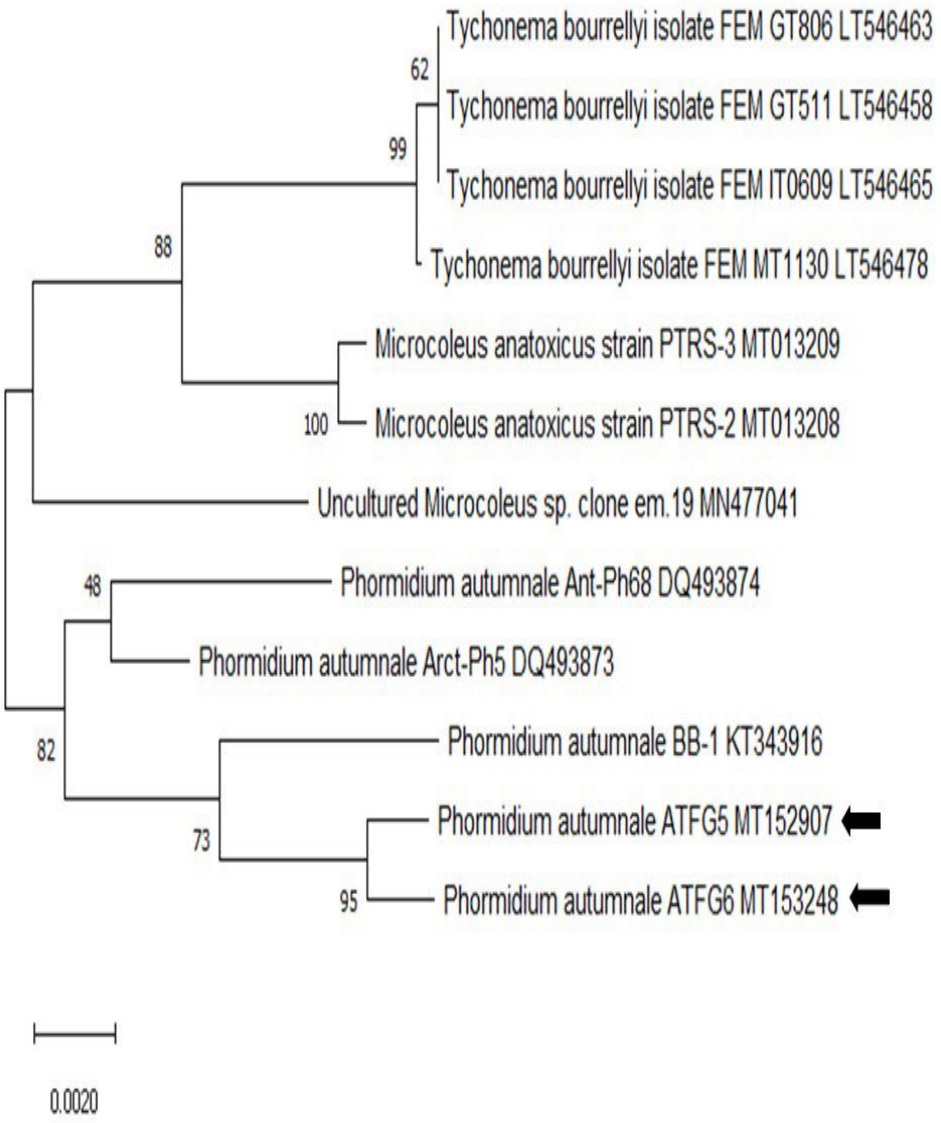

Figure 3-13 Phylogenetic tree for the aligned sequences of both *Phormidium autumnale* ATFG5 and *Phormidium autumnale* ATFG6, inferred using the Neighbour-Joining method. Significant bootstrap values (>50%) are based on 500 replicates and reported to the corresponding internodes. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura-Nei method. Arrows refer to the strains characterised in this work.

3.5. Discussion

Identification of microalgae using molecular markers has become a necessity as traditional morphological identification alone is insufficient to reflect their phylogenetic lineages (Smith, 2016; Kunrunmi, Adesalu and Kumar, 2017; Ferro, Gentili and Funk, 2018). In his study to identify a *Chlorella*-like organism, Smith (2016) revealed that although morphological characterization of this organism is clearly pointing out its similarity to members of the genus *Chlorella* but applying the molecular identification using a combination of both the SSU rDNA and ITS molecular markers indicated that the organism was in fact a species of *Micractinium inermum*. In the current study a combination of five primers is together used to identify the partial sequence of the SSU rDNA and the complete sequence of the ITS regions for the isolated microalgal strains. Although the morphological identification indicated some preliminary features for the isolated microalgae, the genetic identification aided more accurate and confident genus and/or species level identification.

The discriminative power of the ITS regions as a useful molecular marker for genetic identification of green microalgae on the genus and species level is well established (Ferrigo *et al.*, 2015; Smith, 2016; Ferro, Gentili and Funk, 2018). Although, the 18S rDNA sequencing was commonly used as a DNA marker for algal genetic identification, it was found that coupling it with ITS region sequencing yielded more confident and accurate identification. In this study, two primers (18S Huss and 18S Lim) are used to identify the sequence of the SSU rDNA and three primers (ITS1, 5.8S and ITS2) are used to reveal the sequence of the internal transcribed spacer regions. The contig of the resultant partial 18S sequence and the complete ITS sequence is analysed in the NCBI database. The results suggested two strains to be *Chlorella vulgaris*, one to be a *Chlorococcum* species and one *Scotiellopsis reticulata*. However, two strains could not be identified using these markers, but were successfully identified using 16S rDNA primers yielding sequences that suggested they belonged to the prokaryote cyanobacteria group. The two cyanobacterial strains were identified based on the NCBI BLAST search as *Phormidium autumnale.*

Phylogenetic analysis is conducted to support the primary BLAST identification. The evolutionary lineage study reflected by the constructed phylogenetic trees for the two isolated *Chlorella vulgaris* strains is robust. The phylogenetic tree (Figure 3-10) illustrated that both *C. vulgaris* strains ATFG1 and ATFG2 were amongst the *Chlorella vulgaris* clade, and this was supported by mostly high bootstrap values. Nevertheless, apart from *C. vulgaris* SAG211-11BX13688, the isolated strains are clearly separated from other *Chlorella*, *Pseudochlorella* and *Micractinium* strains which was supported by good bootstrap values. Ferro, Gentili and Funk (2018) also expressed confidence in their results with their isolated *Chlorella* strain, the phylogeny was supported by high bootstrap values indicating the clear separation of their isolated *C. vulgaris* strain compared to other strains of *Chlorella* and *Micractinium*. It might be worth mentioning that clear and confident results shown in the phylogenetic studies of the *Chlorella* genus in general and *C. vulgaris* species in particular might possibly be attributed to the extensive studies for this green microalga which has some outstanding applications in different useful fields.

The third isolated strain is identified as a *Chlorococcum* species based on the NCBI BLAST search, which was further supported by the phylogenetic analysis performed (Figure 3-11). The *Chlorococcum* sp. ATFG, although grouped in the same clade with other *Chlorococcum* species, was not supported by high bootstrap values and thus could not provide an identification at the species level. The uncertainty of species identification in the *Chlorococcum* genus is in agreement with the findings of Kunrunmi, Adesalu and Kumar (2017). Although, Kunrunmi *et al*. used the 18S rDNA as a molecular marker in their genetic identification attempt and, in this study, both 18S rDNA and ITS regions were collectively used for identification, the identity of the isolated *Chlorococcum* strains could not be ascertained and further investigations are required.

Molecular analysis of the fourth green algal strain suggested it is a *Scotiellopsis reticulata* strain, which is strongly supported by the phylogenetic tree Figure 3-12 with strong statistical confidence (100% bootstrap value). The phylogeny of the isolated strain *S. reticulata* ATFG clearly differentiated it from other clades of *Scenedesmus* and

Tetradesmus, and it grouped with *S. reticulata* strain CCALA 474 JX513885 with strong bootstrap value support. Hereby we suggest the importance of coupling the study of both 18S rDNA and ITS sequences which is believed to yield better differentiation for such a group of green algae. Ferro, Gentili and Funk (2018) tried to genetically identify a locally isolated strain of *S. reticulata* using the molecular marker ITS and the plastid marker 23S. However, their data did not support the separation of the isolated *S. reticulata* strain in the phylogenetic tree and it was clustered in the same clade with *Scenedesmus*.

Nevertheless, grouping *Scotiellopsis* strains with other members from the *Scenedesmus* family is not surprising and this might be attributed to the relatively complex taxonomic history of the genus *Scotiellopsis* (Kaufnerová and Eliáš, 2013). In their study, Kaufnerová and Eliáš (2013) untangled some of the complexity surrounding the species *S. reticulata* indicating its close relationship to *Scenedesmus rubescens* depending on the sequence study of the 18S rDNA and ITS2 molecular markers.

Applying the genetic identification using the molecular marker 18S rDNA primers did not yield any meaningful data for the remaining two filamentous strains. After microscopic examination of these strains the decision was made to use the 16S rDNA sequence amplification and when analysing the results against NCBI BLAST the two filamentous organisms are revealed to be, in fact, cyanobacterial organisms belonging to the species *Phormidium autumnale*. These results are supported by the phylogeny study of the two isolated strains of *P. autumnale* (ATFG5 and ATFG6), which indicated that these strains are grouped in the same clade with *P. autumnale* BB-1KT343916 with good statistical confidence (bootstrap support 73). They are in the same group with other *Phormidium* strains supported by good confidence (bootstrap value 82), Figure 3-13.

Finding species of *Chlorella* in landfill leachate samples is not surprising as *Chlorella* species are well known for their ability to tolerate stress conditions (Ferrigo *et al.*, 2015; Cheah *et al.*, 2016; Nordin, Yusof and Samsudin, 2017; El Ouaer, 2020). Species of *Chlorella* have been shown to effectively remove total phosphorus, ammonia-N, nitrate-N, COD, BOD and heavy metals from landfill leachate (Thongpinyochai, 2014).

Although a *Chlorococcum* sp. was previously reported to present in wastewater (Renuka *et al.*, 2015), there is limited research on its ability for landfill leachate treatment (Mustafa, Phang and Chu, 2012). On the other hand, it has been demonstrated in some studies that some strains within the *Scenedesmus* genus have been reported for their ability to tolerate heavy metals (Ferrigo *et al.*, 2015; Cheah *et al.*, 2016) and grow well in 100% landfill leachate when pH is regulated (Edmundson and Wilkie, 2013). There are far fewer studies on the ability of the closely related *S. reticulata* species to treat wastewater although its isolation from a municipal wastewater source has been reported by Ferro, Gentili and Funk (2018). To the best of our knowledge, the current work is the first report of the isolation of a *S. reticulata* strain from a landfill leachate treatment site in the United Kingdom.

Phorimidium autumnale species are reported to exist widely in wet soils as well as aquatic microbial mats (Strunecký, Komárek and Elster, 2012). Olguín (2003) mentioned in her review article about phycoremediation studies revealing the capability of some *Phormidium* strains to effectively remove nutrients from wastewater suggesting their potential ability to become good candidates for wastewater treatment in different climate conditions. However, they will not be used in any further experiments in the current study because of their reported cyanotoxins production (McAllister, Wood and Hawes, 2016).

3.6. Conclusion

Four green microalgae and two cyanobacterial strains from a local landfill leachate treatment site in Chesterfield, UK, are isolated, purified and genetically identified using different molecular markers, emphasizing the biodiversity of indigenous microalgae and cyanobacteria inhabiting such environment. The four green microalgae identified were as follows: *Chlorella vulgaris* ATFG1, *Chlorella vulgaris* ATFG2, *Chlorococcum* sp. ATFG and *Scotiellopsis reticulata* ATFG. To the best of our knowledge, this is the first report on green algae and cyanobacteria biodiversity in a landfill leachate treatment site in the UK. Also, this is (to the best of our knowledge) the first report on isolation of a strain of *Scotiellopsis reticulata* from a landfill leachate

treatment site in the UK. The potential of these green microalgal strains/isolates for LL treatment is further explored in the following chapters starting with a trial to grow them in different LL concentrations diluted with distilled water (v/v), 5%, 10%, 15%, 20% and 25%, in the next chapter.

CHAPTER FOUR

Determining the growth of the isolated green microalgae

in different concentrations of diluted landfill leachate

4.1. Abstract

Growth profiles of different green microalgae in BG11 medium and different landfill leachate concentrations (5%, 10%, 15%, 20% and 25%) are examined using cell count of algal cells every 3 days over the period of the experiment which was 30 days. The landfill leachate is diluted in distilled water and no BG11 medium is present in the leachate cultures. All the tested green microalgae in this study exhibit higher growth in the growth medium, BG11, than the other tested landfill leachate concentrations. Low concentrations of landfill leachate, 5%, 10% and 15%, allowed significant growth of all the strains (p<0.05) except for *Chlorococcum* sp. However, higher concentrations (≥ 20%) did not support the growth of the tested algae except for the strains *Chlorella vulgaris* ATFG1 and *Chlorella vulgaris* ATFG2 which were the only strains showing tolerance to 20% LL, moreover they showed significant growth (p<0.05) at this challenging LL concentration. The highest concentration tested (25%) did not induce any growth for any of the tested strains and was inhibitory to some of them. These results were the main guide to choose 20% LL concentration for the rest of the studies in order to select the most promising algal isolate for growing at this challenging concentration with the possibility of optimising its growth conditions and LL treatment ability in the future.

4.2. Introduction

Algae are a very diverse and large group of microscopic and macroscopic eukaryotic organisms that carry out oxygenic photosynthesis. Algae are known to be ubiquitous in nature as they inhabit a wide variety of terrestrial and aquatic (marine and fresh

water) habitats which has made them the objects of extensive study by researchers in different fields (Wang and Chen, 2009). Another reason for studying different algal species was their fascinating capability of being useful in different fields such as food and health supplements production, aquaculture support, pharmaceuticals and biofuel production as well as waste water treatment (Cuellar-Bermudez *et al.*, 2017); the latter will be the scope of this study.

The study of microalgae for wastewater treatment purposes has been going for more than two decades (Paskuliakova *et al.*, 2018), hence comes the expression phycoremediation. Phycoremediation has been reported since 1957 (Cuellar-Bermudez *et al.*, 2017) and can be defined as the use of algae to take up and/or biotransform the organic and inorganic pollutants during their growth in wastewaters (Paskuliakova, Tonry and Touzet, 2016). Generally, wastewater can represent a source of available nutrients (especially nitrogen and phosphorus) which can be utilized by algae for growth thus coupling the process of wastewater treatment with the process of biomass production with the possibility of recovery of the desired compounds from the wastewater e.g., heavy metals (Cuellar-Bermudez *et al.*, 2017). However, landfill leachate is a complex, challenging, and costly type of wastewater to treat hence, as previously mentioned, there are less studies on its treatment using microalgae than other types of wastewaters which might be attributed to its potential toxicity as well as complex nature. Usually, raw landfill leachate is highly toxic and inhibitory to algal growth but when diluted and/or pre-treated and/or the algal isolates are laboratory adapted to thrive in high landfill leachate concentrations, it is possible to grow different algal strains in it with the possibility of partial or full removal of different pollutants (Dogaris, Ammar and Philippidis, 2020).

Several studies have reported the ability of different strains of microalgae to grow in diluted and pre-treated landfill leachate with the highest biomass production achieved in diluted landfill leachate concentrations ranging from 10-30% (El Ouaer *et al.*, 2016; Paskuliakova, Tonry and Touzet, 2016; Nordin, Yusof and Samsudin, 2017). Nevertheless, when attempting to use dilution solely (i.e., without pre-treatment) the highest growth achieved, as reported in different studies, was at 10% landfill leachate

concentration (Lin *et al.*, 2007; Paskuliakova, Tonry and Touzet, 2016; El Ouaer *et al.*, 2020).

Diluting landfill leachate (to certain limits) might enhance its ability to be used as growth media for algae as diluting it will decrease the toxic effect of free ammonia nitrogen, the primary cause of toxicity to algae, as well as decreasing the effect of other pollutants which might solely or synergistically have detrimental effects on algal growth in landfill leachate. This idea is strongly supported by various studies in the literature which showed a correlation between lower leachate dilutions and less algal growth and vice versa (Lin *et al.*, 2007; Thongpinyochai, 2014; Paskuliakova, Tonry and Touzet, 2016; El Ouaer *et al.*, 2020).

This chapter will focus on investigating the growth of the four isolated green microalgae in different landfill leachate concentrations (5%, 10%, 15%, 20% and 25%) diluted in distilled water. Algal growth is estimated using cell count by haemocytometer as indicated in the materials and methods section in detail. The aim is to determine the highest concentration of landfill leachate that algae will be able to thrive at, in order to screen which strain would be able to show the best performance in nutrient and contaminant removals from this LL concentration in further experiments, as illustrated in the results section and discussed in the discussion section of the chapter.

4.3. Materials and Methods

4.3.1. Landfill Leachate sample collection

As mentioned in the previous chapter, soil samples were collected in May 2018 from Erin landfill leachate treatment site in Chesterfield, UK, owned by Viridor, a British waste management company (53°14'42.6"N 1°19'45.9"W). Samples were stored in airtight containers until used.

4.3.2. Microalgal strains isolation and maintenance

Green microalgal strains were isolated, purified and genetically identified as described in detail in the previous chapter. The purity of the cultures is regularly checked by microscopic examination under light microscope. The four green

microalgal strains used in the work described in this chapter are: *Chlorella vulgaris* ATFG1, *Chlorella vulgaris* ATFG2, *Chlorococcum* sp. and *Scotiellopsis reticulata*.

Algal cultures that are used as inocula for the following experiment are maintained in Erlenmeyer flasks containing fresh liquid BG11 medium. BG11 medium is prepared according to Stanier *et al.* (1971). Cultures are incubated in a temperature-controlled growth room (25 ± 2^{o}C) in shaking incubators (80 rpm, Stuart orbital shaker). They are grown under continuous illumination provided by daylight fluorescent lamps with a light intensity of 25 ± 5 µmols photons m^{-2} s^{-1} (798 ± 2 lux).

4.3.3. Experimental set up

The experiment is set up in sterile test tubes. Seven different groups are set up to test the growth of each green microalgal strain in each of the tested LL concentration for the time interval of the experiment which was 30 days. Five groups of the experimental set up consist of 8 test tubes each. Each group represent a certain LL dilution i.e., 5%, 10%, 15%, 20% and 25%, diluted in distilled water. There is a duplicate for each concentration for each algal strain (2 test tubes for each strain so 8 in total for each dilution). LL samples are filtered using 0.45 µm filters prior to the experiment and are diluted with sterile distilled water to the required concentration. All the test tubes used in the experiment are sterilised by autoclaving and are sealed with sterile bungs. The last two groups are controls; one of them contains four test tubes each with BG11 media with no LL added but inoculated with the tested microalgae and the other did not have any algae nor media but contains only LL with the tested concentrations. All the test tubes in the six experimental arrangements (except the LL control) are inoculated with an initial algal load of 10 ×10^{4} cells ml^{-1} (counted by haemocytometer) of actively growing algal cells.

4.3.4. Algal growth Determination

Every three days, growth is measured using cell density determination by cell count (using a haemocytometer). Growth rate µ (d^{-1}) is calculated according to the following equation (Lim, Chu and Phang, 2010):

$$\mu\ (day^{-1}) = \frac{\mathrm{Ln}\, N_2 - \mathrm{Ln}\, N_1}{T_2 - T_1}$$

N_1 and N_2 represent the cell density at times T_1 (day 0) and T_2 (day 30) during the experiment, respectively.

Inhibition rate ($I_{\mu i}$) is also determined, according to the following equation (El Ouaer *et al.*, 2020):

$$I_{\mu i} = \frac{\mu_c - \mu_i}{\mu_c}$$

$I_{\mu i}$ is the inhibition rate for the leachate solution (i).

μ_i and μ_c are the growth rates of tested algal strain in the leachate solution (i) and the control BG11 (c), respectively.

4.3.5. Statistical analysis

Statistical analysis is carried out using R v.4.0.5 and R studio v.1.2.5. Growth curve data are presented as mean and standard error. Significant growth comparing day 0 and 30 is tested using paired t-test for each species at each leachate concentration. A p value <0.05 is considered statistically significant.

4.4. Results

Growth of the previously isolated green microalgae is characterised in different landfill leachate concentrations (5%, 10%, 15%, 20% and 25%) as well as in BG11 medium. The difference in the growth trends amongst BG11 medium and the different landfill leachate concentrations for each alga is important to determine the most inhibitory LL concentration for algal growth as well as the least inhibitory one compared to the BG11 medium, which usually support their growth, over a period of 30 days (the time interval of the experiment). Chemical properties of landfill leachate used in this study is characterised in detail in the next chapter (Table **5-1**).

4.4.1. *Chlorella vulgaris* ATFG1

As shown in Figure 4-1, the highest growth for *Chlorella vulgaris* ATFG1 during the 30 days of the experiment is observed in BG11 medium. The cell density of *Chlorella vulgaris* ATFG1 increased by 152.6-fold in BG11 medium whilst the increase in cell

density for the different tested LL concentrations was 33.5, 26, 10, 4.2 and 1 at concentrations 5%, 10%, 15%, 20% and 25% respectively. Nonetheless, concentrations 5%, 10%, 15% and 20% allowed a significant increase in the growth of *Chlorella vulgaris* ATFG1 from day 0 to day 30 of the experiment (p<0.05). The growth rates as shown in Figure 4-2 is highest (0.16 d^{-1}) for BG11 and then decreases as the landfill leachate concentration increases so it is highest at concentration 5% and lowest at concentration 25%. This is also indicated by the inhibition rate illustrated in Figure 4-3, concentration 25% has the highest inhibition rate and 5% has the lowest inhibition rate on the growth of Chlorella *vulgaris* ATFG1.

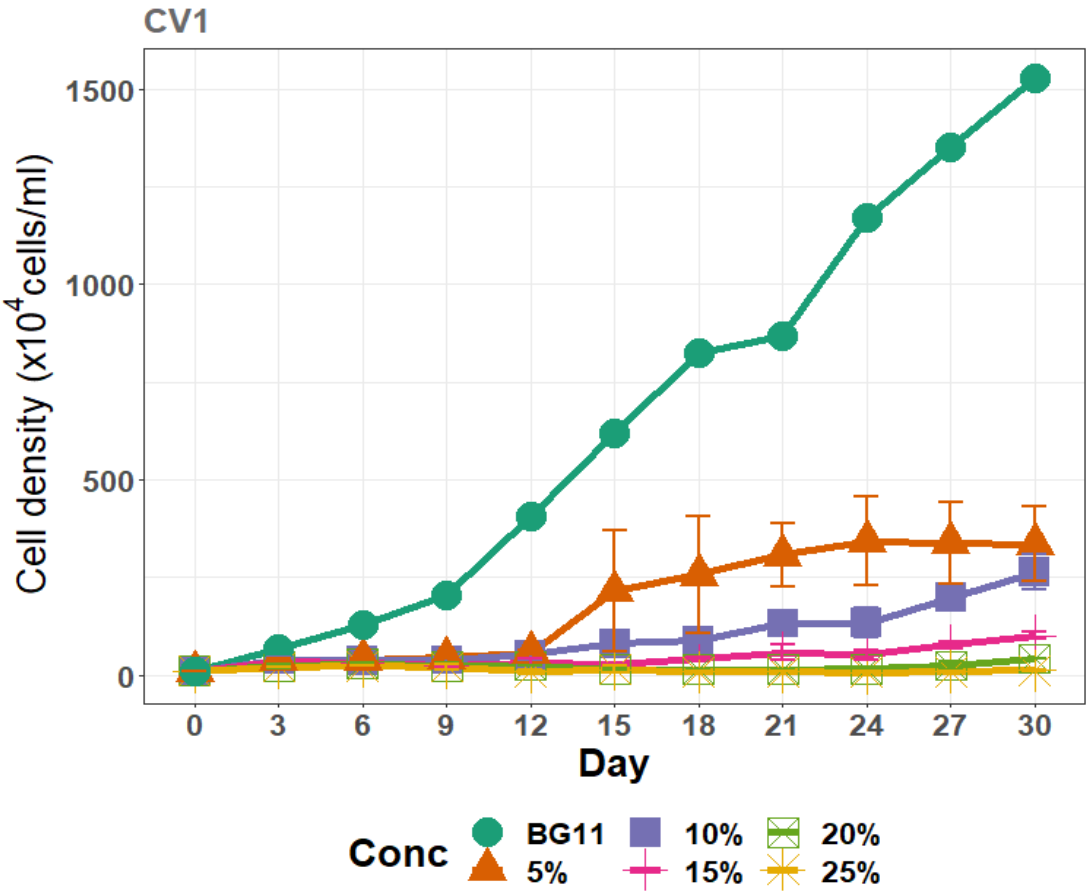

Figure 4-1 Growth curve of the microalga *Chlorella vulgaris* ATFG1 in BG11 medium and different LL concentrations (5%, 10%, 15%, 20% and 25%). Mean and standard error values of two biological replicates are plotted.

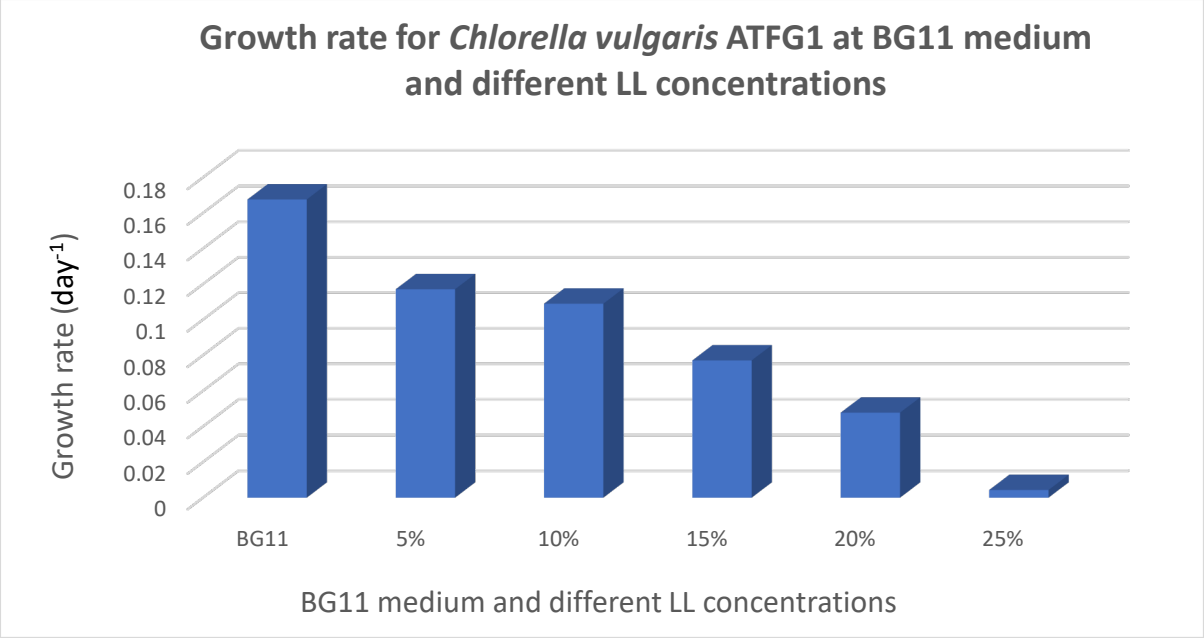

Figure 4-2 Growth rates of the microalga *Chlorella vulgaris* ATFG1 in BG11 medium and different LL concentrations (5%, 10%, 15%, 20% and 25%).

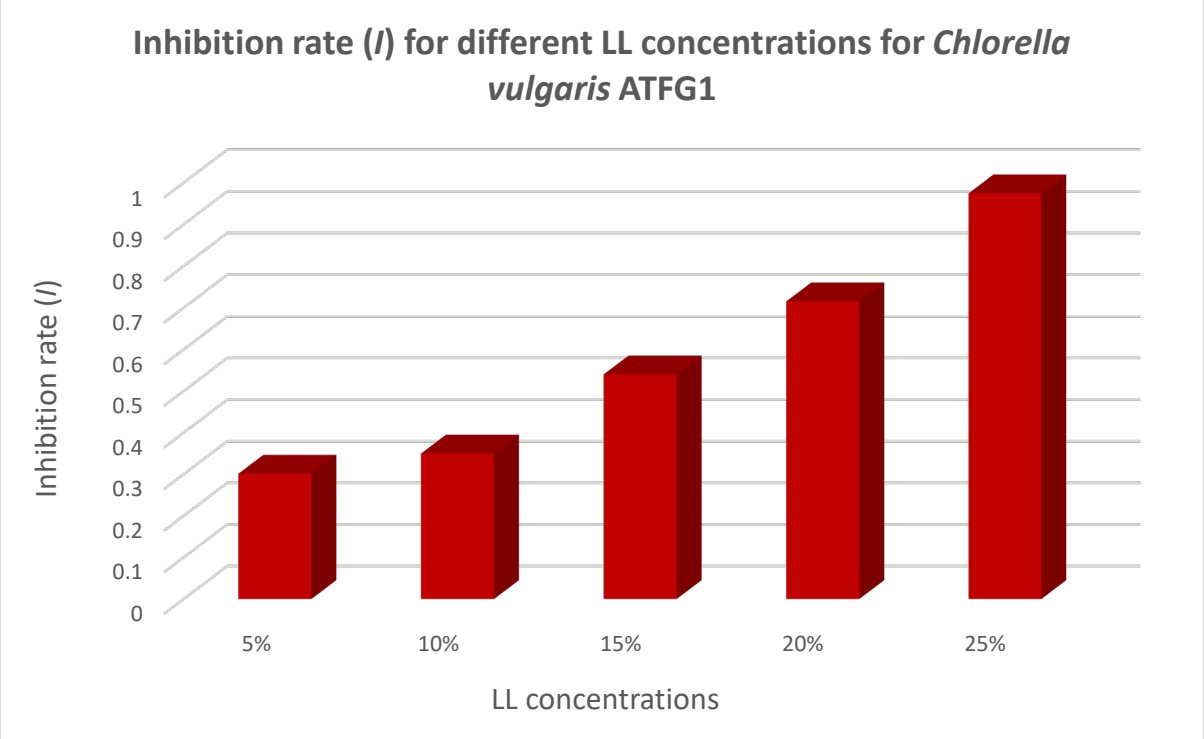

Figure 4-3 Inhibition rates of different LL concentrations (5%, 10%, 15%, 20% and 25%) on the growth of the microalga *Chlorella vulgaris* ATFG1.

4.4.2. *Chlorella vulgaris* ATFG2

Figure 4-4 shows the growth curve of the green microalga *Chlorella vulgaris* ATFG2 in different LL concentrations (5%, 10%, 15%, 20% and 25%) as well as in the BG11 medium during the 30-day experimental period. Like the other *Chlorella* strain, the highest growth is observed in BG11 medium followed by the lowest LL concentration then the next highest (5%, 10%, 15%, 20% and 25%), moreover the increase in cell density was 86.7, 37.3, 19.6, 18.9, 5.2 and 0.4-fold, respectively, which is deemed a significant increase for all the tested concentrations except for 25% ($p < 0.05$). The strain *Chlorella vulgaris* ATFG2 did not grow in the concentration 25%, but it showed different growth rates in the BG11 medium as well as the different tested concentrations of LL (Figure 4-4).

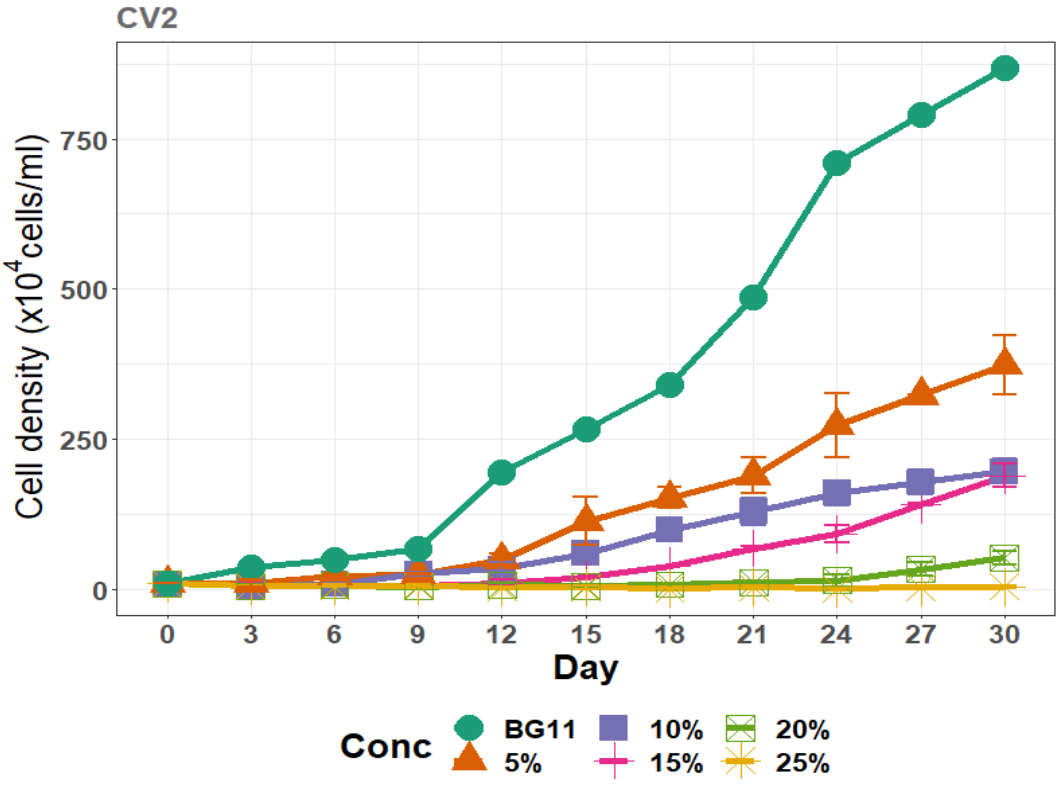

Figure 4-4 Growth curves of the microalga *Chlorella vulgaris* ATFG2 in BG11 medium and different LL concentrations (5%, 10%, 15%, 20% and 25%). Mean and standard error values of two biological replicates are plotted.

Exponential growth rate calculated from cell count is 0.14 d^{-1} in BG11 medium, which decreased to 0.12 d^{-1} in 5% LL concentration and 0.09 d^{-1} in concentrations 10 and 15%. The lowest growth rate is 0.05 d^{-1} at the concentration 20%, whilst no growth is

achieved at concentration 25% (Figure 4-5). Figure 4-6 supports these results by showing values of growth inhibition rates for the tested LL concentrations (5%, 10%, 15%, 20% and 25%).

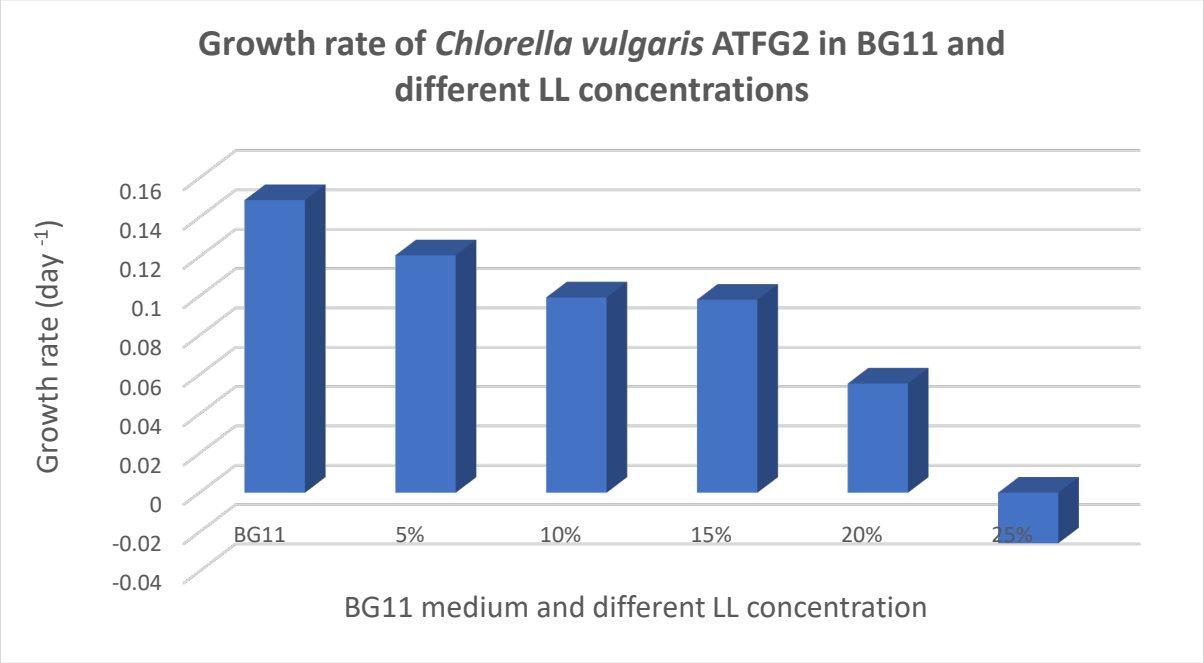

Figure 4-5 Growth rates of the microalga *Chlorella vulgaris* ATFG2 in BG11 medium and different LL concentrations (5%, 10%, 15%, 20% and 25%).

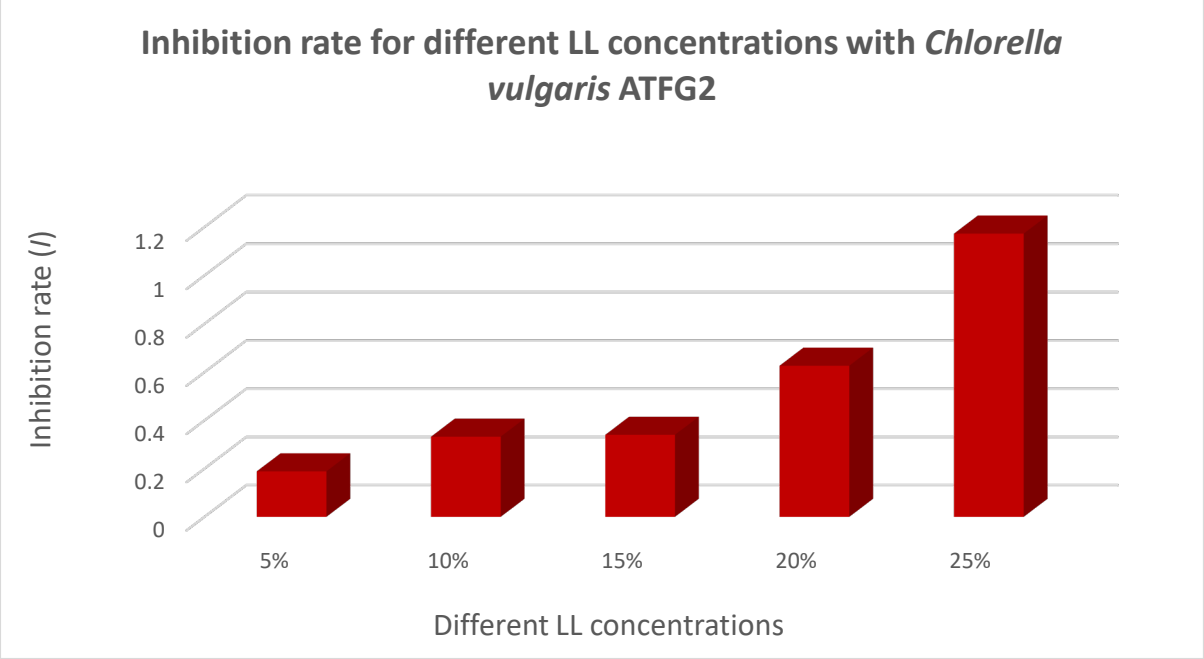

Figure 4-6 Inhibition rates of different LL concentrations (5%, 10%, 15%, 20% and 25%) on the growth of the microalga *Chlorella vulgaris* ATFG2.

4.4.3. *Chlorococcum* sp.

Growth of the microalga *Chlorococcum* sp. increased by 16.9-fold in BG11 medium and by 3.6-fold in 5% LL concentration, whilst it collapsed in the rest of LL concentrations tested (10%, 15%, 20% and 25%) as shown in Figure 4-7.

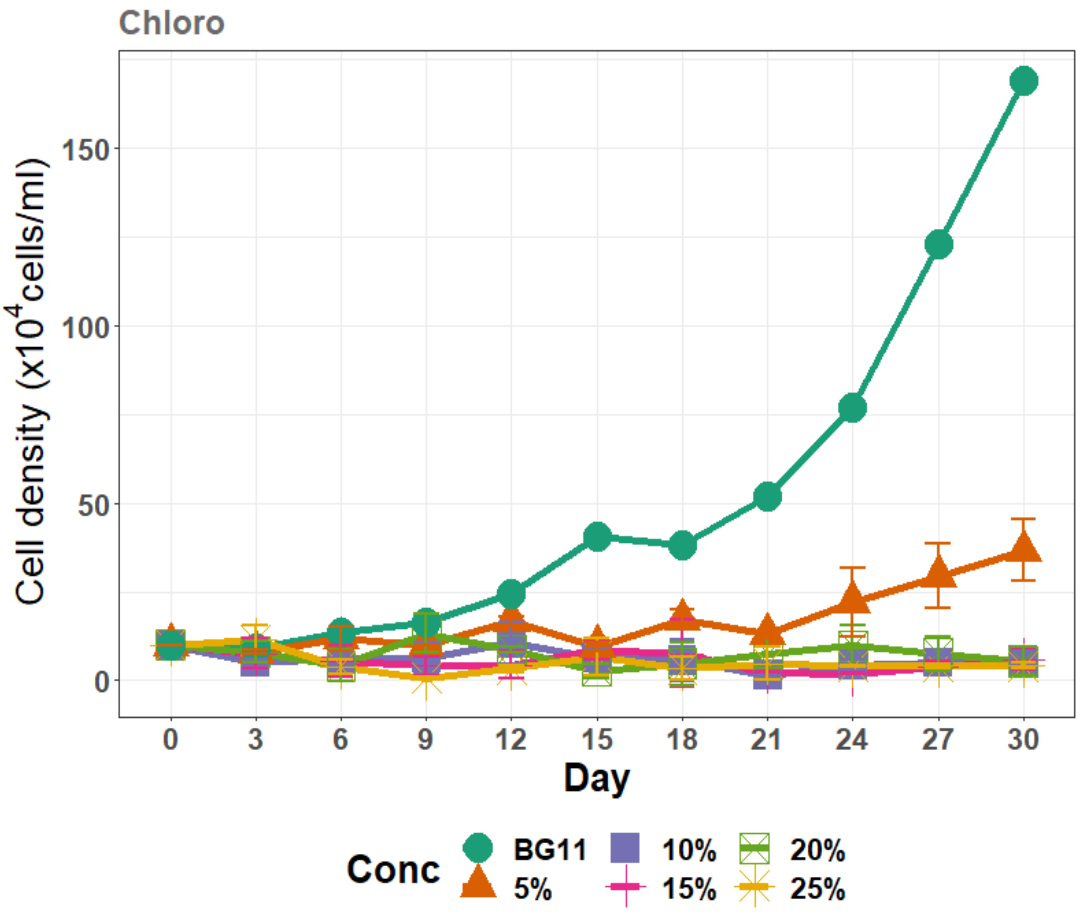

Figure 4-7 Growth curves of the microalga *Chlorococcum sp.* in BG11 medium and different LL concentrations (5%, 10%, 15%, 20% and 25%). Mean and standard error values of two biological replicates are plotted.

The exponential growth rate is calculated as 0.09 d^{-1} for BG11 medium and 0.04 d^{-1} for 5% LL concentration (Figure 4-8). LL concentrations starting from 10% and above were inhibitory for the growth of *Chlorococcum* sp. (Figure 4-9).

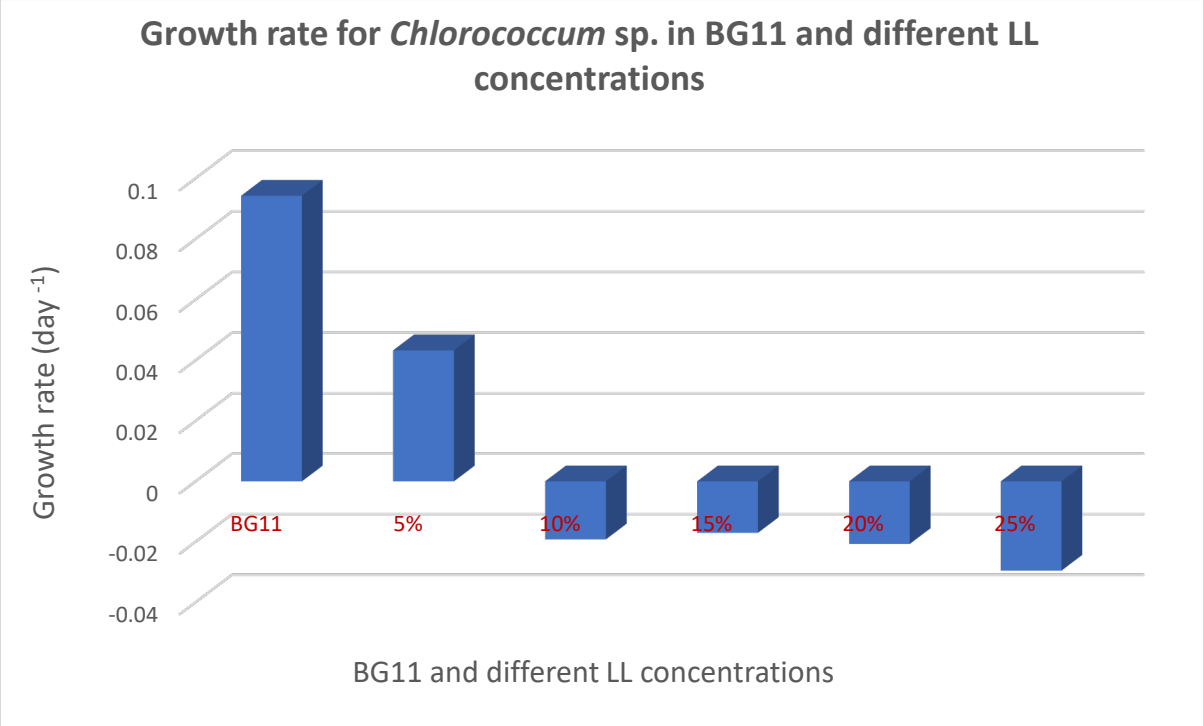

Figure 4-8 Growth rates of the microalga *Chlorococcum* sp. in BG11 medium and different LL concentrations (5%, 10%, 15%, 20% and 25%).

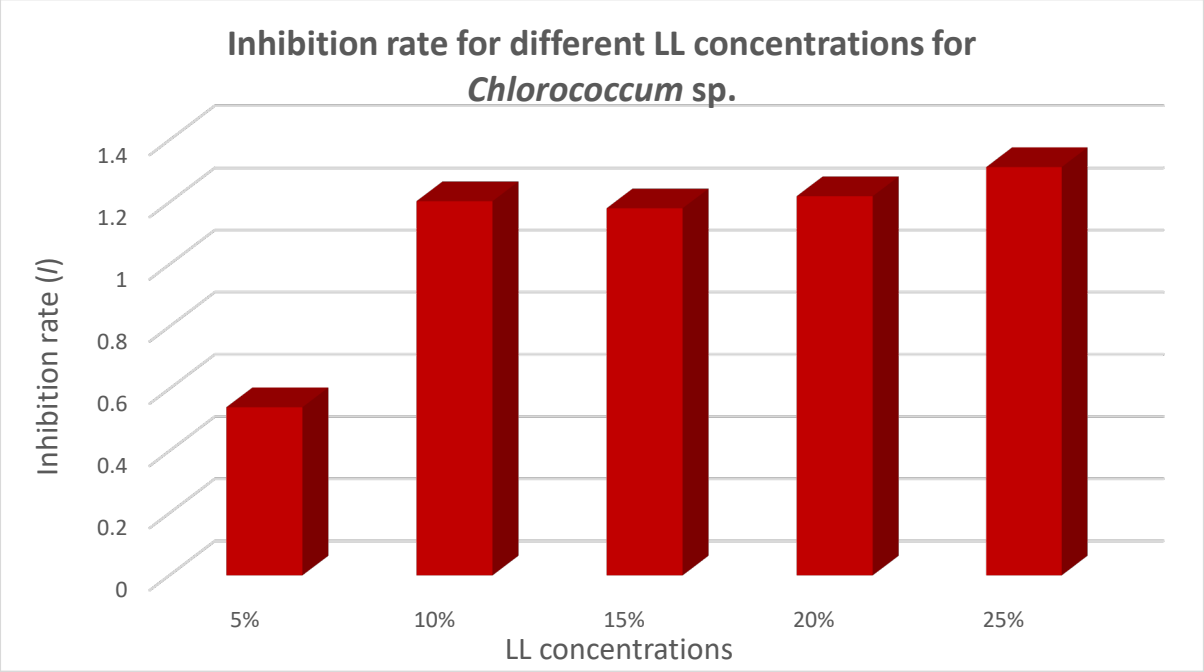

Figure 4-9 Inhibition rates of different LL concentrations (5%, 10%, 15%, 20% and 25%) on the growth of the microalga *Chlorococcum* sp.

4.4.4. *Scotiellopsis reticulata*

Growth is observed in BG11 medium as well as the different tested concentrations of LL (with variation) for the strain *Scotiellopsis reticulata* as shown in Figure 4-10. The highest increase in growth for *Scotiellopsis reticulata* is observed in BG11 medium where an increase by 22.5-fold was achieved. Cell densities increased at the end of the experiment by 11.4, 7.8, 5.2, 2.6 and 1.4-fold for the concentrations 5%, 10%, 15%, 20% and 25%, respectively. Concentrations 5%, 10% and 15% allowed a significant growth increase from day 0 to day 30 of the experiment (p<0.05).

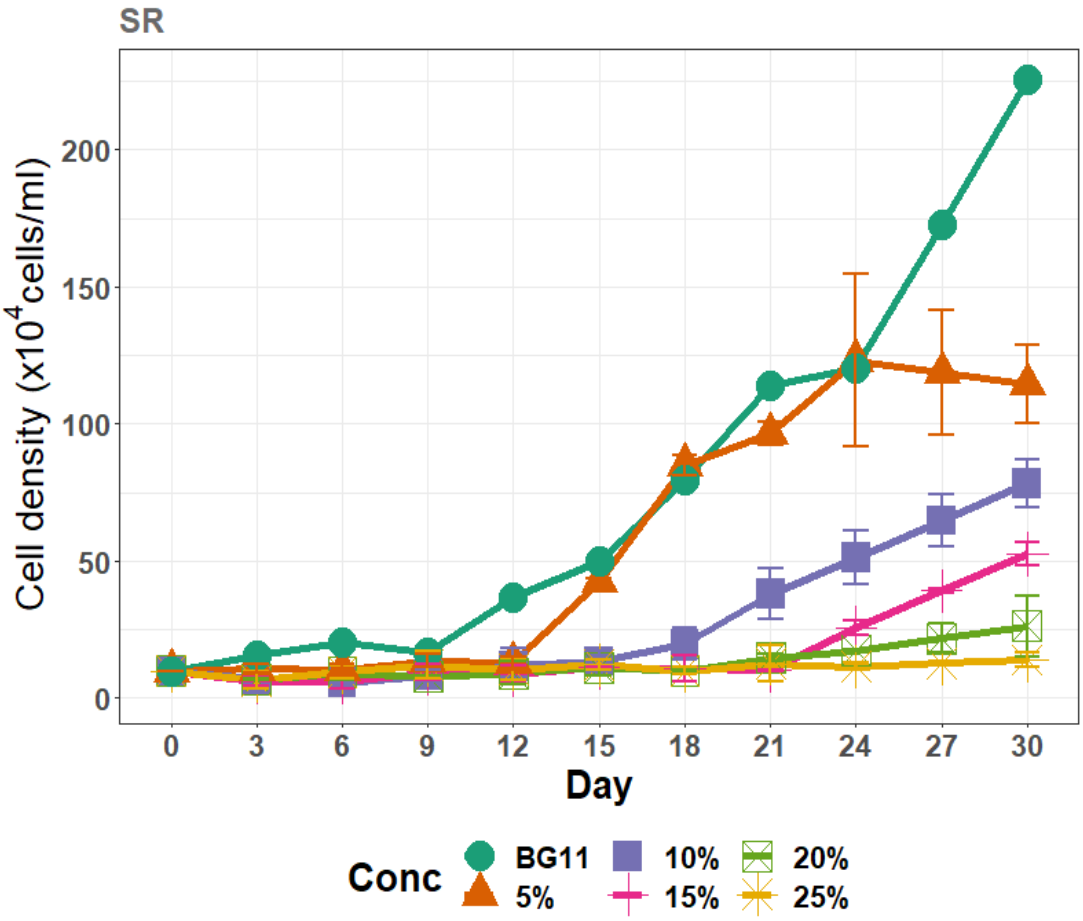

Figure 4-10 Growth curves of the microalga *Scotiellopsis reticulata* in BG11 medium and different LL concentrations (5%, 10%, 15%, 20% and 25%). Mean and standard error values of two biological replicates are plotted.

The growth rates for the green microalga *Scotiellopsis reticulata* in BG11 medium and different LL concentrations is illustrated in Figure 4-11, where the highest growth rate value is 0.1 d^{-1} at BG11, and it decreased as the LL concentration increased in the range of 0.08-0.01 d^{-1} for concentrations 5%-25%.

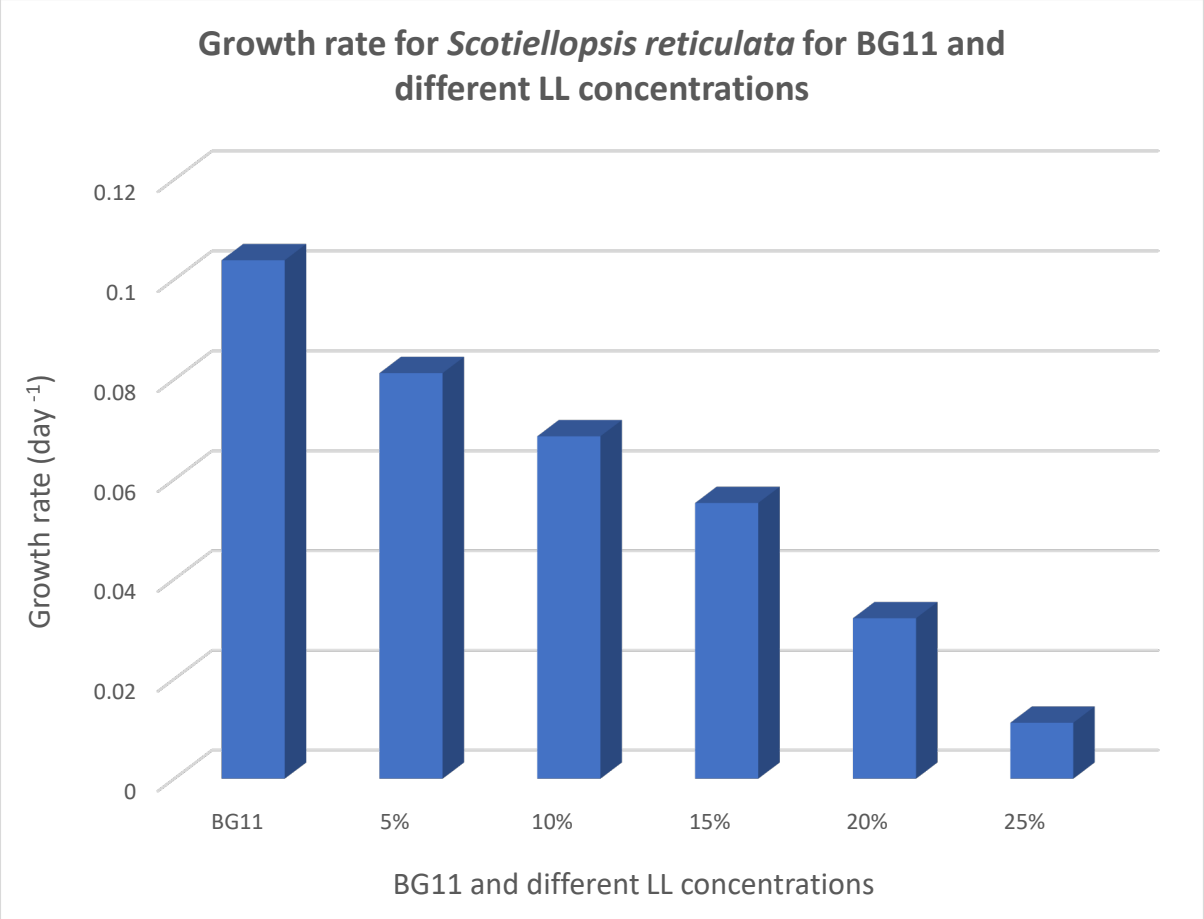

Figure 4-11 Growth rates of the microalga *Scotiellopsis reticulata* in BG11 medium and different LL concentrations (5%, 10%, 15%, 20% and 25%).

Figure 4-12 shows that concentration 25% has the highest inhibitory rate on the growth of *Scotiellopsis reticulata* whilst the inhibitory effect decreases as the concentration of LL decreases, so the lowest inhibitory rate is observed at concentration 5%.

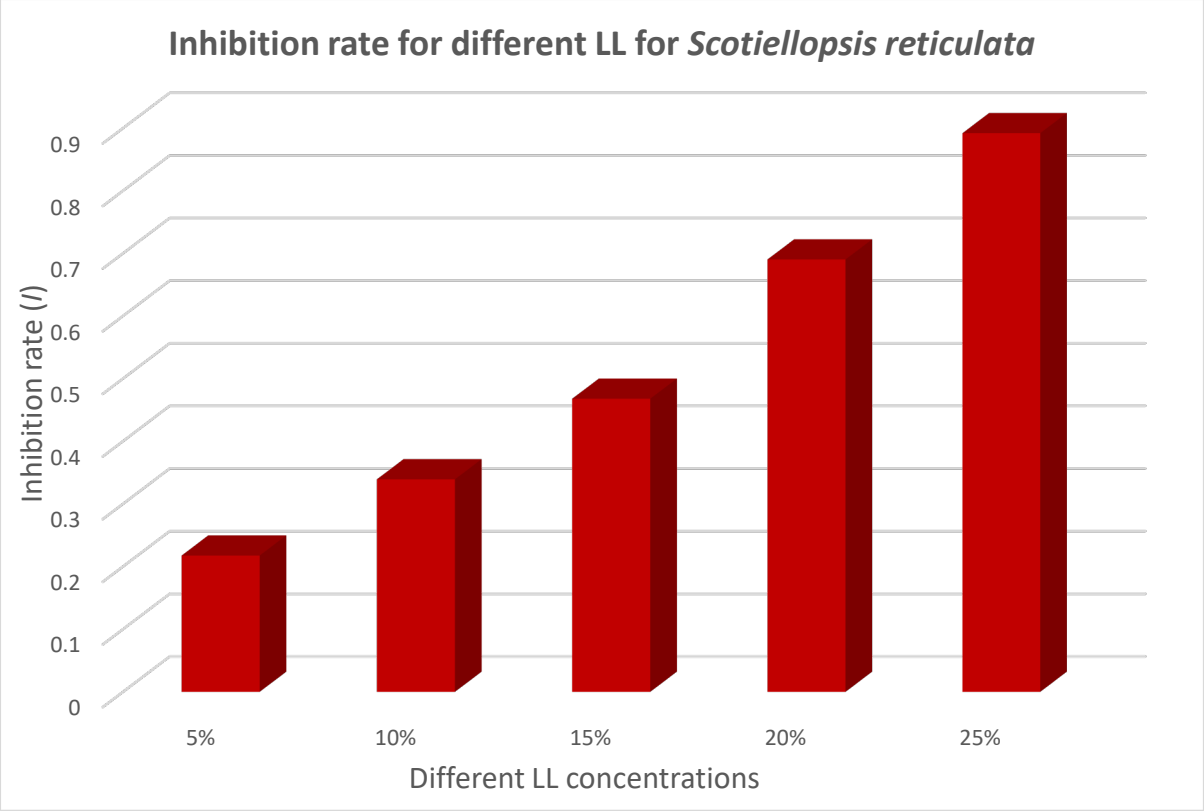

Figure 4-12 Inhibition rates of different LL concentrations (5%, 10%, 15%, 20% and 25%) on the growth of the microalga *Scotiellopsis reticulata.*

Figure 4-13 include all the growth curves of the four tested green microalgae: *Chlorella vulgaris* ATFG1, *Chlorella vulgaris* ATFG2, *Chlorococcum* sp. and *Scotiellopsis reticulata* in BG11 media and different LL concentrations (5%, 10%, 15%, 20% and 25%).

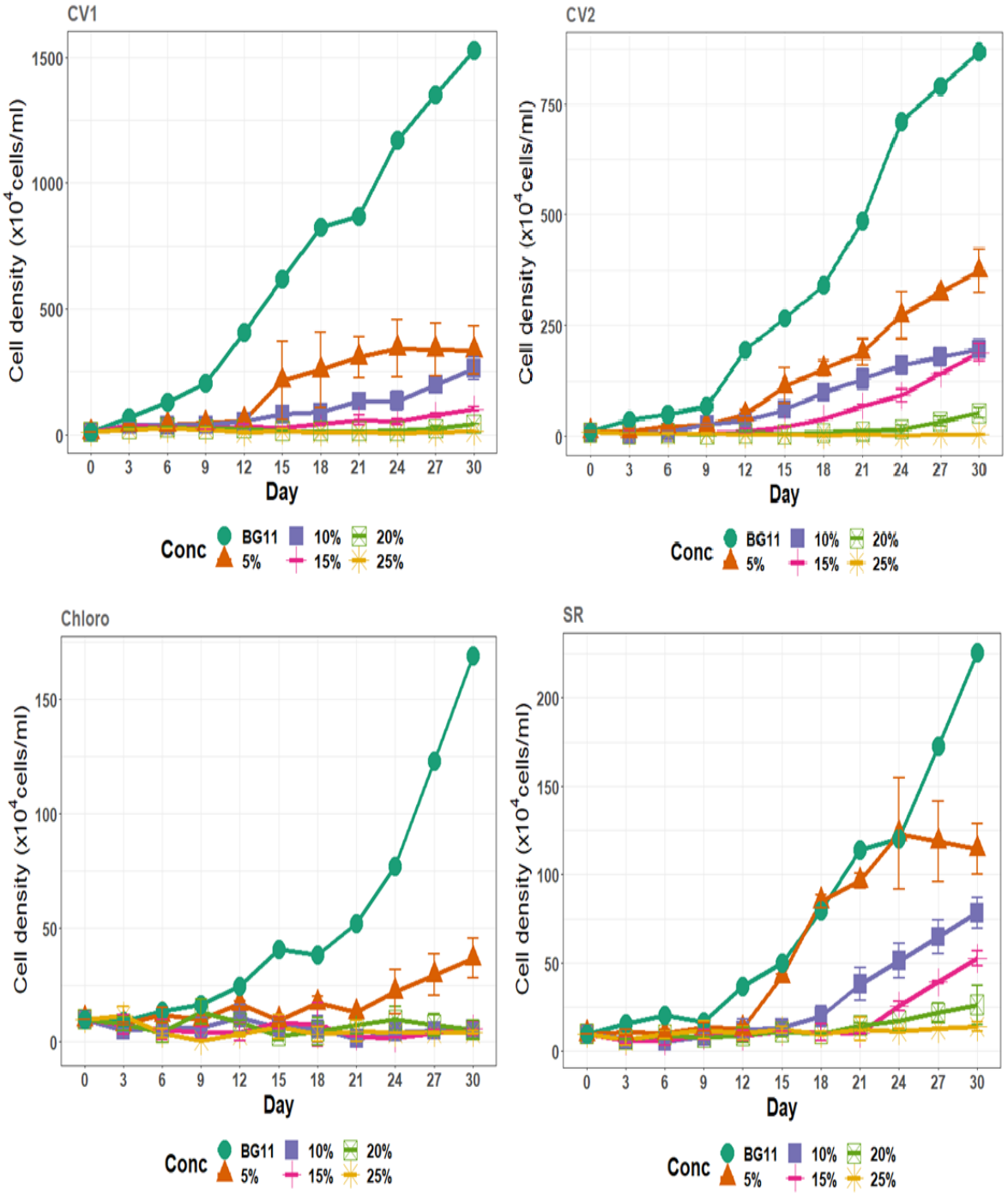

Figure 4-13 Growth curves of the four tested green microalgae in BG11 media and different LL concentrations (5%, 10%, 15%, 20% and 25%). CV1 (*Chlorella vulgaris* ATFG1), CV2 (*Chlorella vulgaris* ATFG2), Chloro (*Chlorococcum* sp.) and SR (*Scotiellopsis reticulata*) . Mean and standard error values of two biological replicates are plotted.

4.5. Discussion

The fact that microalgae, especially chlorophytes, have simple growth requirements and can grow in a wide variety of habitats and environments has paved the way for growing them in wastewaters and recently in landfill leachate for the purpose of treatment coupled with biomass production. Although landfill leachate is considered an extremely complex, toxic and a very challenging medium for the growth of any microorganism, various successful attempts to grow microalgae and/or other microorganisms have been documented in diluted leachate either with autoclaved deionised water, distilled water, or municipal wastewater (Lin *et al.*, 2007; Zhao *et al.*, 2014; Paskuliakova, Tonry and Touzet, 2016; El Ouaer *et al.*, 2020; Okurowska *et al.*, 2021).

The urge to dilute landfill leachate with either distilled/deionised water or with municipal wastewater is due to the toxicity of raw landfill leachate to different living organisms including microalgae. Although, small concentrations of landfill leachate (up to 10%) might have some stimulatory effects on algal growth (Lin *et al.*, 2007; El Ouaer *et al.*, 2020), however, higher concentrations (>10%) usually induce inhibitory effects on algal growth (Lin *et al.*, 2007; Zhao *et al.*, 2014; El Ouaer *et al.*, 2020). The stimulatory effects of lower landfill leachate concentrations for algal growth might be attributed to the presence of some nutrients e.g., organic substances (Cheung, Chu and Wong, 1993) and ammonia in appropriate amounts as higher ammonia concentrations might have adverse effects on algal growth (Lin *et al.*, 2007).

On the other hand, toxicity of higher concentrations of landfill leachate may be attributed to various reasons:

First: Its high ammonia content, although ammonium usually represents a preferred nitrogen source for green algae (Hellebust and Ahmad, 1989) but high levels of ammonium might induce toxicity and/or inhibition for algal growth (Lin *et al.*, 2007). This may be explained by Erickson (1985) who stated that, total ammonia in aqueous solutions exists in two principal forms, the ionized form (ammonium; NH_4^+) and the unionized form (ammonia; NH_3), the relative concentration of these two forms is pH

and temperature-dependent as the ratio of unionized ammonia to ionized ammonium increases by 10-fold for each unit rise in pH and by 2-fold for each 10 ˚C rise in temperature over the 0-30 ˚C range. Leachate is usually characterised by relatively high pH > 8 so the possibility of formation of the more toxic form of ammonia (unionised form; NH_3) might increase especially at temperatures ≥25^{o}C (Cheung, Chu and Wong, 1993). Toxicity of ammonia may be attributed to immediate cell lysis at high ammonia concentrations (Collos and Harrison, 2014) and/or photosynthesis inhibition at pH values over 8.0 for ammonia concentrations over 2.0 mM (Abeliovich and Azov, 1976). Second: The presence of various organic compounds in levels which might be highly toxic to green microalgae; these compounds include volatile fatty acids, recalcitrant hydroxy aromatic compounds and/or polychlorinated biphenyls (PCBs) (Cheung, Chu and Wong, 1993). Third: Leachate usually has higher concentrations of some salts and heavy metals which might represent potential toxicity especially with the relatively high pH of the leachate (≥8) which might favour the formation of toxic forms of trace metals and sulphides (Cheung, Chu and Wong, 1993; Okurowska *et al.*, 2021). Fourth: The strong dark brown colour of raw landfill leachate is also a powerful reason for algal growth inhibition as algae are photosynthetic organisms and they require light to undergo photosynthesis. The colour of LL can strongly reduce the light availability, which is necessary for algal photosynthesis, thus adversely affect algal growth. The amount of light available for algal photosynthesis is also limited with LL dilution, light availability decreases as concentration of LL increases and vice versa(Cheung, Chu and Wong, 1993; Okurowska *et al.*, 2021).

Growth profiles of different green microalgae in BG11 medium and different landfill leachate concentrations (5%, 10%, 15%, 20% and 25%) were determined using cell count of algal cells every 3 days over a 30-day period experiment. All the tested green microalgae in this study exhibited higher growth in the growth medium (BG11) than other tested landfill leachate concentrations. Similar results were obtained in several different studies when comparing algal growth profiles in growth medium (BBM) and different LL concentrations (Lin *et al.*, 2007; Edmundson and Wilkie, 2013; El Ouaer *et al.*, 2016, 2020). Growth media usually support higher algal growth when compared

to different LL concentrations which may be attributed to the potential toxicity of LL to algae as discussed earlier. However, El Ouaer *et al.* (2016) found that the increase in cell densities of *Chlorella* sp. in case of BBM and 10% diluted LL were 25 and 21 times, respectively. On the other hand, the increase in cell densities of the two tested *Chlorella vulgaris* strains (ATFG1 and ATFG2) in this study were 152.6 and 86.7 in case of BG11 whilst 26 and 19.6 in case of the dilution 10% LL, respectively.

To make it easier to discuss, we will divide the studied concentrations of landfill leachate into 3 sections: ≤15%, 20% and ≥25%. Relatively low landfill leachate concentrations might have stimulatory effect on algal growth rather than higher concentrations which might be partially or completely inhibitory for the growth of algae. Our results revealed that the growth of *Chlorella vulgaris* ATFG1, *Chlorella vulgaris* ATFG2 and *Scotiellopsis reticulata*, showed significant increase in cell density for concentrations 5%, 10% and 15% (p<0.05). Similar results were obtained by Cheung, Chu and Wong (1993) who found that the growth of *Chlorella pyrenoidosa* and *Chlorella vulgaris* increased significantly at concentrations 5%, 10% and 15% in GDB landfill leachate. They also demonstrated that *Chlorella pyrenoidosa* exhibited significant increase in growth at the same concentrations of another more toxic type of landfill leachate examined (JB leachate). Our results also revealed that on the contrary of the increase in growth densities for *Chlorella vulgaris* ATFG1, *Chlorella vulgaris* ATFG2 and *Scotiellopsis reticulata*, the increase in growth of *Chlorococcum sp*. at concentration 5% was not significant and its growth was inhibited by higher concentrations. In a 30-day experiment conducted by Paskuliakova, Tonry and Touzet (2016), four strains of green microalgae were grown in a 10% raw leachate and microalgae biovolume was determined every 5 days, while one strain (*Chlamydomonas sp.* SW15arl) was able to grow extensively at this concentration, the other three were not able to grow with one collapsing at day 8 (*Chlamydomonas sp.* SW13als) and the other two (*Scenedesmus sp.* OT08aTL and *Scenedesmus sp.* OT11aTL) did not show any growth although appeared to be surviving.

Despite the LL concentration 10% being the concentration usually enhancing the highest algal growth in several studies (Lin *et al.*, 2007; Zhao *et al.*, 2014; El Ouaer *et*

al., 2020) our results indicated that the concentration 5% LL was the concentration that supported the maximum growth represented by cell density, the highest growth rate, and the lowest inhibition rate for all the tested green microalgal strains compared to other higher LL concentrations tested in this study. This may be attributed to the presence of some nutrients e.g., organic compounds and ammonia in appropriate amounts i.e., diluted to less toxic levels which is not inhibitory and/or in some cases even enhances the algal growth (Cheung, Chu and Wong, 1993; Lin *et al.*, 2007).

Regarding the second section: our experiment results indicated that a concentration of 20% supported significant increase in growth (p<0.05) of only *Chlorella vulgaris* (both strains: ATFG1 and ATFG2), whilst it inhibited the growth of *Chlorococcum* sp. and neither inhibited nor induced significant increase in growth of *Scotiellopsis reticulata*. Cheung, Chu and Wong (1993) also reported that 20% LL concentration from two different landfills (JB and GDB) had different enhancing and inhibitory effects on different tested microalgae. They demonstrated that 20% concentration of the JB landfill leachate induced significant increase in growth of *Chlorella pyrenoidosa* whilst significantly decreased the growth of *Chlorella vulgaris* and *Scenedesmus* sp. and completely supressed the growth of *Dunaliella tertiolecta*. The other tested leachate which was the GDB leachate also increased the growth of *Chlorella pyrenoidosa* and unlike JB LL it induced significant increase in the growth of *Chlorella vulgaris*, however, it did not support the growth of *Scenedesmus* sp. and *Dunaliella tertiolecta*. Zhao *et al.* (2014) also studied the effect of municipal wastewater spiked with different concentrations of LL on the growth of a microalgae-bacterium consortium and demonstrated that the 20% leachate spike ratio was somehow inhibitory to algal growth, and this was observed in the chlorophyll a measurement which exhibited a 6-day lag phase before starting to show some increase indicating the increase in growth of the microalga *Chlorella pyrenoidosa* (FACHB-9).

The highest tested LL concentration in this study was 25% and it did not support the growth of any of the examined microalgal strains for the time of the experiment, which was 30 days, in fact it suppressed the growth of *Chlorella vulgaris* ATFG2 and *Chlorococcum sp.* Our results agree with Cheung, Chu and Wong (1993), as they found

that concentration ≥25% JB Landfill leachate was significantly inhibitory for the growth of *Chlorella vulgaris*, *Scenedesmus* sp. and *Dunaliella tertiolecta*. On the other contrary, the growth of *Chlorella pyrenoidosa* showed significant increase at concentration 25% of two types of landfill leachates (JB and GDB). With few exceptions, concentrations > 25% of LL that is not subjected to any treatment are inhibitory to algal growth due its high toxicity as discussed previously (Cheung, Chu and Wong, 1993; Lin *et al.*, 2007; Edmundson and Wilkie, 2013; El Ouaer *et al.*, 2016, 2020).

Different algal strains may exhibit different growth patterns in the same landfill leachate concentration which may indicate differences in algal resistance depending on the strain as well as the toxicity and/or strength level of landfill leachate (Cheung, Chu and Wong, 1993). Although, in this study all the isolated strains were isolated from the same landfill leachate treatment plant, nevertheless, it could be concluded that some of them might show individual resistance more than the others, in other words when they were in their natural habitat they were living as a community which might account for their total resistance to LL toxicity. Whilst for the purpose of this study, they were isolated and purified which might affect their resistance to the same LL as individuals, this theory must be further examined scientifically in the future.

4.6. Conclusion

The growth profiles of four green microalgae strains in different concentrations of landfill leachate are examined. In general, low concentrations (5%, 10% and 15%) allowed a significant growth of all the strains except for *Chlorococcum* sp. However, higher concentrations (≥ 20%) did not support the growth of the tested algae with the exception of strains *Chlorella vulgaris* ATFG1 and *Chlorella vulgaris* ATFG2 that were the only strains showing tolerance to the 20% LL concentration, moreover they showed significant increase in their growth at this challenging concentration. The highest concentration tested (25%) did not induce any growth for any of the tested strains and was inhibitory to some of them. Based on these results, the highest LL concentration (20%), to which some of the tested strains showed tolerance, was chosen

to be used in further screening experiments to detect which green microalga will be able to grow and uptake some of the major pollutants in this tested LL concentration for the purpose of algal treatment in the future.

CHAPTER FIVE

Screening green microalgae
for growth and bioremediation of 20% diluted Landfill Leachate

5.1. Abstract

In this chapter, an experiment was carried out to screen the four strains of green microalgae, previously isolated and identified, for their ability to (solely or collectively) grow and remove various nutrients from 20% (v/v) LL diluted with distilled water as mentioned in the previous chapter. The tested concentration of LL was supplemented by K_2HPO_4. The experiment is conducted over a period of 30 days under continuous illumination (surface intensity of 1272.5 ± 19 Lux) and continuous stirring at room temperature. The results indicate a significantly high growth for one of the *Chlorella vulgaris* cultures tested (C.v.1.1) as measured by spectrophotometry every 5 days (OD_{595}) with a 4.5-fold increase in growth compared to the other tested *Chlorella vulgaris* culture which came next in order, for significant growth increase. The biomass produced from C.v.1.1 has final density 0.284 g/L. C.v.1.1 also recorded the highest ammonia-N removal compared to other treatments by 86.7% with a significant decrease in the pH accompanying the ammonia-N uptake. *As*, *Cr*, *Pb* and *Cu* uptake are evaluated at the end of the experiment. *As* removal is significantly higher than the control treatment in case of *Chlorella vulgaris* cultures (C.v.1.1 and C.v.1.2) while no significant removal is recorded for any of the tested treatments compared to the control in case of the other metals tested. *Chlorella vulgaris* culture (C.v.1.1) might be further explored as a potential candidate for bioremediation of 20% diluted LL.

5.2. Introduction

The ability of microalgae to survive in landfill leachate underscores their likely tolerance to various toxic pollutants in landfill leachate. Consequently, they should possess capability to utilise and/or remove these pollutants as they grow, so producing algal biomass and remediating landfill leachate. Landfill leachate, as mentioned previously, consists of a vast array of pollutants e.g., dissolved organic matter (DOM), inorganic macro-compounds, heavy metals, and xenobiotic organic compounds (Wijekoon et al., 2022). Classical parameters that are usually used to characterise landfill leachate could be summarised as follows: chemical oxygen demand (COD), biochemical oxygen demand (BOD), total organic carbon (TOC), suspended solids, pH, ammonium nitrogen (NH_4^+-N) and heavy metals composition (Teng et al., 2021).

Toxicity of landfill leachate to the environment, different living organisms and human beings with the potential carcinogenicity and genotoxicity hazards are widely reported in the literature (Clément and Merlin, 1995; Dogaris, Ammar and Philippidis, 2020; Nawaz et al., 2020; Teng et al., 2021; Wijekoon et al., 2022) The toxic effects of each pollutant in the landfill leachate might vary according to different parameters such as the pH of the leachate, the organic/mineral complex matrix as well as the interaction effects (antagonism or synergism) amongst different constituents of the leachate (Clément and Merlin, 1995; Wijekoon et al., 2022). Herein, we will outline the detrimental effects of some of the main toxic components of landfill leachate and their potential hazards to the ecosystem and living organisms to emphasize the importance of their removal by algal biomass. Toxic effects of different components of landfill leachate involve:

1. Dissolved organic matter (DOM): dissolved organic matter present in landfill leachate includes amino acids, volatile fatty acids, hydrophilic acids, fulvic-like and humic-like compounds. Their toxic effects might be attributed mainly to their effect on microbial activity which might in turn cause fouling of membranes and decrease the effluent quality of the coagulation process (one of the physical-chemical methods for landfill leachate treatment). The functional groups in the DOM could possibly

interact with other contaminants in the leachate. They might form complexes with heavy metals present in the leachate which might further influence their transportation, stability, and bioavailability. They also account for the leachate's dark brown colour. DOM can be characterised using several bulk parameters e.g., COD, BOD, BOD/COD ratio, TOC, VFA and other individual compounds, i.e., methane (Teng et al., 2021; Wijekoon et al., 2022).

2. Inorganic macro-compounds: amongst different inorganic entities in landfill leachate, ammonia stands out as one of the most significant components of landfill leachate in the long term (Wijekoon et al., 2022). Although ammonium is considered as a preferred nitrogen source for algae to uptake and assimilate, nevertheless high ammonia concentrations may have very toxic effects on different living organisms as well as the environment (Hellebust and Ahmad, 1989; Wijekoon et al., 2022). The distribution of ammonia in leachate in the form of ammonium (NH_4^+) or free ammonia (NH_3) is a temperature and pH dependent phenomenon. Ammonia in its unionised free form is reported to be more toxic to different living organisms especially in high concentrations, its acute toxicity and sometimes lethal effect to fish, duckweed, algae, and other microorganisms is well reported in the literature (Cheung, Chu and Wong, 1993; Clément and Merlin, 1995; Francis-Floyd et al., 2009; Wijekoon et al., 2022).

3. pH: pH is one of the most important parameters influencing leachate toxicity. High pH values (≥ 8) induce ammonia toxicity by enhancing the formation of the more toxic form of ammonia (NH_3) as well as precipitation of some compounds with some toxic effects e.g., iron and zinc hydroxides. On the other hand, low pH values are likely to cause changes in the ionic forms of some compounds causing them to exist in more toxic forms, sulphides and cyanides are more toxic at low pH values (Cameron, 1980).

4. Heavy metals: the concentrations of heavy metals tend to decrease with landfill aging as the high pH values of old landfills decreases the solubility of heavy metals (Teng et al., 2021). However, heavy metals are known to have deleterious effects on both environment and food chain which result in acute toxicity, carcinogenicity, and genotoxicity in human beings (Wijekoon et al., 2022).

In this chapter, the general characteristics of raw and diluted landfill leachate is determined. The growth of different green microalgae at 20% landfill leachate

concentration is estimated (Results section 5.4.2.I.). COD, ammonia-nitrogen, pH and heavy metals are determined in order to screen different algal strains for the most powerful candidate in landfill leachate treatment with the highest growth at this particular concentration (20%) (Results section 5.4.2.II).

5.3. Materials and Methods

5.3.1. Chemical analysis of landfill leachate

Leachate samples to be tested were kept in airtight containers and the samples used in each experiment are withdrawn from the container after mixing it by shaking vigorously several times. Leachate samples for the screening experiment (2 Litres) are then collected and passed through 0.2 µm disposable sterile filters (Steritop, Millipore) driven by a vacuum pump N840FT.18 (KNF Laboport, Freiburg, Germany) and the flow through (filtrate) was then collected in autoclave-sterilised 1L Duran bottles. The bottles are then stored at 4^{o}C until experimental set-up and further analysis.

The filtered leachate is chemically analysed in duplicate for pH, COD, NH_3-N and heavy metals analysis as described in detail herein:

I. pH: pH of the landfill leachate samples is measured using a pH electrode; FiveEasyTMFE20 (Mettler-Toledo AG, Schwerzenbach, Switzerland).
II. COD: The chemical oxygen demand of the leachate samples is measured using COD cuvette test kit LCK014 for the raw landfill leachate and LCK514 for the diluted landfill leachate samples (HACH Lange GMBH, Düsseldorf, Germany) following the instructions written in the manual provided by the company. The readings are done using HACH DR2800 Laboratory Spectrophotometer (HACH Lange, Germany).
III. NH_3-N: NH_3-N content in landfill leachate samples is measured using the modified Nessler method (Jeong, Park and Kim, 2013; Okurowska et al., 2021).Steps for ammonia determination in the sample are as follows:

- A Poly Vinyl Alcohol solution (PVA) with concentration 0.135% is prepared by dissolving 0.135 g of PVA (Acros Organics, Spain) in 100 ml DI water.

- 10 Ammonia standards are prepared using 1000 mg/l Ammonium standard solution (Merck KGaA, Darmstadt, Germany).
- Each ammonia standard is measured for absorbance in triplicate at wavelength 425 nm (after treatment) using a Spectrophotometer (Jenway 6715 UV/Vis., UK) and a standard curve of concentration vs. optical density was created (Appendix 6).
- Landfill leachate samples as well as ammonia standards to be measured for ammonia content are prepared as follows:
    - Samples (1 ml) are filtered using 0.2 µm syringe filter as well as DI water to be used as a Blank.
    - 20 µl of Mineral Stabiliser (HACH, Loveland, USA) is added to each sample after filtration and mixed by shaking.
    - 20 µl of PVA solution (prepared earlier) is added to each sample and mixed well.
    - 40 µl of Nessler Reagent (HACH, Loveland, USA) is added and mixed well, a yellow colour is developed in samples with ammonia content and the intensity of the colour increases as the concentration of the ammonia in the sample increases.
    - Absorbance is then measured for samples at 425 nm using a Spectrophotometer (Jenway 6715 UV/Vis., UK).

N.B. Raw leachate samples are diluted 100x before treatment for ammonia measurements, only, due to the sensitive range of the ammonia analyser.

IV. Heavy metals analysis: Filtered samples are acidified to 2% (v/v) HNO_3 and analysed using ICP emission spectrometer ICAP 6000 series (Thermo Scientific). Calibration curves of at least 5 points are obtained both with a multi-element standard (Inorganic Ventures, traceable standard). Measurement's uncertainty is determined as the percentage (%) of relative standard deviation (%RSD) of each measurement.

5.3.2. Experimental set-up

The experiment is set up in duplicate 1 L autoclave-sterilised Duran bottles. Two bottles are used for each of the four algae, and two for all the four algae together as a group to test the synergistic effect of all of them together, and two are used as control i.e., no algae are inoculated. Each bottle is filled with 500 ml diluted leachate (20% v/v) with sterile distilled water, supplemented with 5 ml of an inorganic phosphate source (5.2 g/L $K_2HPO_4.3H_2O$) which is equivalent to the same amount added for preparation of 500 ml BG11.

Each Duran bottle is inoculated with 20% (v/v) algal inoculum (100 ml) of 29 days old algal culture. Regarding the group culture, which contains all the tested algal strains together, it was set up as 5% (v/v) for each alga so the total algal inocula is 20% (v/v). All inocula are measured at 595 nm using a spectrophotometer (Jenway 6715 UV/Vis., UK) and their OD measurements are adjusted to 0.36-0.39.

Twelve autoclave-sterilised magnets are placed into the twelve tested Duran bottles which are then each placed onto a magnetic stirrer plate to be continuously stirred and mixed during the duration of the experiment (30 days) at medium speed.

The Experiment is set up at room temperature and the temperature is monitored continuously (22^{o}C ±2), the Duran bottles were subjected to continuous light from fluorescent light tubes at surface intensity of 1272.5 ± 19 Lux, measured by a Luxmeter (Fisher Scientific) throughout the duration of the experiment.

Every five days, samples are collected from the experimental Duran bottles to determine algal growth as described below and then filtered by 0.2 µm syringe filters, where the filtrate is used for the chemical analysis of the leachate.

I. Algal growth measurements:

- Algal growth is measured by optical density (OD) determination every five days, using a Spectrophotometer (Jenway 6715 UV/Vis., UK) at 595 nm. The blank used during measurements was diluted 20% v/v landfill leachate filtered through 0.2 µm syringe filter.

- At the end of the experiment (after 30 days), the dry biomass for the algal cultures was determined. 200 ml of each Duran bottle was collected (after being well mixed) and centrifuged at 3900 rpm for 10 minutes using a centrifuge (Eppendorf 5810R) after which the pellet was left to dry on a pre-weighed Petri dish overnight at 30-55^{o}C.

II. Chemical analysis of leachate:

- Aliquots of leachate collected every five days are passed through 0.2 µm syringe filters after algal growth determination to be analysed for pH and COD as mentioned previously.
- Filtered samples are stored at -20^{o}C until the end of experiment after which they are analysed for ammonia content as described previously in detail. Samples from days 0, 5, 10, 15, 20 and 25 are diluted 50x prior to analysis whilst samples from day 30 were diluted 25x prior to the ammonia analysis by the Nessler method described above.
- Heavy metals content is determined at the beginning and the end of the experiment (Day 0 and Day 30) as described previously for the filtered samples of the leachate.

5.3.3. Statistical analysis

Statistical analysis is carried out using R v.4.0.5 and R studio v.1.2.5. Data are presented as mean and standard deviation. Significance testing for the different measurements is carried out using paired t-test when comparing day 0 and day 30 for each treatment, while ANOVA and post Hoc tests are used when comparing the different treatments. A p value <0.05 was considered statistically significant.

5.4. Results

5.4.1. Chemical analysis of landfill leachate

Landfill leachate is a complex and toxic type of wastewater, which is characterised by a dark-brown colour. Raw and 20% diluted landfill leachate samples used in the

following experiment were chemically analysed for their different parameters as shown in Table 5-1.

Table 5-1 Chemical properties of raw and 20% diluted landfill leachate used in this study.

Parameter	Raw LL	Diluted LL (20%)
pH	8.175±0.015	8.295±0.015
COD	2272 ± 8 mg/L	694.5 ± 4.5 mg/L
NH_3-N	1474.7 ± 64.17 mg/L	435.9 ± 15.6 mg/L
Arsenic (As)	0.0725 mg/L	0.0235 ± 0.003 mg/L
Copper (Cu)	0.0722 mg/L	0.0212 ± 0.001 mg/L
Chromium (Cr)	0.5195 mg/L	0.1429 mg/L
Lead (Pb)	0.061 mg/L	0.0172 mg/L

5.4.2. Experimental set-up

Four green microalgae (*Chlorella vulgaris* ATFG1, *Chlorella vulgaris* ATFG2, *Chlorococcum* sp. and *Scotiellopsis reticulata*) are all tested individually and collectively for growth and pollutants removal in 20% diluted LL (v/v) for 30 days in duplicate 1L Duran bottle as well as a control with no algae inoculated. Figure 5-1 shows the start of the experiment (Day 0) and the end of it (Day 30).

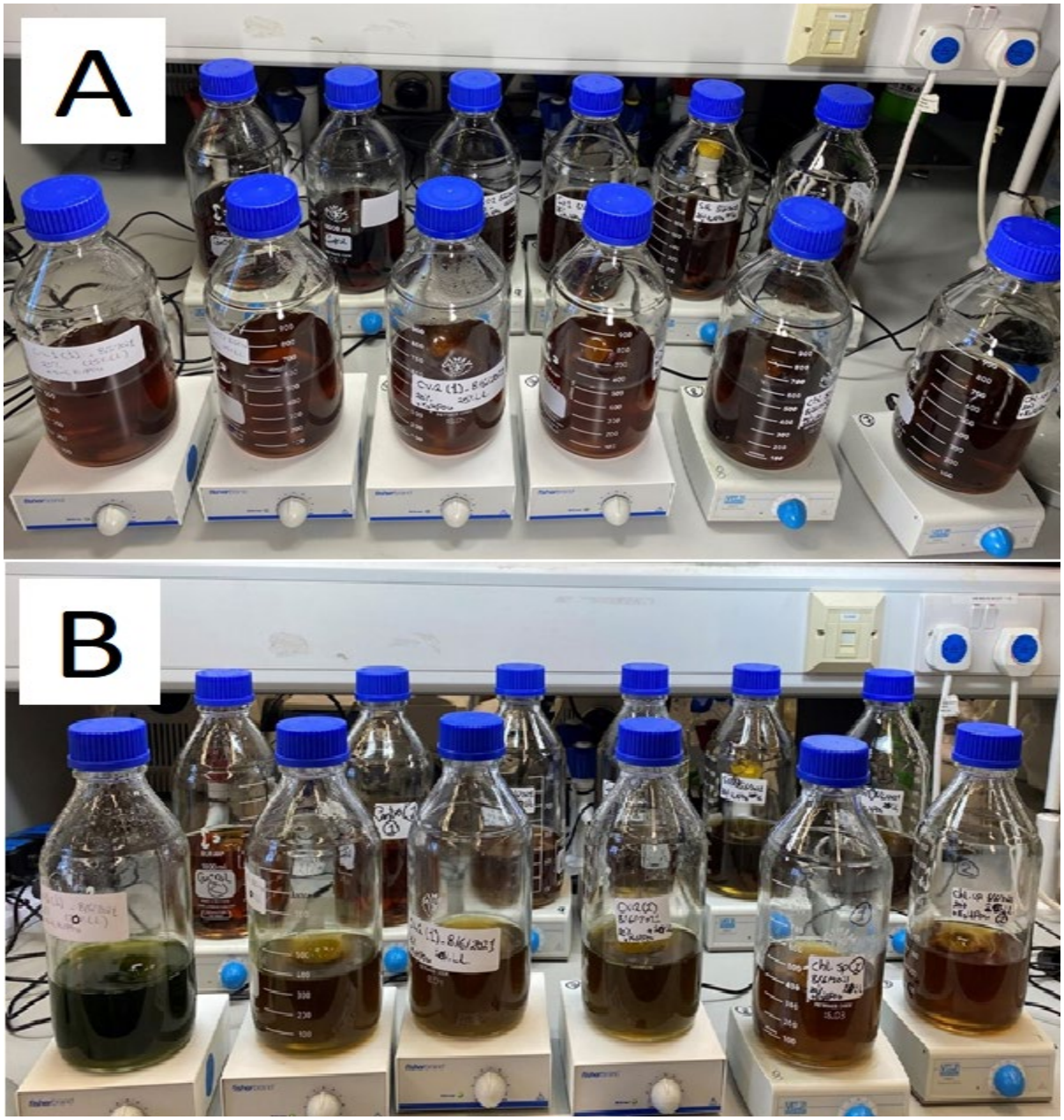

Figure 5-1 A picture showing the experimental set up (A) at the start of the experiment at Day 0; (B) at the end of the experiment at day 30.

Surprisingly, one of the replicates of *Chlorella vulgaris* ATFG1 (C.v.1.1) started growing profusely compared to the other replicate (C.v.1.2) with a much denser green colour as shown in Figure 5-2.

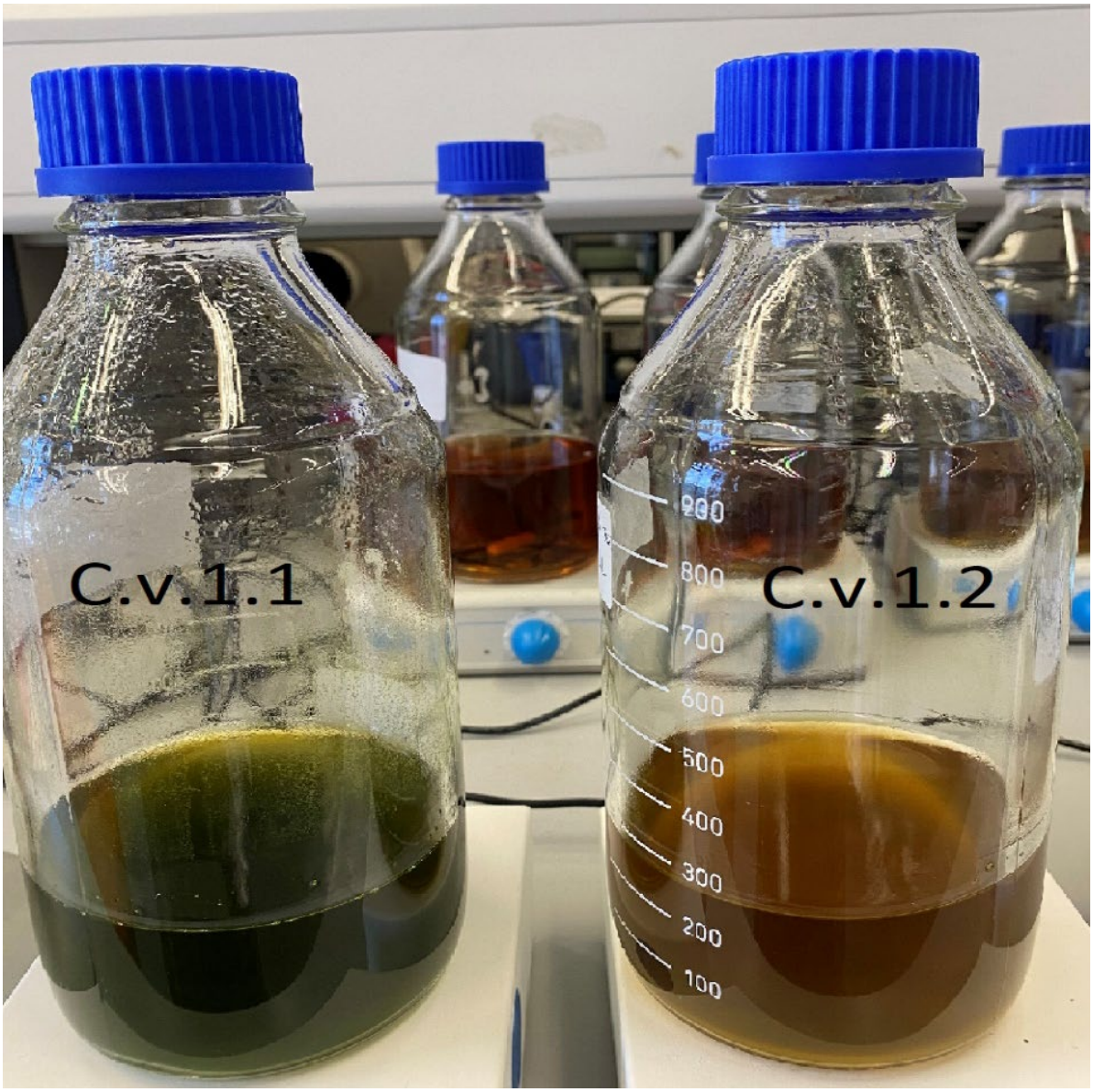

Figure 5-2 A picture showing both replicates of the alga *Chlorella vulgaris* ATFG1 (C.v.1.1 and C.v.1.2) at the end of the experiment (Day 30) with a much denser green colour can be obvious for the replicate C.v.1.1 compared to C.v.1.2.

Microscopic examination is undertaken at Day 23 and Day 24 of the screening experiment to check the purity of the two replicate cultures of *Chlorella vulgaris* ATFG1 (C.v.1.1. and C.v.1.2), after a change in colouration of the culture C.v.1.1 as well as growth profile and nutrients removal uptake were evident (Figure 5-3). Microscopic examination is conducted using multiple samples of both cultures. It is concluded that cells of the two cultures (C.v.1.1 and C.v.1.2) did not exhibit morphological difference under light microscopic examination with no evidence of contamination by other

microorganisms (Magnification 40X), however, DNA sequencing for both cultures will be the key step to confirm whether a genotype change has happened, which will be explored in the following chapter.

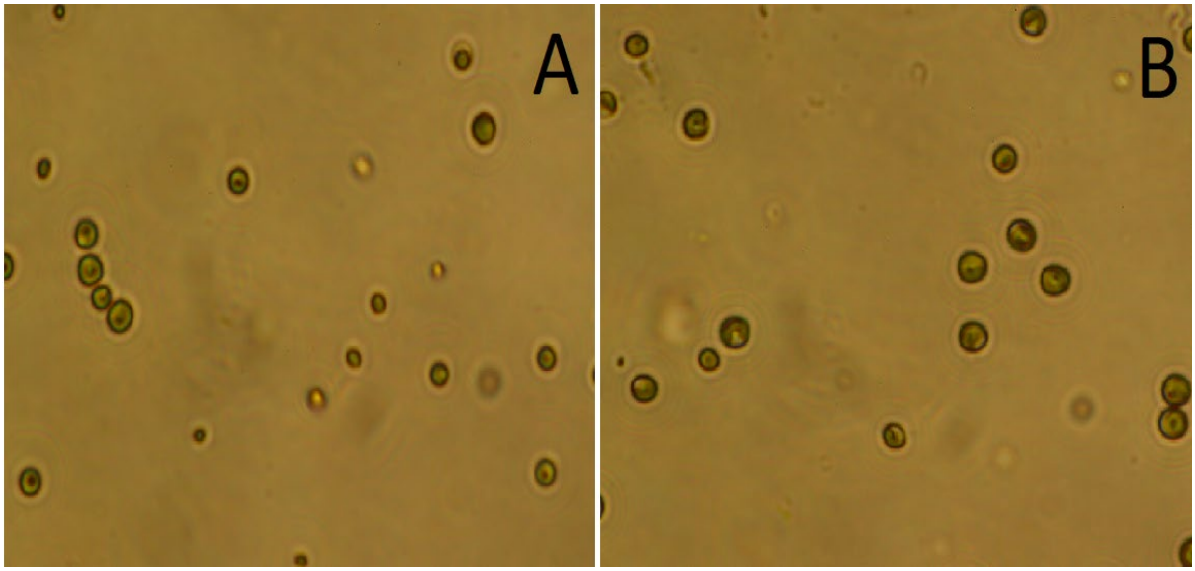

Figure 5-3 A picture showing light microscopic examination for both replicates of the green microalga *Chlorella vulgaris* ATFG1 at Day 23/24 of the screening experiment. A) C.v.1.1; B) C.v.1.2. Magnification: 40X.

I. Algal Growth measurements

- OD measurements every five days

Growth of different algal strains individually and collectively is measured every five days by spectrophotometry as optical density (OD) at 595 nm to determine the strain with the highest growth at the tested LL concentration (Figure 5-3). The growth of strains C.v.1.1 and C.v.1.2 increased significantly from day 0 to day 30 (p<0.01). However, the highest growth is observed for C.v.1.1, as it is significantly higher than C.v.1.2, C.v.2, *Chlorococcum* sp., *Scotiellopsis reticulata* and all four algae grown together (Gp) ($p < 0.01$) as shown in Figure 5-4.

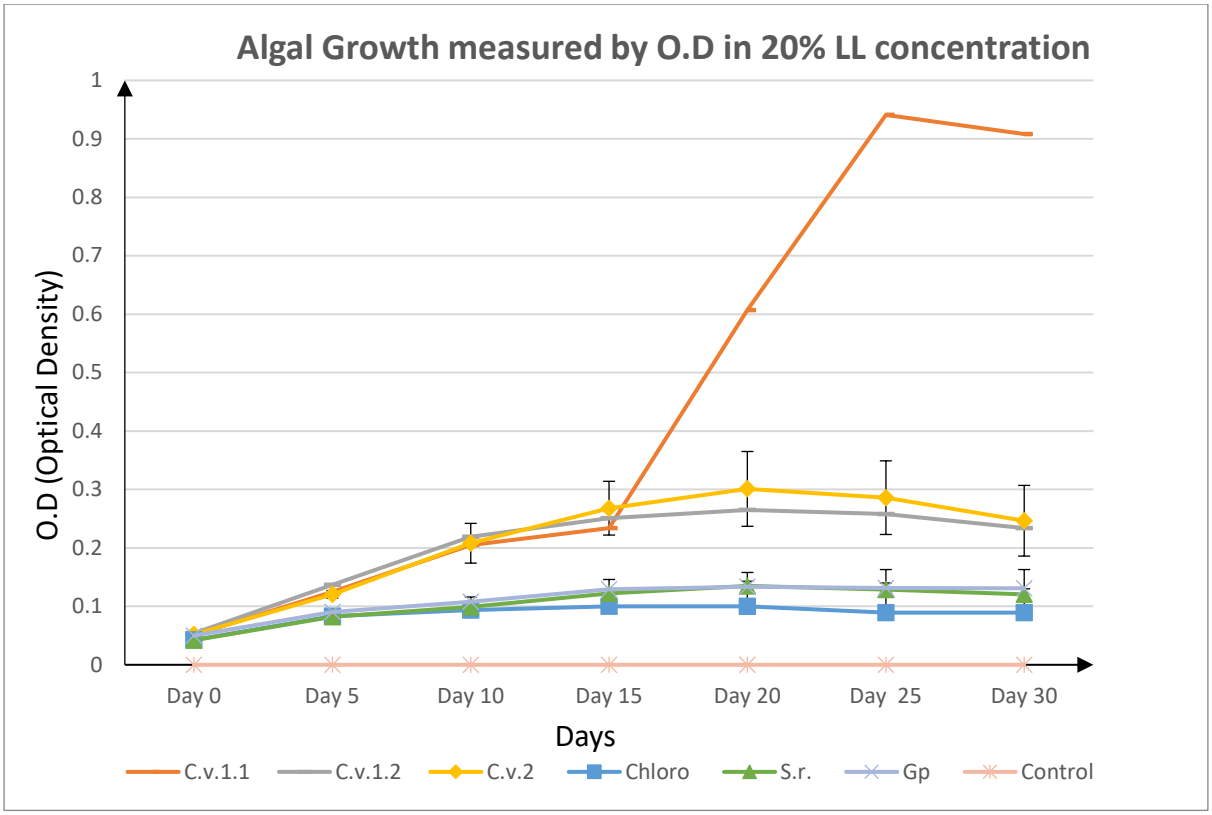

Figure 5-4 Comparing growth of different algal strains tested individually and collectively in 20% LL concentration with C.v.1.1 showing the highest growth. C.v.1.1 (*Chlorella vulgaris* 1.1), C.v.1.2 (*Chlorella vulgaris* 1.2), C.v.2 (*Chlorella vulgaris* 2), Chloro (*Chlorococcum* sp.), S.r. (*Scotiellopsis reticulata*), Gp (group of all algal strains together), Control (control with no algae). Mean and standard deviation values of two biological replicates are plotted.

- Algal dry biomass determined at day 30

Figure 5-5 shows the different dry biomasses, measured as g/L for each of the tested strains individually and collectively. The highest dry biomass is observed for C.v.1.1 with a significant difference compared to the other strains (p<0.01). The dry biomass achieved at the end of the experiment for C.v.1.1 was 0.284 g/L as shown in Figure 5-5.

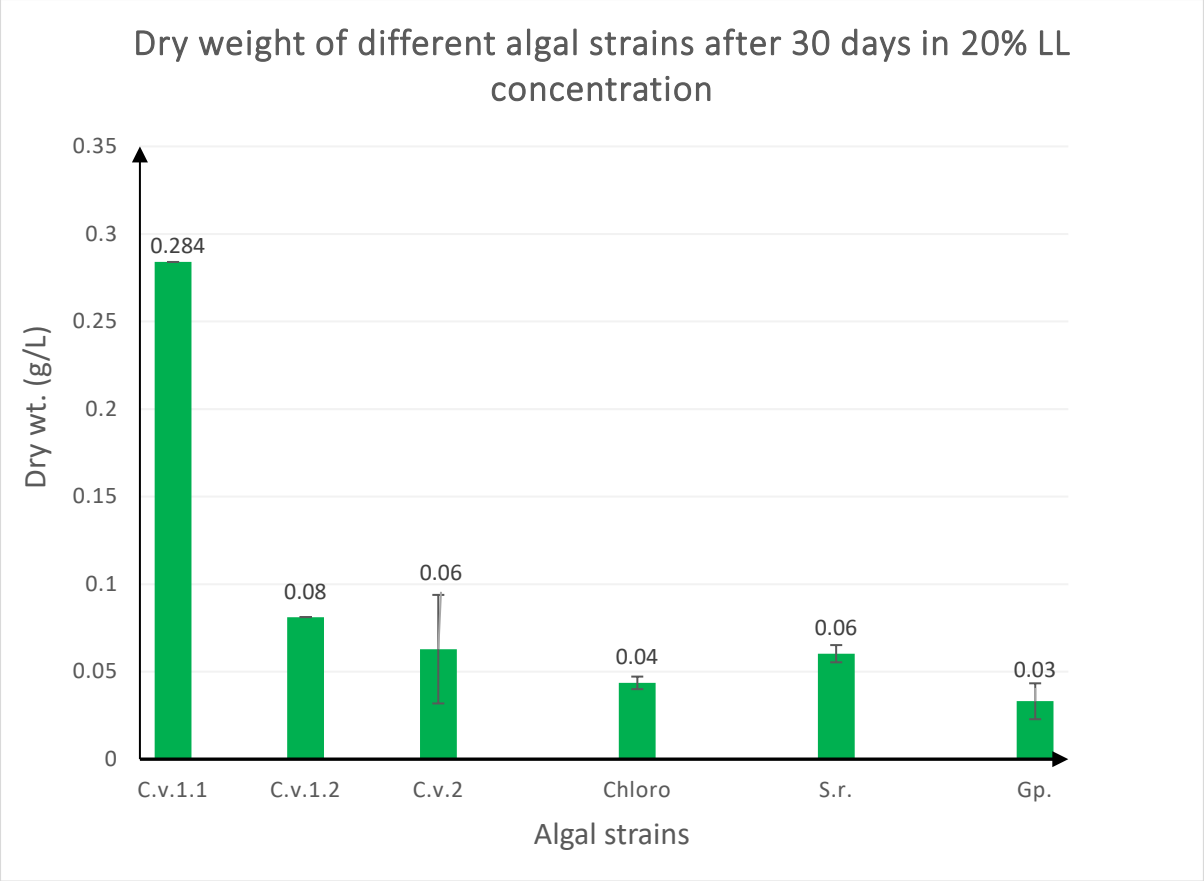

Figure 5-5 Dry biomass determined at the end of the screening experiment (Day 30), showing the highest biomass produced by C.v.1.1. C.v.1.1 (*Chlorella vulgaris* 1.1), C.v.1.2 (*Chlorella vulgaris* 1.2), C.v.2 (*Chlorella vulgaris* 2), Chloro (*Chlorococcum* sp.), S.r. (*Scotiellopsis reticulata*), Gp (group of all algal strains together). Mean and standard deviation values of two biological replicates are plotted.

II. Chemical analysis of LL during the screening experiment

- pH determination every five days

pH dynamics throughout the period of the experiment was illustrated in Figure 5-6. A significant increase in the pH values at the end of the experiment is observed for strains C.v1.2, C.v.2, *Scotiellopsis reticulata* and the Gp. (the tested algal strains altogether), whilst a significant decrease in the value of the pH was observed for the highest growing strain C.v.1.1 at the end of the experiment (pH value 4.8) (p<0.05). The change in the pH values for both the *Chlorococcum* sp. and the control treatment is not significant (p<0.05).

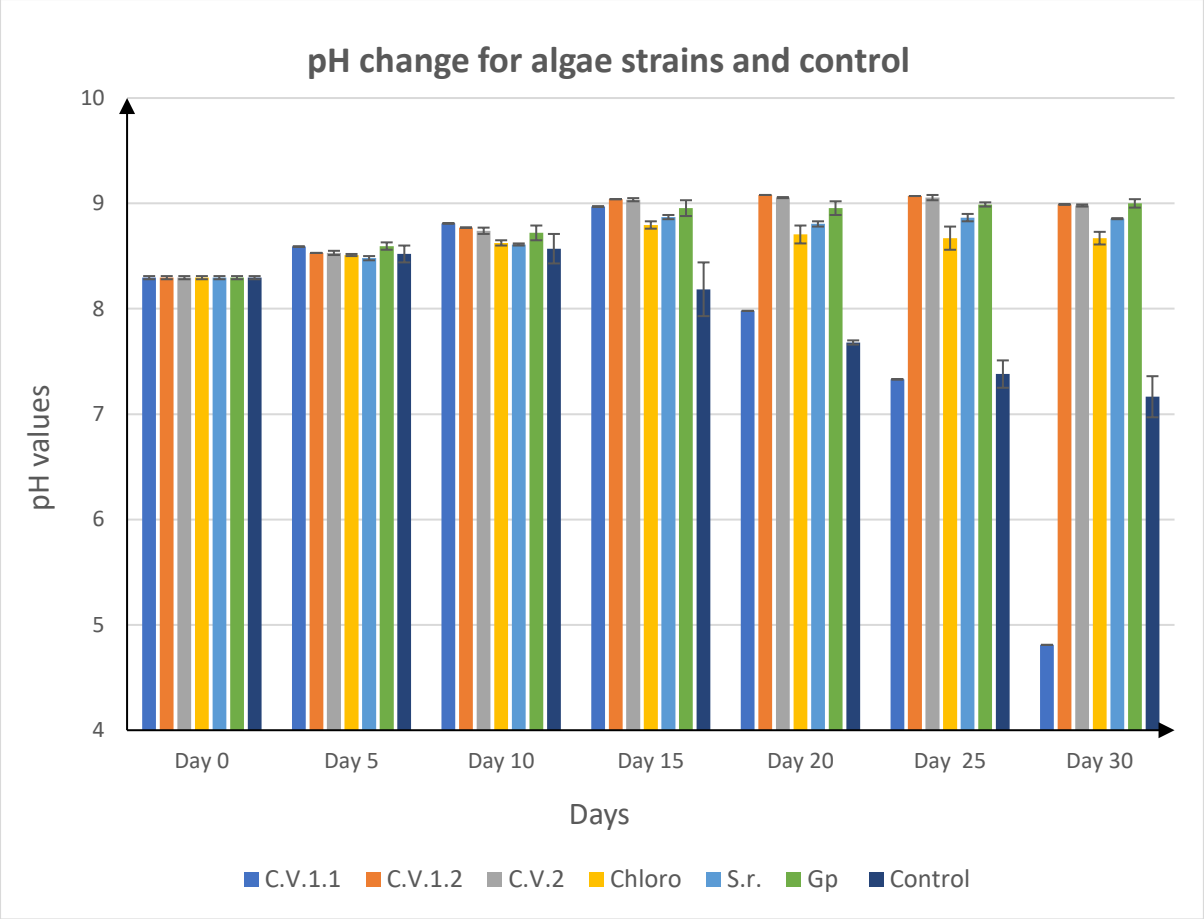

Figure 5-6 pH changes for different algal strains tested individually and collectively during the time interval of the screening experiment (30 days) in 20% LL concentration. C.v.1.1 (*Chlorella vulgaris* 1.1), C.v.1.2 (*Chlorella vulgaris* 1.2), C.v.2 (*Chlorella vulgaris* 2), Chloro (*Chlorococcum* sp.), S.r. (*Scotiellopsis reticulata*), Gp (group of all algal strains together), Control (control with no algae). Mean and standard deviation values of two biological replicates are plotted.

- COD determination every five days

The change in the chemical oxygen demand (COD) throughout the time interval of the experiment is monitored and plotted in Figure 5-7. It was observed that, the COD values decreased in case of all the treatments at Day 5 after which it followed different patterns for the different treatments. The COD value increased significantly in case of C.v.1.1 from day 0 to day 30 (p<0.01) and is significantly higher (p<0.01) when compared to the rest of the treatments at day 30. On the other hand, the COD values

decreased significantly (p<0.05) for C.v.1.2, C.v.2, *Scotiellopsis reticulata* (S.r.) and the All the tested algae together (Gp.) treatments from day 0 to day 30. Although, the control treatment increased throughout the period of the experiment, but its increase is not significant (p>0.05).

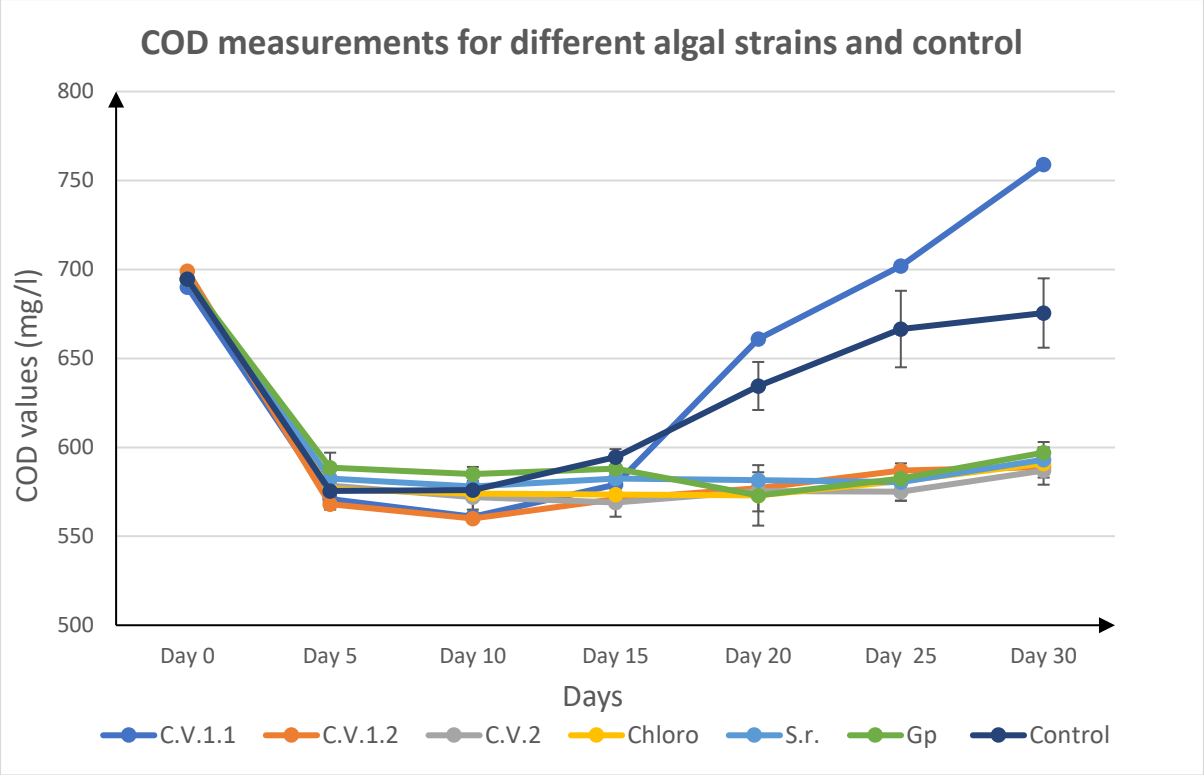

Figure 5-7 COD change during the screening experiment for the different algal treatments and the control in the 20% diluted LL. C.v.1.1 (*Chlorella vulgaris* 1.1), C.v.1.2 (*Chlorella vulgaris* 1.2), C.v.2 (*Chlorella vulgaris* 2), Chloro (*Chlorococcum* sp.), S.r. (*Scotiellopsis reticulata*), Gp (group of all algal strains together), Control (control with no algae). Mean and standard deviation values of two biological replicates are plotted.

- NH_3-N removal throughout the time interval of the experiment

The removal of NH_3-N is recorded during the screening experiment as one of the most important pollutants in the LL (Figure 5-8). The concentration of ammonia-N decreases significantly from Day 0 to Day 30 in case of all the treatments except for C.v.2 and the *Chlorococcum* sp. (p<0.01). However, the highest reduction in the

ammonia-N concentration is recorded for C.v.1.1, where concentration decreases from 435.9 mg/L (Day 0) to 57.9 mg/L (Day 30) as shown in Figure 5-8.

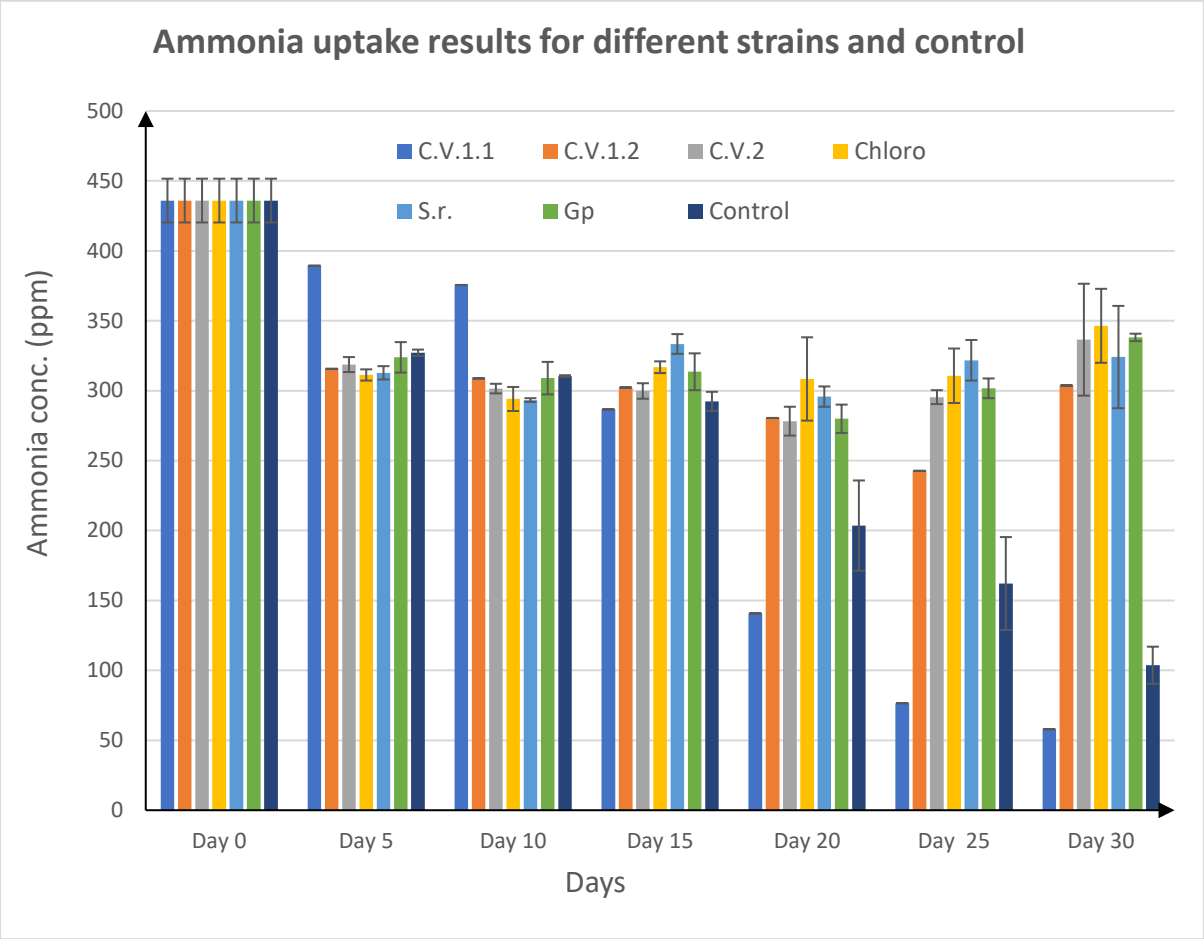

Figure 5-8 Ammonia-N removal throughout the screening experiment by the different algal treatments and the control in 20% diluted LL. C.v.1.1 (*Chlorella vulgaris* 1.1), C.v.1.2 (*Chlorella vulgaris* 1.2), C.v.2 (*Chlorella vulgaris* 2), Chloro (*Chlorococcum* sp.), S.r. (*Scotiellopsis reticulata*), Gp (group of all algal strains together), Control (control with no algae). Mean and standard deviation values of two biological replicates are plotted.

- Ammonia-N removal expressed as %

Removal of NH_3-N after 30 days of the experiment is recorded as removal percentage, compared to Day 0, between all the tested algae strains individually and altogether as well as the control (Figure 5-9). It is worth mentioning that the removal percentage of NH_3-N at the end of the experiment is significantly higher (p<0.01) in case of C.v.1.1 as well as the control. The removal efficiency of the other tested treatments is not significant. The amount of NH_3-N decreases by an average of 76.2% in the control

treatment, however the highest decrease in NH_3-N is recorded for C.v.1.1 by 86.7% , as shown in Figure 5-9.

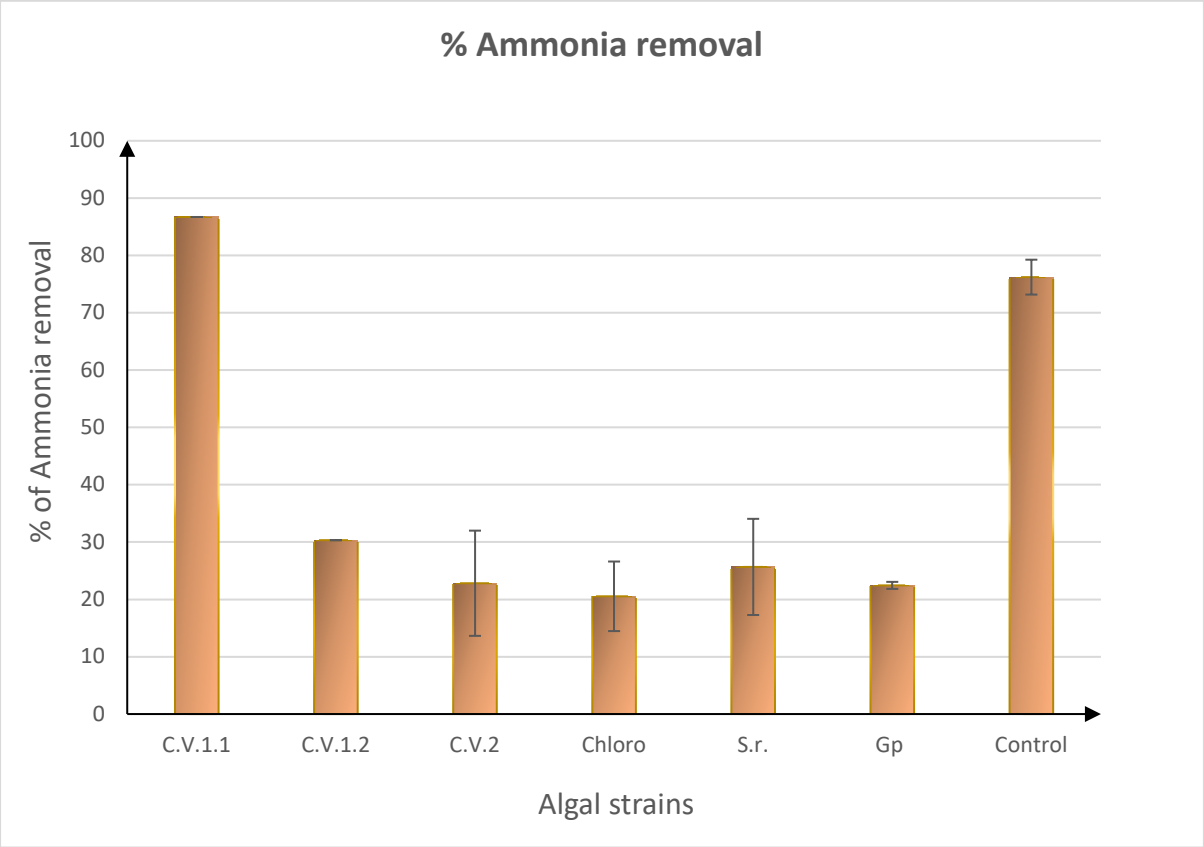

Figure 5-9 Removal of NH_3-N expressed as percentage of removal amongst the different tested algal strains individually and collectively in 20% LL concentration at the end of the screening experiment (Day 30). C.v.1.1 (*Chlorella vulgaris* 1.1), C.v.1.2 (*Chlorella vulgaris* 1.2), C.v.2 (*Chlorella vulgaris* 2), Chloro (*Chlorococcum* sp.), S.r. (*Scotiellopsis reticulata*), Gp (group of all algal strains together), Control (control with no algae). Mean and standard deviation values of two biological replicates are plotted.

- Heavy metals analysis

Prior to acid digestion, filtered LL samples are quantitatively analysed for four heavy metals (*As*, *Cu*, *Cr* and *Pb*) using the ICP-ES as described earlier. The percentages of removal of the tested heavy metals at the end of the experiment (Day 30) compared to Day 0 are represented in the following Figures: Figure **5-10**, Figure **5-11**, Figure **5-12**, Figure **5-13**. Figure **5-10**. shows the removal percentage of Arsenic (*As*) amongst

different algal treatments in the screening experiment. Both C.v.1.1 and C.v.1.2 decrease the amount of Arsenic in the tested diluted 20% LL samples by 31.2% and 32.9%, respectively, which is significantly higher than the decrease observed in the control (p<0.01).

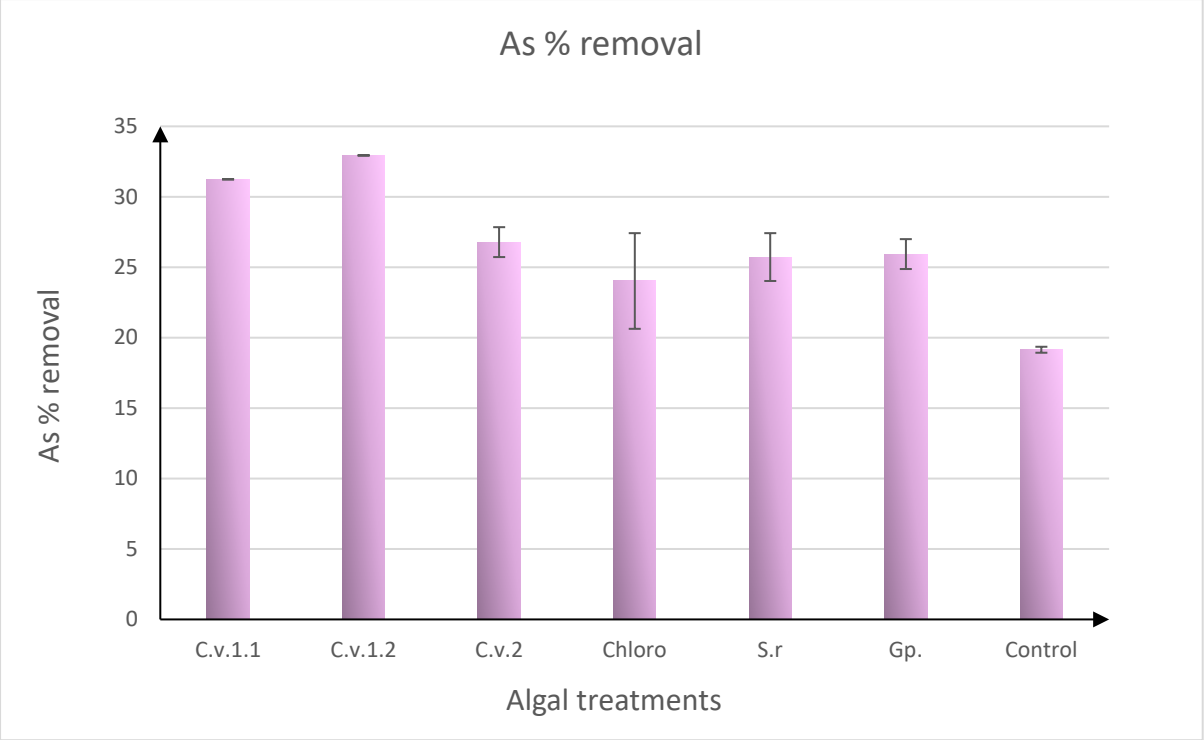

Figure 5-10 Arsenic uptake expressed as removal percentage between different algal treatments and the control in 20% diluted LL. C.v.1.1 (*Chlorella vulgaris* 1.1), C.v.1.2 (*Chlorella vulgaris* 1.2), C.v.2 (*Chlorella vulgaris* 2), Chloro (*Chlorococcum* sp.), S.r. (*Scotiellopsis reticulata*), Gp (group of all algal strains together), Control (control with no algae). Mean and standard deviation values of two biological replicates are plotted.

The decrease in the Chromium (*Cr*) concentration between the tested algal treatments and the control measured at the end of the experiment (Figure 5-11) does not show significant removal for Chromium in any of the tested treatments (p<0.05).

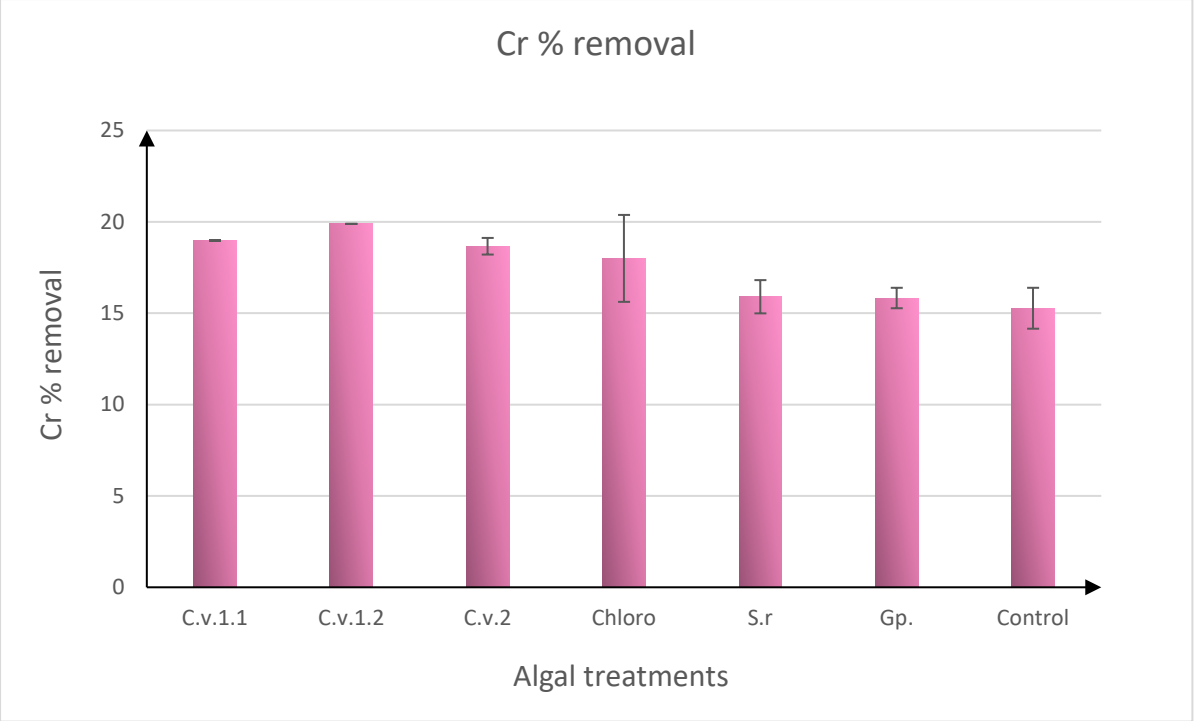

Figure 5-11 Chromium uptake expressed as removal percentage between different algal treatments and the control in 20% diluted LL. C.v.1.1 (*Chlorella vulgaris* 1.1), C.v.1.2 (*Chlorella vulgaris* 1.2), C.v.2 (*Chlorella vulgaris* 2), Chloro (*Chlorococcum* sp.), S.r. (*Scotiellopsis reticulata*), Gp (group of all algal strains together), Control (control with no algae). Mean and standard deviation values of two biological replicates are plotted.

Reduction in Copper (*Cu*) concentration in the tested algal treatments and the control is expressed as removal percentage in Figure 5-12. Although, the reduction of Copper concentration observed in case of C.v..1.1 was significantly higher than the other algal treatments, nevertheless the removal percentage of the control is concurrently significantly higher than the other tested algal treatments as well ($p \leq 0.05$).

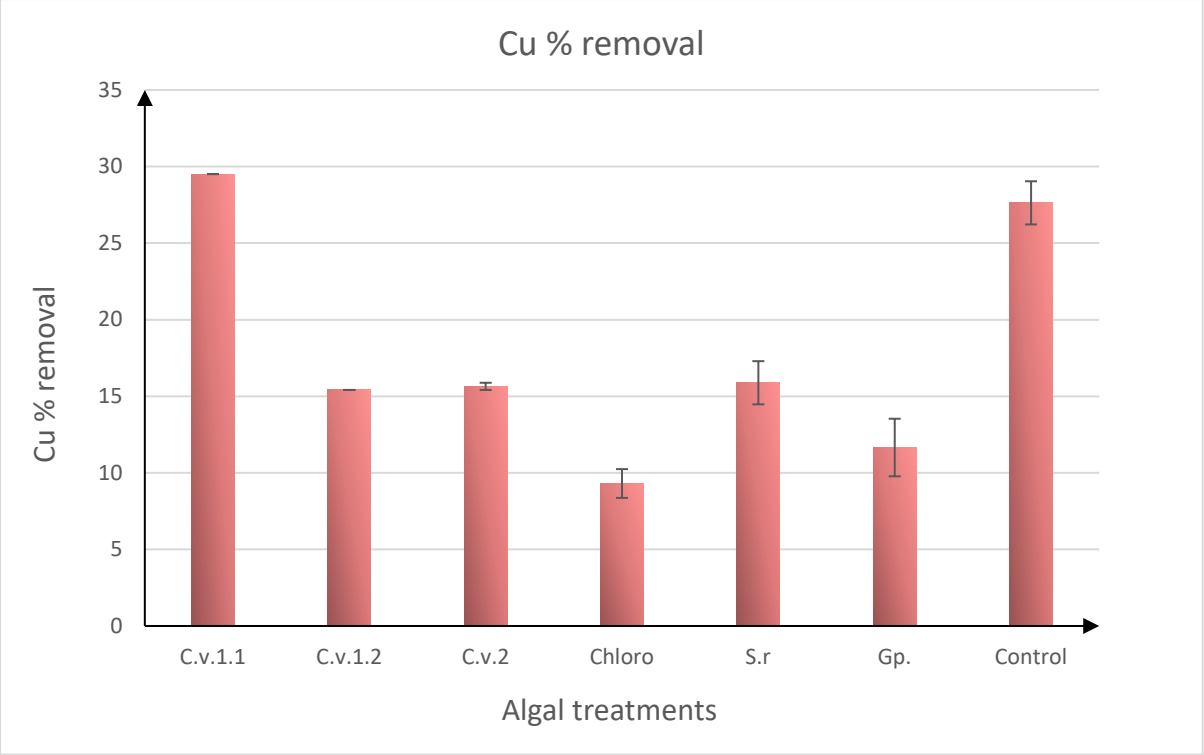

Figure 5-12 Copper uptake expressed as removal percentage between different algal treatments and the control in 20% diluted LL. C.v.1.1 (*Chlorella vulgaris* 1.1), C.v.1.2 (*Chlorella vulgaris* 1.2), C.v.2 (*Chlorella vulgaris* 2), Chloro (*Chlorococcum* sp.), S.r. (*Scotiellopsis reticulata*), Gp (group of all algal strains together), Control (control with no algae). Mean and standard deviation values of two biological replicates are plotted.

Figure 5-13 illustrates the removal percentage of Lead (*Pb*) at the end of the screening experiment for the different tested algal treatments as well as the control. There is no statistically significant difference between the Lead removal percentage amongst the different algal treatments and the control.

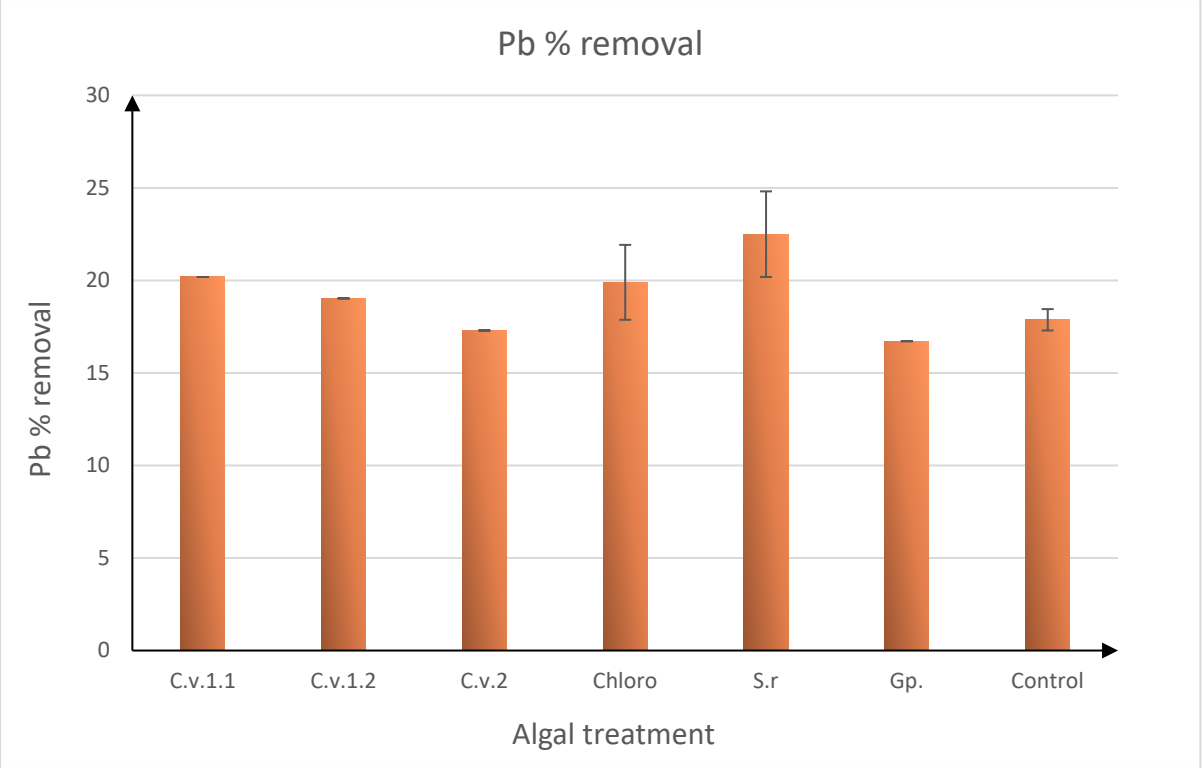

Figure 5-13 Lead uptake expressed as removal percentage between different algal treatments and the control in 20% diluted LL. C.v.1.1 (*Chlorella vulgaris* 1.1), C.v.1.2 (*Chlorella vulgaris* 1.2), C.v.2 (*Chlorella vulgaris* 2), Chloro (*Chlorococcum* sp.), S.r. (*Scotiellopsis reticulata*), Gp (group of all algal strains together), Control (control with no algae). Mean and standard deviation values of two biological replicates are plotted.

5.5. Discussion

Landfill leachate characteristics may vary according to several factors e.g., age of landfill, weather conditions, precipitations as well as the composition and types of wastes. Landfill age, in particular, is a key factor influencing the landfill leachate composition (Li, Zhou and Hua, 2010). The chemical analysis for the landfill leachate used in this study might indicate it is relatively old (stabilised) landfill leachate. It is characterised by slightly high pH value (> 8) with COD values 2272 ± 8 mg/L and relatively high ammonia content (1474.7 ± 64.17 mg/L) in addition to relatively low heavy metals content. With exception to the Copper and Chromium contents, the analysed characteristics of the LL used in this study are more or less similar to the one used by Hernández-García *et al.* (2019). On the contrary, the COD and the total nitrogen values in a study conducted by Viegas *et al.* (2021) are 9600 (± 571) mg O_2 L^{-1}

and 3295 (± 438) mg L^{-1}, respectively, which are relatively higher than the values recorded in this study. However, the Chromium content recorded in the study carried out by Viegas *et al.* (2021) is (0.2 ± 1 mg L^{-1}) less than the one recorded in this study (0.5195 mg/L). This is consistent with LL compositional variations, according to different factors, as mentioned previously.

One replicate (C.v.1.1) shows sudden onset of outstanding growth, compared to its other replicate (C.v.1.2) as well as the rest of the treatments with an observed darker green colour. Purity of both replicates is checked by microscopic examination which did not reveal any observed contamination nor change in the morphological characters between both replicates which made a DNA sequencing step a necessity to be able to explain the change in growth pattern and pollutants uptake exhibited by C.v.1.1 which is further discussed below.

Supplementation with five ml of an inorganic phosphate source (5.2 g/L $K_2HP0_4.3H_2O$) is carried out, which is equivalent to the same amount added for preparation of 500 ml BG11. Similar step was also undertaken by Okurowska *et al.* (2021). In in their study, Okurowska *et al.* (2021) used LL from the same source as the one used in this study (Erin landfill site, Chesterfield, UK) as they reported adding phosphate due to low levels of phosphorus source in the tested LL. Paskuliakova, Tonry and Touzet (2016) also highlighted the importance of phosphate supplementation to tested LL, they found that a higher biomass production together with a 90.7% reduction in ammonia levels was obtained when phosphate was supplemented to a culture of *Chlamydomonas* sp. SW15aRL, tested for growth and pollutants removal in 10% diluted raw LL.

A screening experiment is conducted to determine the highest growth and nutrient removal efficiency amongst four green microalgal strains (individually and collectively) previously isolated from a LL treatment site in Chesterfield, UK, in the challenging concentration of 20% LL. The growth profiles of all the different treatments indicate that both C.v.1.1 and C.v.1.2 showed significant increase in growth (based on optical density) from Day 0 to Day 30 (p<0.01). However, the strain C.v.1.1 manifests the highest growth when compared to the other treatments with a

significant increase over the rest of the treatments (p<0.01). The growth of C.v.1.1 is 4.5-fold higher than the other tested *Chlorella vulgaris* strain C.v.1.2. This was also further shown, in the final dry biomass measured for all the treatments where C.v.1.1 is significantly higher than the other tested treatments (p<0.01) with a biomass production of 0.28 g/L after 30 days in 20% LL.

The ability of Chlorophytes especially *Chlorella* species to thrive and grow in diluted 10% raw LL was reported in different studies (Lin *et al.*, 2007; Zhao *et al.*, 2014; Paskuliakova, Tonry and Touzet, 2016; El Ouaer *et al.*, 2020; Okurowska *et al.*, 2021). Nevertheless, as the concentration of raw, untreated LL increases, the viability of microalgal growth in this media becomes more challenging, as the concentration of toxic substances e.g., ammonia becomes higher as well. However, there are some successful attempts for growing *Chlorella vulgaris* in raw diluted untreated LL with concentration ≥20% (v/v). Okurowska *et al.* (2021) reported a 3-fold increase in the growth rate (based on cell count) from 0.07 d^{-1} to 0.2 d^{-1} when an algal-bacterial consortium obtained from a landfill leachate treatment site (with the *Chlorella vulgaris* being the dominant algal strain) is subjected to 24 months of adaptive laboratory evolution. Although, we have not attempted laboratory adaptation in this study, nonetheless our results could be comparable to those obtained by Cheung, Chu and Wong (1993) where the *Chlorella vulgaris* strain they used is able to grow successfully, with a significant increase, in 20% and 25% diluted LL. Although, the *Chlorella vulgaris* strain used in Cheung, Chu and Wong (1993) study is obtained from the American Type Culture Collection, another two strains, *Scenedesmus* sp. and *Chlorella pyrenoidosa* isolated from a drain in an arable local land in Hong Kong and a wet surface with leachate runoff in Junk Bay landfill in Hong Kong, respectively, are able to grow significantly and/or tolerate LL concentrations 5-50%. Cheung, Chu and Wong (1993) attributed these results to two main reasons concurrently: (i) the inhibitory effect of the LL used in the study (two types of LL were used and one was concluded to be less toxic than the other); (ii) the tolerance and/or resistance of the algal species used. It was concluded that the algal isolates from the field are more resistant than the one from the culture collection. This agrees with our results, as all our strains are indigenous microalgae that were isolated from a local LL treatment site. The potential

tolerance and/or resistance of naturally occurring microalgae in a LL site might be higher than those of microalgal isolates obtained from a culture collection. Surprisingly, C.v.1.1 growth in 20% LL outperforms other strains tested (individually and altogether), with the microscopic examination eliminating the possibility of contamination and with the evident darker greener colour of the C.v.1.1 culture compared to the other treatments. This might signal for a developed tolerance or resistance towards the 20% LL concentration. A hypothesis is developed to explain this phenomenon, predicting the possibility of a developed mutation. To test this hypothesis, further experiments as well as genome sequencing are carried out.

pH is one of the most important factors to be considered when dealing with LL, it could highly influence growth of microalgae in LL (Edmundson and Wilkie, 2013) as well as different nutrients removal from wastewaters in general (Zheng *et al.*, 2019). Since there is a close relationship between ammonia uptake and the change in the pH dynamics (Shi, Zhang and Chen, 2000), so both pH results and ammonia-N results will be discussed simultaneously for a better understanding for both in the current study. In this experiment, the pH decreases significantly by day 30 in case of C.v.1.1 which also recorded the highest significant growth amongst all the treatments. These results come in line with the ammonia removal percentage results whereas the highest ammonia-N removal was carried out by C.v.1.1 as the ammonia-N decreases significantly by 86.7% which is significantly higher than the rest of algal treatments. Our results are similar to those reported by Paskuliakova, Tonry and Touzet (2016), in which the strain, *Chlamydomonas* sp.SW15aRL was the only actively growing strain in 10% raw LL and was also the most efficient in ammonia-N removal, with a significant removal for the total ammonia-N by 91% after 24 days. It is worth mentioning that a significant reduction in the ammonia-N content by 86.7% was consistent with a dramatic drop in the pH of the C.v.1.1 culture (from 8.3 to 4.8). These results might be explained by Shi, Zhang and Chen (2000), who pointed out the possibility of severe drop in the pH accompanying the utilisation of ammonia as the main nitrogen source by actively growing algae where the rapid utilisation of the ammonium ions eventually causes a dramatic pH drop. On the other hand, the control treatment also exhibited a significant ammonia-N removal by 76.2% which was also

concurrent with a decrease in the pH from 8.3 to 7.2. Although, the control did not show any algal growth, nevertheless the ammonia removal could be attributed to the stripping effect resulting from the aeration (caused by magnetic stirring in our study) which was reported to possibly account for ammonia loss in other studies (Zhao *et al.*, 2014; Paskuliakova, Tonry and Touzet, 2016). Therefore, the ammonia-N removal caused by C.v.1.1 in this study could not be fully attributed to the biological uptake by the microalga *Chlorella vulgaris* 1.1, in fact it could be a result of both algal uptake and ammonia volatilisation or stripping which will be further investigated in the next chapter. On the contrary, although significant decrease is recorded in the ammonia-N from Day 0 to Day 30 for the treatments; C.v.1.2, S.r and Gp., however, pH increases significantly in these treatments. Additionally, the biomass production is moderate and not significantly high, such as that reported by C.v.1.1. These results are actually comparable to findings in other studies (Lin *et al.*, 2007; Paskuliakova, Tonry and Touzet, 2016) where considerable and/or significant ammonia-N removal is recorded accompanied by almost no gain in biomass for the tested algal treatments, however, no clear explanations are provided in both studies for these observations. A possible explanation for the ammonia removal in C.v.1.2, S.r and Gp is the stripping effect caused by continuous aeration throughout the experiment. Whilst a proposed explanation for the significant increase (instead of decrease) in the pH values observed in these treatments compared to the control treatment is the presence of algal growth which might affect the pH by consuming carbon dioxide causing pH increase (Lin *et al.*, 2007). Interestingly, Zheng *et al.* (2019) highlighted the toxic effect of increasing ammonia concentration when the microalga *Chlorella vulgaris* used in their study is tested for growth in high-strength ammonium wastewater. Zheng *et al.* (2019) reported the threshold of ammonia toxicity in their study is 110 mg/L for the tested *Chlorella vulgaris*, concentrations higher than that significantly reduced the biomass concentration. This actually refers to an interesting point in our study where the strain C.v.1.1 is able to thrive and grow profusely with a significant 3-fold increase in O.D, significantly higher biomass production than other treatments in addition to significant ammonia-N removal percentage when the ammonia concentration is 435.9 mg/L at the start of the experiment, which might suggest an interesting highly-

ammonia tolerant strain that could be a possible candidate in LL treatment. Optimisation attempts for the growth and LL treatment using this strain is conducted in the following chapter.

Chemical oxygen demand (COD) could be defined as the amount of a specified oxidant that reacts with the sample under controlled conditions. The quantity consumed from the oxidant is expressed in terms of its oxygen equivalence. Examples of the most commonly used specified oxidants in COD measurements is the dichromate ion ($Cr_2O_7^{2-}$). Although, COD values can reflect the concentration of both organic and inorganic components of the samples, which are subjected to oxidation, but usually the organic components are dominant and most interesting (APHA, 2017). Our results showed significant decrease in the COD values for C.v.1.2, C.v.2, S.r and Gp treatments which might be the result of the moderate algal growth in these treatments. Our results agree with Lin et al. (2007), who reported >60% removal rate for COD for the strains *Chlorella pyrenoidosa* (LK), *Chlorella pyrenoidosa* (P) and *Chlamydomonas snowiae* (LK) at 30% LL concentration, a concentration that allowed poor algal growth for all strains especially *Chlamydomonas snowiae* (LK) which showed negative growth at this concentration. On the other hand, C.v.1.1 that shows the highest growth amongst all the other treatments, exhibits a significant *increase* in COD at the end of the experiment from Day 0 to day 30. These results hereby are contradictory to other studies (El Ouaer *et al.*, 2016, 2020) who reported concomitant algal growth increase and COD removal or decrease. However, our results are supported by Zhao *et al.* (2014) who also reported significant increase in the DOC (Dissolved Organic Carbon) for different leachate spike ratios (0%, 5%, 10%, 15% and 20%) throughout the 12-days of the experiment. Desai (2015) found that the COD concentrations in some treatments had exceeded the control at the end of the experiment where he was using a *Chlorella* sp. in LL treatment using different concentrations of diluted LL (10%, 25%, 50% and 85%). The significant increase in the COD or the dissolved organic carbon might be an indication of the microalgal growth. When microalgae perform autotrophic metabolism (photosynthesis) they convert inorganic carbon to biomass which will in turn account for the increase in organic matter and thus increase in COD levels rather than accounting for organic carbon

removal (Zhao *et al.*, 2014). Another possibility for the COD increase accompanying high algal growth is the production of extracellular organic matter as the algae grow (Zhao *et al.*, 2014; Desai, 2015). Hulatt and Thomas (2010) estimate the mean maximum amount of dissolved organic carbon (DOC) released as a culture of *Chlorella vulgaris* grows in a photobioreactor to be 6.4% of the total organic carbon in the culture whilst Zhao *et al.* (2014) in their study report the extracellular organic matter to account for 1.6%-9.5% of the total captured carbon in the culture of *Chlorella pyrenoidosa* used in the study. An increase in the COD level at the end of the experiment for the control treatment is observed, although not significant but it is not exactly known why this increase happened, LL is a very complex type of wastewater with many factors contributing to its complexity. Zheng *et al.* (2019) conclude that nutrients removal e.g., COD from a high-strength ammonia wastewater (manure-free piggery wastewater) using a culture of *Chlorella vulgaris* are found to be affected by pH, ammonium concentrations and carbon/nitrogen ratios in the cultures. Although, the main reason behind increase in the COD level for the control is not confirmed, yet the effect of one or more of the factors mentioned earlier (either solely or synergistically) together with the conditions of the experiment, could represent a possible platform for the reasons that require further investigation in the future.

The concentration of heavy metals (HMs) in LL is generally low, however, the HMs load in landfill leachate may vary from one landfill to another according to different factors e.g., landfill age, waste type and topography. Heavy metals solubility/availability, on the other hand, in a particular landfill leachate is influenced by its degradation phase, pH, dissolved organic matter (DOM) content as well as sulphide and carbonate-influenced precipitation. High pH, characterising old stabilised LL, may induce complexation which in turn decreases the concentration of HMs in a landfill leachate, the opposite is observed in young LL where pH is low (due to organic acids production) and this results in high solubility of heavy metals thus increasing heavy metals concentration during this stage (Wijekoon *et al.*, 2022). Some heavy metals, even at very low concentrations, might represent a threat to different living organisms (Kaplan, 2013). The results obtained from this study focused mainly on determining the heavy metals concentrations at Day 0 and day 30 of the experiment

for all the different treatments including the control for comparability reasons. Our results show the significant removal of only one heavy metal: Arsenic. Arsenic is removed by 31.2% and 32.9%, in treatments C.v.1.1 and C.v.1.2, respectively, which was significantly higher than the control removal percentage ($p<0.01$). No significant removal was observed (compared to the control treatment) for the other tested metals (Cu, Pb and Cr). Different results were obtained by Kumari, Ghosh and Thakur (2016) whose bacto-algal treatment was able to successfully remove 91.5% and 74.9% of *Cr* and *Pb*, respectively. Studies on arsenic removal from LL by microalgae is limited but a study conducted by Mustafa, Phang and Chu (2012), a consortium of five species of microalgae, *Chlorella vulgaris*, *Scenedesmus quadricauda*, *Euglena gracilis*, *Ankistrodesmus convolutus* and *Chlorococcum oviforme*, reduced the amount of arsenic to the permissible discharged levels according to the Department of Environment Malaysia with the ratio of the amount of *As* accumulated in algal cells compared to the effluent is 22:1. Our biosystem manifests a decrease in the concentration of *As* from 23.56 ppb (0.023 mg/L) in Day 0 of the experiment to 16.2 ppb (0.0162 mg/L) and 15.8 ppb (0.0158 mg/L) in Day 30 of the experiment by C.v.1.1 and C.v.1.2, respectively. According to Mohan and Pittman Jr (2007), maximum permissible limits for *As* (which is a highly toxic HM) in drinking water vary among different countries, where some adopt the WHO guidelines of 10 ppb (0.01 mg/L) and others still adopt the earlier WHO standards of 50 ppb (0.05 mg/L). In this context, the decrease in *As* concentration achieved by this study is considered sufficient and acceptable especially that standards for drinking water would usually be higher than those for wastewater.

5.6. Conclusions

In an attempt to screen the growth and nutrient removal potential of the four green microalgae in the scope of this study, individually and collectively, a 20% diluted LL supplemented with K_2HPO_4 is used as a growth medium for the tested algae for 30 days, during which the algal growth and the nutrient removal ability is regularly determined. Our results indicate an interesting finding of a *Chlorella vulgaris* culture (C.v.1.1) that outperforms the other treatments in growth, showing the highest significant growth at the end of the experiment, regarding both optical density

measurements and dry biomass calculation. This interesting strain also exhibited a significantly high ammonia-N removal percentage at the end of the experiment concomitant with an observed drop in the pH at the end of the experiment. A significant arsenic removal from the tested LL was also observed in the case of C.v.1.1, when compared to the other treatments. Surprisingly, the COD showed a significant increase in case of this particular treatment which might also be attributed to the highest growth it manifested throughout the experiment. To make sure the results could be reproducible, an experiment to test the difference between the two *Chlorella vulgaris* replicates (C.v.1.1 and C.v.1.2) is conducted in the following chapter. This is carried out simultaneously with a trial to explore combining algal treatment in case of the two tested strains with a plasma/UV pre-treatment step to investigate its effect on algal growth and nutrient removal ability from the tested 20% diluted landfill leachate.

CHAPTER SIX

Effect of UV/Plasma pre-treatment

on *Chlorella vulgaris* strains (C.v.1.1 and C.v.1.2) growth and nutrient uptake in 20% LL

6.1. Abstract

In this chapter, the outperforming *Chlorella vulgaris* strain C.v.1.1 (hereafter referred to as C.V.M*) (CCAP 211/141) as well as the *Chlorella vulgaris* strain C.v.1.2 (hereafter referred to as C.V.N) were further tested for growth and treatment of 20% diluted LL in order to determine the reproducibility of the results obtained in the previous chapter. Simultaneously, physical pre-treatment for the tested LL was carried out using plasma/UV treatments compared to the untreated LL to investigate the efficiency of integrating a physical pre-treatment step prior to the biological treatment of LL in terms of enhancing the algal growth as well as the nutrient removal/uptake from the leachate. An experiment was set up to compare the growth and nutrient removal/uptake of both *Chlorella vulgaris* strains in plasma pre-treated, UV pre-treated and untreated 20% LL for 30 days under continuous illumination and stirring. Results indicated the superiority of C.V.M* over C.V.N regrading growth and ammonia-N uptake in all treatments of LL. However, the plasma pre-treatment observed to be the best pre-treatment method, amongst other tested methods, to be used prior to the biotreatment with C.V.M* as they together showed a total ammonia-N removal of 79% with a total biomass production of 0.38 g/L at the end of the experiment. Genome

sequencing of both strains revealed a high degree of similarity but also revealed different variants (SNPs and indels) which might suggest the possibility of occurrence of mutation or sexual reproduction.

6.2. Introduction

The continuously rising production of municipal solid wastes (MSW), as human population grows, is accompanied by an escalating increase in landfilling. The increase in landfilling and the increasing production of landfill leachate are concomitant. This in turn poses many hazardous effects on humans, different living organisms as well as the environment if the produced landfill leachate infiltrates surface and/or ground water resources (Dogaris, Ammar and Philippidis, 2020; Nawaz *et al.*, 2020) Landfill leachate treatment, therefore, is a worldwide challenge and it is considered a necessary safeguard to strictly meet the landfill leachate discharge measures before being safely released into the environment (Luo *et al.*, 2020). In this context, several studies have reported the different types of LL treatment approaches including conventional methods, biological methods and physical-chemical methods, nonetheless, these studies also highlighted the fact that no method was capable alone to meet the discharge standards, not to mention the drawbacks of each method individually, thus integrating two or more treatment steps was concluded to be potentially superior to a single method treatment regarding efficiency, sustainability as well as cost-efficiency (Dogaris, Ammar and Philippidis, 2020; Luo *et al.*, 2020; Teng *et al.*, 2021; Wijekoon *et al.*, 2022). Current LL treatment is conducted by a combination of different technologies i.e., physical-chemical, and microbial (Dogaris, Ammar and Philippidis, 2020).

Algae-based treatment technologies, in general, are considered relatively novel amongst other biological LL treatment methods with the potential addition of several other advantages e.g., cost-efficiency, sustainability, resources recovery, in addition to the possibility of coupling LL treatment with the production of valuable biomass which might further be used to produce biofuels as well as different other high-value

bioproducts (Dogaris, Ammar and Philippidis, 2020; Nawaz *et al.*, 2020). However, one of the biggest challenges facing LL treatment using microalgae is the limitation of algal growth and nutrients/pollutants uptake by raw LL due to its high toxicity and the need to perform pre-treatment steps e.g., adaptation of algal strains for a long period of time, adjusting pH of raw LL or diluting it to usually very small concentrations (~10%) to minimise its toxic effects on microalgae (Lin *et al.*, 2007; Edmundson and Wilkie, 2013; Zhao *et al.*, 2014; El Ouaer *et al.*, 2020; Okurowska *et al.*, 2021). Hence, integrating algae-based treatment with a suitable treatment/pre-treatment technology might improve its efficiency as well as combining the merits of the two approaches, perhaps with coupled synergies, creating the possibility of overcoming the shortcomings of both technologies. Modularity often optimizes each individual step, at great cost. Synergies could allow for far from optimal individual operation of the staged modular approaches.

Integrating both biological and physical-chemical treatment methods is reported to improve the overall contaminants removal efficiency in LL, including removal of NH_3-N, COD, and heavy metals (Luo *et al.*, 2020; Wijekoon *et al.*, 2022). Different physical-chemical methods for LL treatment are well reported in the literature (Luo *et al.*, 2020; Teng *et al.*, 2021; Wijekoon *et al.*, 2022). Physical-chemical treatment techniques are usually used for the removal of non-biodegradables (e.g., humic and fulvic acids) and/or other compounds (e.g., heavy metals). Physical-chemical treatments include coagulation-flocculation, chemical precipitation, adsorption, membrane filtration, ion exchange, air stripping, chemical oxidation/advanced oxidation processes and electrochemical treatment (Luo *et al.*, 2020). Amongst different types of physical-chemical treatment methods, advanced oxidation processes (AOPs) stand out as an effective method for degradation of different refractory compounds in LL. AOPs involve ozone-based oxidation, Fenton oxidation as well as electrochemical oxidation (Li, Zhou and Hua, 2010). Ozone-based oxidation processes were deemed an excellent option to apply before biological treatment which is mainly attributed to their ability to enhance the biodegradability of LL as well as their successful decolourisation potential (Wu *et al.*, 2004). Ozonation processes also possess the advantage of the ozone high oxidative power (Li, Zhou and Hua, 2010)

without the disadvantage of iron sludge generation of other efficient oxidation treatments (Fenton oxidation) that increase the environmental burden of safe disposal afterwards. This in turn inspired us to implement plasma/UV in this study as plasma is well known to include ozone amongst other reactive species produced when plasma is generated (Zhao *et al.*, 2011; Pankaj and Keener, 2017) and the UV lamp used in this study generates ozone as well.

Plasma technology for landfill leachate treatment is considered an efficient approach in removal of ammonia-N (Zhao *et al.*, 2011) as well as some harmful organics (Singh *et al.*, 2021) from landfill leachate, in addition to effective decolourisation of leachate in relatively short times (Zhao *et al.*, 2011; Singh *et al.*, 2021) besides having the advantage of being a clean green technology (Siswanto *et al.*, 2018). UV technology also represents an effective method for landfill leachate treatment especially when coupled with ozone/H_2O_2 as reported in a few studies (Wu *et al.*, 2004; Shu *et al.*, 2006). However, there is hardly any research on coupling plasma/UV with microalgae in landfill leachate treatment. The closest study was that conducted by Quan *et al.* (2020) where they reported that Ozonisation of LL, as a pre-treatment step, was found to enhance the subsequent microalgal growth and nitrogen removal efficiency.

In this chapter, pre-treatment of LL using plasma/UV technologies will be conducted for both strains C.v.1.1 and C.v.1.2 (hereafter referred to as C.V.M* (CCAP 211/141) and C.V.N, respectively), with the non-treated LL as a control, to test the effect of plasma/UV pre-treatment vs without treatment on both algal growth as well as nutrients/pollutants uptake efficiency in 20% LL (v/v).

6.3. Materials and Methods

6.3.1. Landfill Leachate pre-treatment

The same batch of landfill leachate used in the previous experiments was also used for this experiment after dilution with sterile distilled water to a concentration of 20% (v/v). However, chemical analysis for this diluted LL was also carried out at the start of this experiment i.e., pH, COD, NH_3-N and heavy metals measurements.

The diluted LL used in this experiment was divided into three groups; one group was pre-treated by plasma, one group was pre-treated by UV and one group was left untreated.

I. Plasma pre-treatment:

This part was carried out in collaboration with Dr Thomas D. Holmes (University of Sheffield) who kindly designed the plasma pre-treatment reactor to be applied to the tested LL (20%).

A dielectric barrier discharge reactor for plasma generation was used which incorporates the novel fluidic oscillator microbubble technology (Zimmerman *et al.*, 2009). The plasma pre-treatment experimental set up was prepared as follows:

- Compressed air (from the departmental compressor) was blown into a glass tube at 0.2 bar pressure, the flow rate was measured by rotameter and was estimated to be 10 L/m ± 1.
- A steel rod 1 mm in diameter was positioned down the middle of the glass tube. This was connected to the live output of a high voltage amplifier, driven by a sinusoidal waveform of approximately 50 kHz.
- Aluminium tape was applied to the outer surface of this glass tube, and this was connected to the neutral cable of the high voltage amplifier.
- The peak voltages given out by the high voltage amplifier were around 2 kV (0 to peak).
- The plasma voltage was measured by a Tektronix high voltage probe, and the plasma current was measured by taking the voltage across a monitoring capacitor (of 6.8 nF capacitance) using a standard voltage probe. Both these probes were connected to Picoscope USB oscilloscope, which was set up to continuously capture the waveform data every few microseconds. The data was analysed by converting the waveform data into lissasjous plots.
- When inserted into the leachate (as shown in Figure 6-1) the plasma was observed to be present right up to the end of the glass tube where the outlet of the tube would be in contact with the liquid. This suggests that a greater number of short-

lived plasma species would reach the liquid than would be that case if the plasma was positioned further away from this point of contact.

- The output of this plasma was investigated using a Hiden QGA quadrupole mass spectrometer.

The plasma pre-treatment for the diluted LL in the Drechsel bottle, as shown in Figure 6-1, was carried out for three hours until a change in the colour of the leachate from brown to yellow was evident. The amount of diluted LL that was subjected to plasma pre-treatment was then filtered using 0.2 µm disposable sterile filters (Steritop, Millipore) driven by a vacuum pump N840FT.18 (KNF Laboport, Freiburg, Germany) and the filtrate was collected in autoclave-sterilised Duran bottles. Plasma pre-treated filtered LL was then used for the experiment, where part of it was used as a control (no algae were inoculated) and the rest was used for algal inoculation (C.v.1.1 or C.v.1.2).

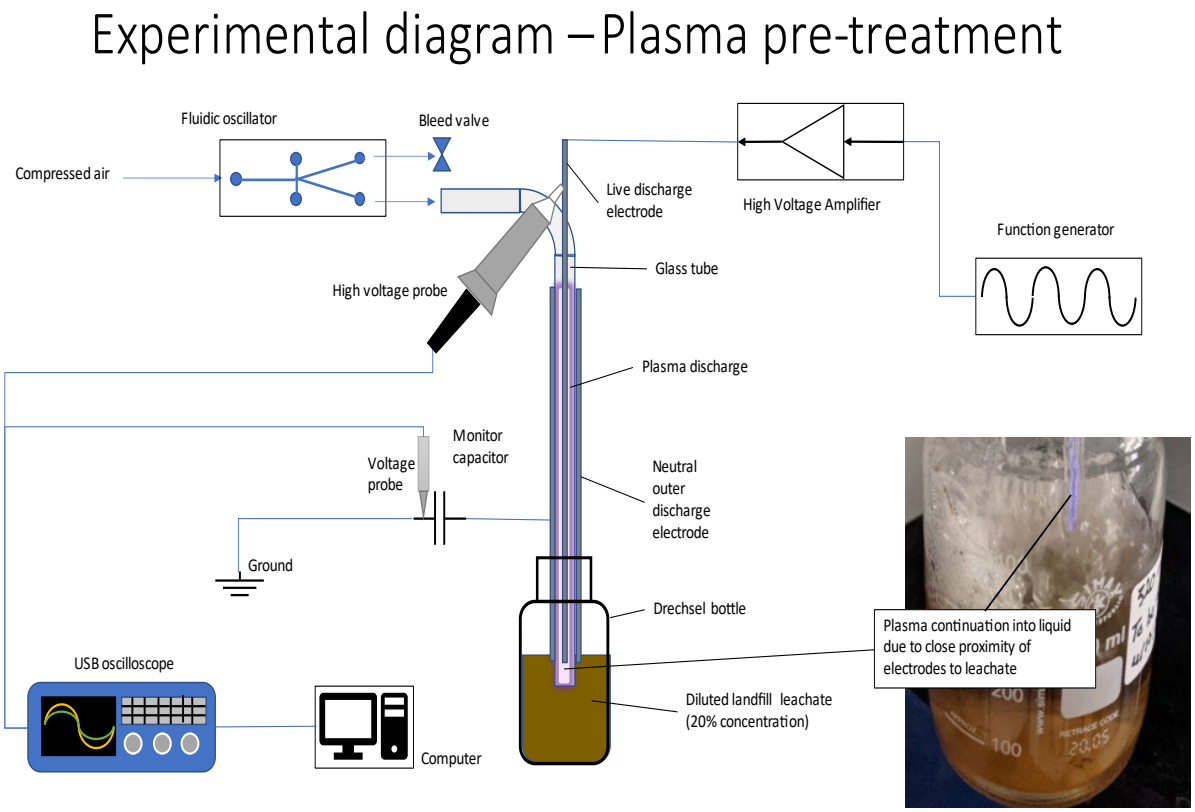

Figure 6-1 Experimental diagram showing the plasma set up for the pre-treatment of the 20% LL used in the current experiment.

II. UV pre-treatment:

This lot of diluted LL (20%) (v/v) was pre-treated by UV prior to the experiment. The set up for the UV pre-treatment (shown in Figure 6-2) was carried out as follows:

- A box was used for the apparatus to be enclosed in, thus achieving dual functions of preventing the UV light as well as the ozone generated from the UV lamp from causing any harmful effects to the user/s in addition to achieving the maximum effect regarding the exposure of LL to the UV light and ozone generated from it, according to the instruction manual of the device. The top of the box was slightly closed to allow the escape of some air to avoid the harmful build-up of the ozone gas inside the box.
- A 250 ml beaker containing 200 ml of the diluted LL to be pre-treated was used in the experiment, the beaker was placed on a magnetic stirrer operating at a medium speed to allow the equal exposure of the treated LL to the UV light and the generated ozone.
- The UV device used is a purchased UV sterilisation apparatus, Smart UV Sterilizer U80 (CE FC RoHS, China) that uses both UVC and ozone for sterilization. This device is equipped with a low-pressure mercury lamp design, it produces UV light (185-254 nm, Power 5 W) together with ozone which is generated at the same time when the lamp works. The lamp was positioned on the top of the beaker (containing LL) at 3.4 ± 1 cm, as measured by a ruler.

The diluted LL to be pre-treated was subjected to a total of 3 hours of UV/ozone lamp exposure, after which it was filtered using 0.2 µm disposable sterile filters (Steritop, Millipore) driven by a vacuum pump N840FT.18 (KNF Laboport, Freiburg, Germany) and the filtrate was collected in autoclave-sterilised Duran bottles. The filtered UV/ozone pre-treated LL was then used for the experiment where part of it was used as a control (no algae were inoculated) and the rest was used for algal inoculation (C.v.1.1 or C.v.1.2).

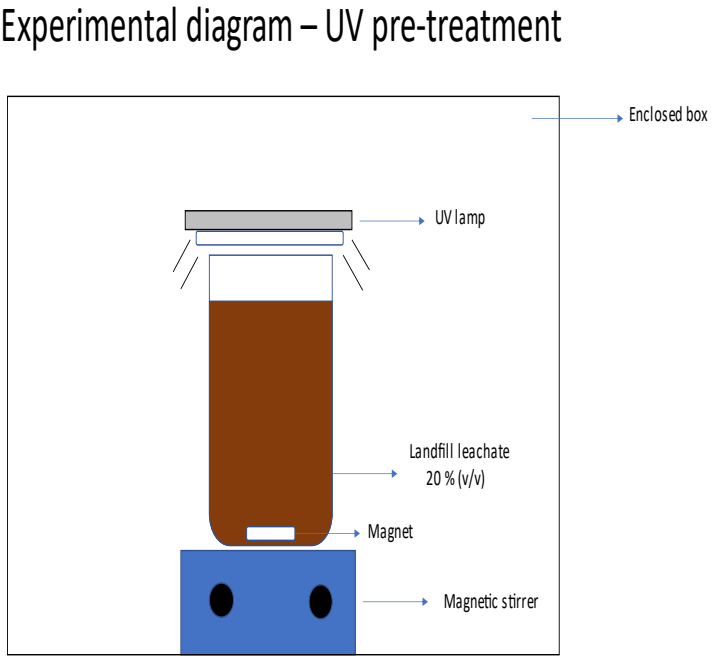

Figure 6-2 An experimental diagram showing the set up for the UV pre-treatment of the diluted landfill leachate 20% (v/v).

III. No treatment (untreated LL):

The final lot/group of diluted LL 20% (v/v) was not subjected to any pre-treatments and was considered as a control group. It was filtered by passing through 0.2 µm disposable sterile filters (Steritop, Millipore) driven by a vacuum pump N840FT.18 (KNF Laboport, Freiburg, Germany) and the filtrate was collected in autoclave-sterilised Duran bottles. The filtered untreated LL was then used for the experiment where part of it was used as a control (no algae were inoculated) and the rest was used for algal inoculation (C.v.1.1 or C.v.1.2).

6.3.2. Experimental set up

The experiment was set up in duplicate 250 ml autoclave sterilised Simax bottles. Two bottles were used for each pre-treatment/no treatment for each alga. The two tested algae were C.v.1.1 and C.v.1.2. Each bottle was filled with 100 ml diluted leachate (20% v/v) with sterile distilled water, supplemented with 1 ml of an inorganic phosphate source (5.2 g/L $K_2HP0_4.3H_2O$) which is equivalent to the same amount added for preparation of 100 ml BG11. Each Duran bottle was inoculated with 20% (v/v) algal

inoculum (20 ml). The OD for all the inocula were measured at 595 nm using a spectrophotometer (Jenway 6715 UV/Vis., UK) and the measurements were adjusted at 0.24-0.25.

Fifteen autoclave-sterilised magnets were placed into the fifteen tested Duran bottles which were then each placed onto a magnetic stirrer plate to be continuously stirred and mixed during the duration of the experiment (30 days) at medium speed. The experiment was set up at room temperature and the temperature was monitored continuously (22.45^{o}C ± 0.85), the Duran bottles were subjected to continuous light from fluorescent light tubes at surface intensity of 968.5 ± 42.8 Lux, measured by a Luxmeter (Fisher Scientific) throughout the duration of the experiment. Every five days, samples (6 ml) were collected from the experimental Duran bottles to determine algal growth as described below and then filtered by 0.2 μm syringe filters, where the filtrate is used for the chemical analysis of the leachate.

6.3.3. Algal growth measurements & chemical analysis of LL:

I. Algal growth measurements:

- Algal growth was monitored by optical density (OD) determination every five days, using a Spectrophotometer (Jenway 6715 UV/Vis., UK) at 595 nm. Three types of blank were used during measurements i.e., one for each treatment group; for the plasma pre-treatment group the blank used was plasma pre-treated filtered 20% LL, for the UV pre-treated group the blank used was UV pre-treated filtered 20% LL and for the untreated group the blank used was filtered untreated 20% LL.
- At the end of the experiment (after 30 days), the dry biomass for the algal cultures was determined. 25 ml of each Duran bottle was collected (after being well homogenised) and centrifuged at 3900 rpm for 10 minutes using a centrifuge (Eppendorf 5810R) after which the pellet was left to dry on a pre-weighed Petri dish overnight at 50^{o}C.

II. Chemical analysis of landfill leachate:

- Aliquots of leachate collected every five days were filtered through 0.2 µm syringe filters after algal growth determination to be analysed for pH and COD as mentioned in the previous chapter.
- Filtered samples were stored at -20^{o}C until the end of experiment after which they were analysed for ammonia content as described in the previous chapter in detail. Samples from days 0, 5, 10, 15, 20, 25 and 30 were diluted 50x prior to the NH_3-N analysis by the Nessler method described previously in detail (calibration curve in Appendix 7).
- Heavy metals content was determined at the beginning and the end of the experiment (Day 0 and Day 30), as described in the previous chapter, for the filtered samples of the leachate.

6.3.4. Genome sequencing for C.V.M* & C.V.N:

DNA extraction for the two strains (C.V.M* and C.V.N) were carried out as explained in detail in chapter one, after which the extracted genomic DNA of the two samples was sent to the sequencing facility Novogene (Cambridge, UK). After which the following was carried out:

- Sample Quality Control
- Library Construction, Quality Control and Sequencing

The genomic DNA was randomly sheared into short fragments. The obtained fragments were end repaired, A-tailed, and further ligated with Illumina adapter. The fragments with adapters were PCR amplified, size selected, and purified. The experimental procedures of DNA library preparation are shown in Figure 6-3.

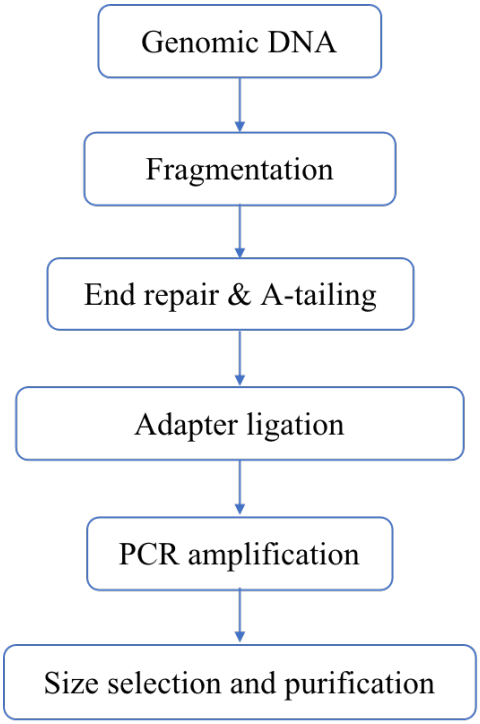

Figure 6-3 Workflow of library construction

The library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection. Quantified libraries were pooled and sequenced on Illumina platforms, according to effective library concentration and data amount required.

The strain C.V.M* was submitted to the Culture Collection of Algae and Protozoa (CCAP) SAMS Limited, UK and was given an accession number: CCAP 211/141 *Chlorella vulgaris*.

6.3.5. Statistical analysis:

Statistical analysis was carried out using R v.4.0.5 and R studio v.1.2.5. Data were presented as mean and standard deviation. Significance testing for the different measurements was carried out using paired t-test when comparing day 0 and 30 for each treatment, while ANOVA and post Hoc tests were used when comparing between the different treatments. A p value <0.05 was considered statistically significant.

6.4. Results

6.4.1. Landfill Leachate pre-treatment

The chemical characters of plasma pre-treated LL, UV pre-treated LL and untreated LL were measured at the start of the experiment (before algae inoculation) and recorded in Table 6-1. There was no significant change in the pH values between untreated and plasma/UV treated LL. COD change showed statistically significant difference between all of the different treatments, with the lowest value recorded for the plasma pre-treated LL (514.5 mg/L). The ammonia-N content in all treatments showed a significant difference, where the highest ammonia concentration was recorded for untreated LL (290.7 mg/L), this concentration decreased significantly after UV treatment (219.4 mg/L) and the lowest concentration (with a significant reduction) was observed in case of the plasma pre-treatment (151.4 mg/L).

Table 6-1 Comparing chemical characteristics of landfill leachate with and without pre-treatments at Day 0 of the experiment.

Parameter	Plasma pre-treated LL (20%)	UV pre-treated LL (20%)	Untreated LL (20%)	P value
pH	8.6±0.015	8.6±0.015	8.4±0.015	0.982
COD	514.5±1.5 mg/L	578±1 mg/L	576±1 mg/L	<0.001[a]
NH_3-N	151.38±0 mg/L	219.43±0 mg/L	290.73±0 mg/L	<0.001[a]
Arsenic (As)	0.0235±0.0013 mg/L	0.0194±0.0012 mg/L	0.0197±0.0005 mg/L	0.017[bc]
Copper (Cu)	0.0267±0.0005 mg/L	0.025±0.0003 mg/L	0.0231±0.0001 mg/L	<0.001[a]
Chromium (Cr)	0.1461±0.0002 mg/L	0.1345±0.001 mg/L	0.1367±0.0009 mg/L	<0.001[bc]
Lead (Pb)	0.014±0.0004 mg/L	0.0123±0.0003 mg/L	0.0126±0.0005 mg/L	0.016[bc]

Values are presented as mean ± standard deviation. ANOVA and Tukey post Hoc test were used for significance testing. A p value<0.005 is considered statistically significant. A Significantly different between all groups, b Significantly different between Plasma pre-treated LL and UV pre-treated LL c Significantly different between Plasma pre-treated LL and untreated LL.

In terms of the tested heavy metals content (*As*, *Cu*, *Cr* and *Pb*), there was a significant difference between the *As* content in the plasma pre-treated LL compared to the UV treated and the untreated LL, the *As* content was significantly higher in the plasma pre-treated LL than other treatments. Similar results were obtained for Cr and Pb, whilst in case of Cu content there was a significant difference between all the tested treatments (Table 6-1).

I. Plasma pre-treatment:

The generated plasma, as shown in Figure 6-4, was used as a pre-treatment step for the 20% diluted landfill leachate (v/v) for a period of three hours, after which a dramatic change in the dark brown colour of the LL was evident. The dark brown colour of LL changed to a yellow colour (Figure 6-5) after being subjected to the three-hour pre-treatment course of plasma induced oxidation.

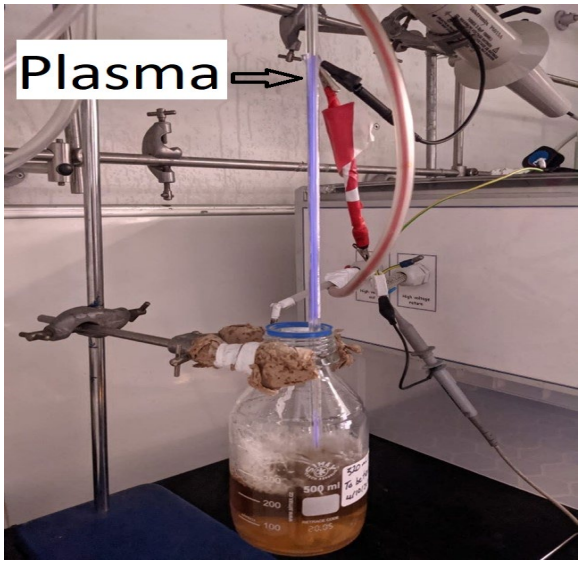

Figure 6-4 A picture showing the plasma discharged during the pre-treatment process of the 20% diluted LL.

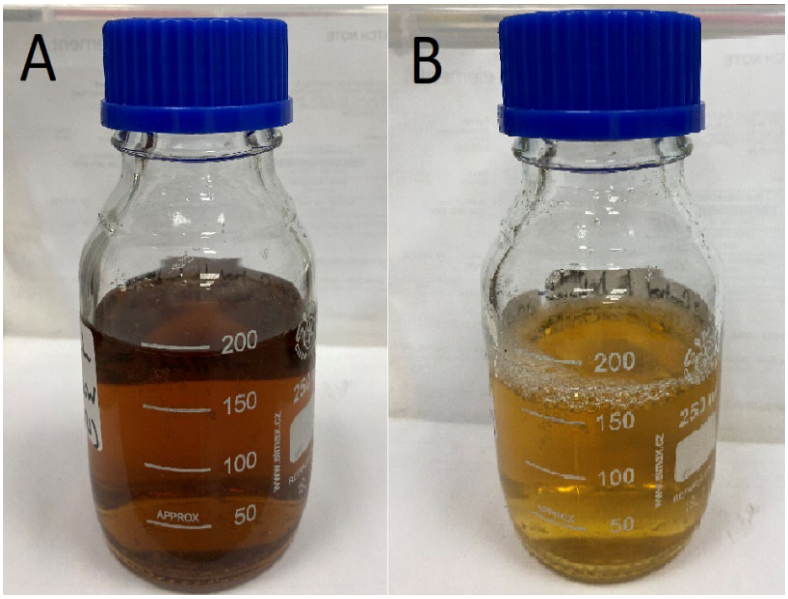

Figure 6-5 A picture showing the pre-treated LL before and after plasma pre-treatment: A) before plasma pre-treatment B) after three hours of plasma pre-treatment.

II. UV pre-treatment:

The batch of diluted 20% LL (v/v) to be pre-treated by UV/ozone lamp was subjected to a similar three-hour pre-treatment step, yet no evident change in the dark brown colour of the LL was observed (Figure 6-6), on the contrary to the plasma pre-treatment where the change in colour was clear.

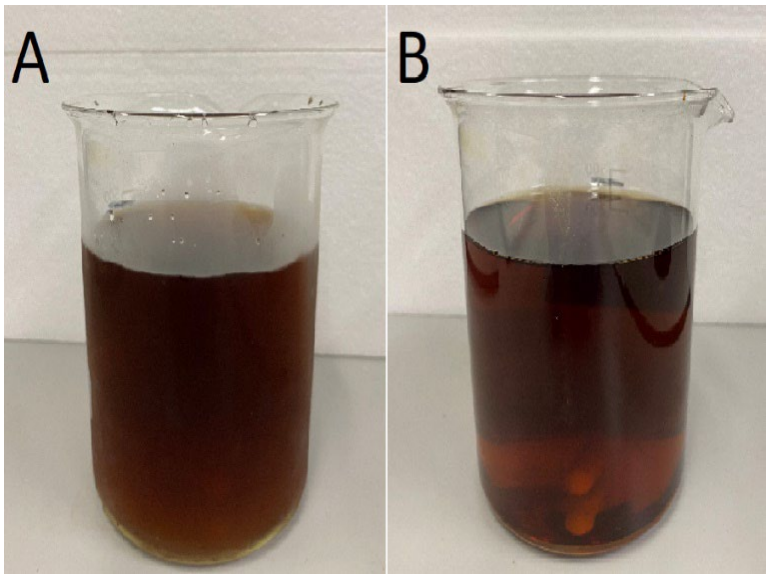

Figure 6-6 A picture showing the UV pre-treated diluted LL before and after treatment: A) before UV pre-treatment B) after three hours of UV pre-treatment.

6.4.2. Experimental set up:

The growth and bioremediation capability of two strains of indigenous *Chlorella vulgaris* (C.v.1.1 & C.v.1.2) in 20% diluted LL was tested. The tested LL was divided into three groups; one subjected to plasma pre-treatment; one to UV pre-treatment and one was left untreated. Each of the *Chlorella vulgaris* strains was inoculated into each of the three groups in duplicate. Figure 6-7 shows a picture showing the start of the experiment at Day 0 and the end of the experiment at Day 30.

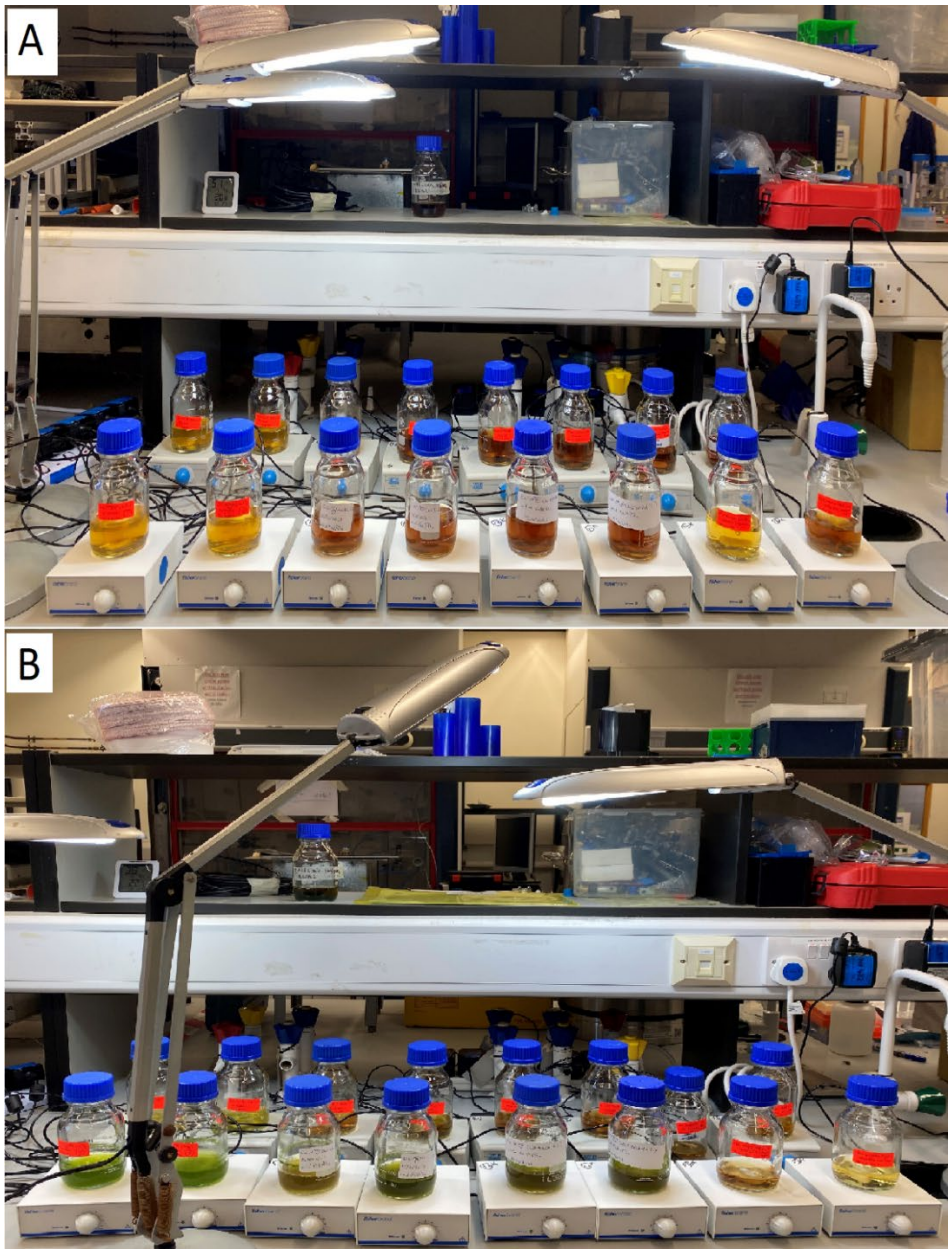

Figure 6-7 A picture showing the plasma/UV pre-treatment experiment at the beginning and the end of the experiment A) at Day 0 of the experiment B) at Day 30 of the experiment.

6.4.3. Algal growth measurements & chemical analysis of LL

6.4.3.1. Algal growth measurements:

The growth of both tested strains of the green microalga *Chlorella vulgaris* was determined by continuous measurements of OD throughout the experiment (every five days), as well as by dry weight determination at the end of the experiment i.e., after 30 days and the results are shown below.

I. Growth measurements by OD at 595 nm:

The dramatic increase in the growth of strain C.v.1.1. (C.V.M*) in case of the plasma/UV pre-treated and even in the untreated LL was obvious (Figure 6-8) and significant from Day 0 to day 30 (p<0.05) for the C.v.1.1. (C.V.M*) growing in the plasma pre-treated and the untreated LL. Whilst the other *Chlorella vulgaris* strain C.v.1.2 (C.V.N) did not show any significant growth increase from Day 0 to Day 30 except for C.V.N growing in the UV treated LL, which exhibited a significant increase in the growth as determined by OD from Day 0 to Day 30.

By comparing the growth of the two strains in different treatments at the end of the experiment at Day 30, it was found that the highest growth was achieved by the strain C.v.1.1 (C.V.M*) in plasma pre-treated LL and it was significantly higher than the other strain C.v.1.2 (C.V.N) in all the tested treatments ($p < 0.05$). However, it was comparable to the growth achieved by C.v.1.1 (C.V.M*) in the UV pre-treated and untreated LL which implies that pre-treatment did not induce a significant change in growth, but it was the strain C.v.1.1 (C.V.M*) that showed a significant growth increase compared to its peer C.v.1.2 (C.V.N).

The growth of C.v.1.1 (C.V.M*) in plasma pre-treated LL was 6.8-fold, 3.7-fold, and 18.7-fold higher than that achieved by C.v.1.2 (C.V.N) in plasma pre-treated LL, in UV pre-treated LL and in untreated LL, respectively. On the other hand, the growth of C.V.M* in untreated LL was significantly higher than that of C.V.N in untreated LL by 18.7-fold at the end of the experiment (Day 30).

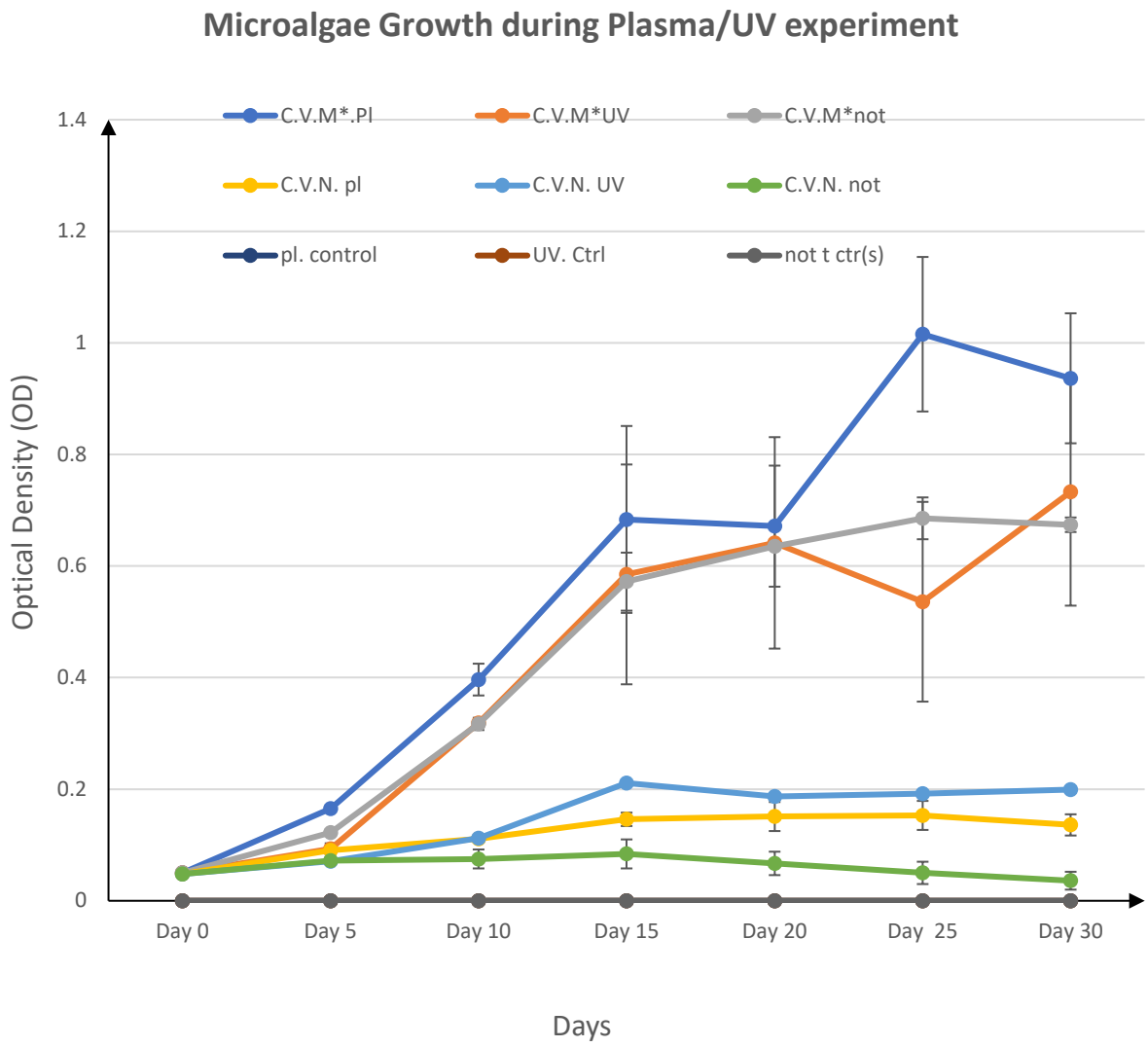

Figure 6-8 Growth of the two tested strains of *Chlorella vulgaris* C.v.1.1. (C.V.M*) and C.v.1.2. (C.V.N) in plasma pre-treated, UV pre-treated and untreated LL. C.V.M*.PL (C.V.M*plasma pre-treated), C.V.N.pl (C.V.N plasma pre-treated), pl. control (plasma pre-treated control), C.V.M*UV (C.V.M* UV pre-treated), C.V.N.UV (C.V.N UV pre-treated), UV. Ctrl (UV pre-treated control), C.V.M*. not (C.V.M* untreated), C.V.N. not (C.V.N untreated), not t ctr(s) (untreated control). Mean and standard deviation values of two biological replicates are plotted.

The dry weight (g/L) was determined for the two tested strains of *Chlorella vulgaris* for all the tested treatments at the end of the experiment (Day 30). Results showed that the highest dry weight was obtained by the strain C.V.M* grown in plasma pre-treated LL (0.38 g/L) as shown in Figure 6-9 A significant increase in the dry weight produced

by the strain C.V.M* in the plasma pre-treated LL was obvious compared to that produced by C.V.N in all the three treatments, where the biomass produced by C.V.M* in plasma pre-treated LL at the end of the experiment was an average of 0.38 g/L which is 4.9, 5.75 and 7.5 times higher than the dry weight produced by the strain C.V.N in plasma pre-treated, UV pre-treated and untreated LL, respectively. The dry weight obtained by the strain C.V.M* in untreated LL was also significantly higher than that of C.V.N in untreated LL by 7.5-fold. Nevertheless, there was no significant difference (p<0.05) between the different LL treatments tested in terms of each individual algae.

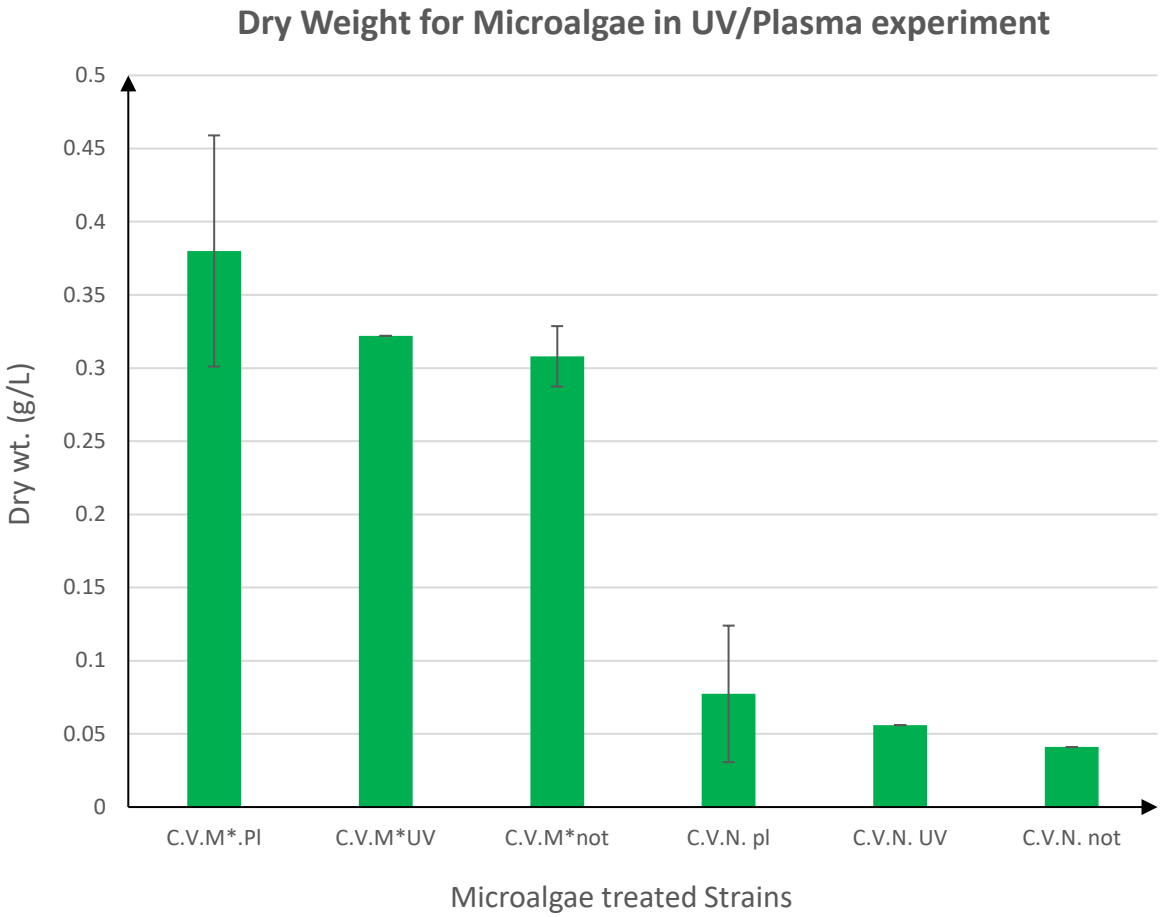

Figure 6-9 The dry weight (g/L) at the end of the UV/plasma experiment (Day 30). C.V.M*.PL (C.V.M*plasma pre-treated), C.V.N.pl (C.V.N plasma pre-treated), C.V.M*UV (C.V.M* UV pre-treated), C.V.N.UV (C.V.N UV pre-treated), C.V.M*. not (C.V.M* untreated), C.V.N. not (C.V.N untreated). Mean and standard deviation values of two biological replicates are plotted.

The growth of the two tested strains (C.V.M* and C.V.N) at the end of the experiment (after 30 days) is shown in Figure 6-10.

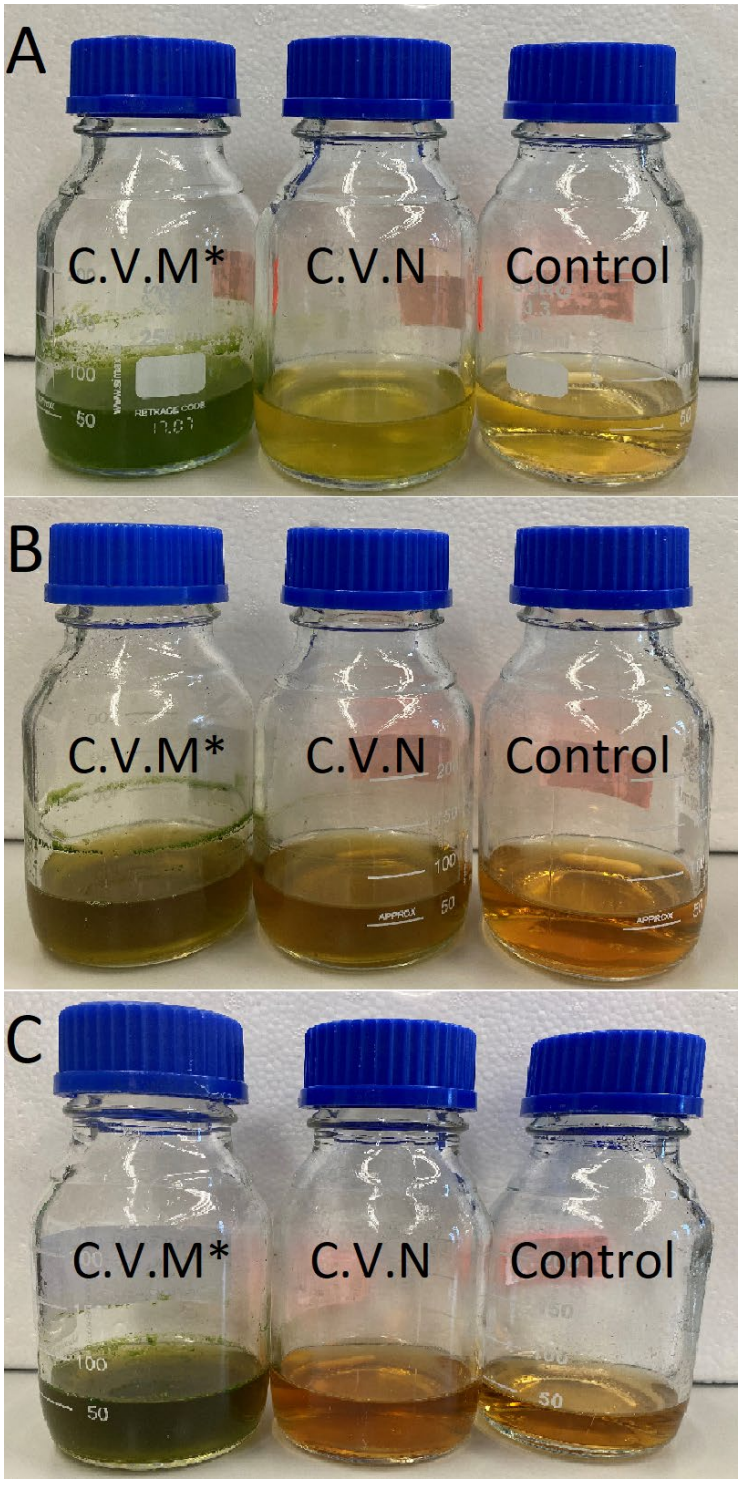

Figure 6-10 A picture showing the growth of C.V.M* and C.V.N in all the different treatments; A) plasma pre-treatment LL; B) UV pre-treatment LL C) untreated LL.

6.4.3.2. Chemical analysis of landfill leachate

Every five days, samples from each treatment were collected, filtered, and analysed for ammonia-N content, pH, and COD. The heavy metals analysis was carried out at the beginning (Day 0) and the end of the experiment (Day 30).

I. Ammonia-N analysis:

Measurements of the ammonia-N content for the 20% diluted LL (v/v); untreated, plasma pre-treated, and UV pre-treated, at the beginning of the experiment (Day 0) revealed that the highest ammonia-N content was recorded for the untreated LL (290.7 mg/L), followed by the UV-treated LL (219.4 mg/L) whereas the lowest ammonia-N concentration was recorded for the plasma pre-treated LL (151.3 mg/L). A dramatic reduction in the amount of ammonia-N content in the plasma pre-treated LL by 1.9-fold compared to the ammonia-N concentration in the untreated LL was significant ($p < 0.001$) as shown in Figure 6-11.

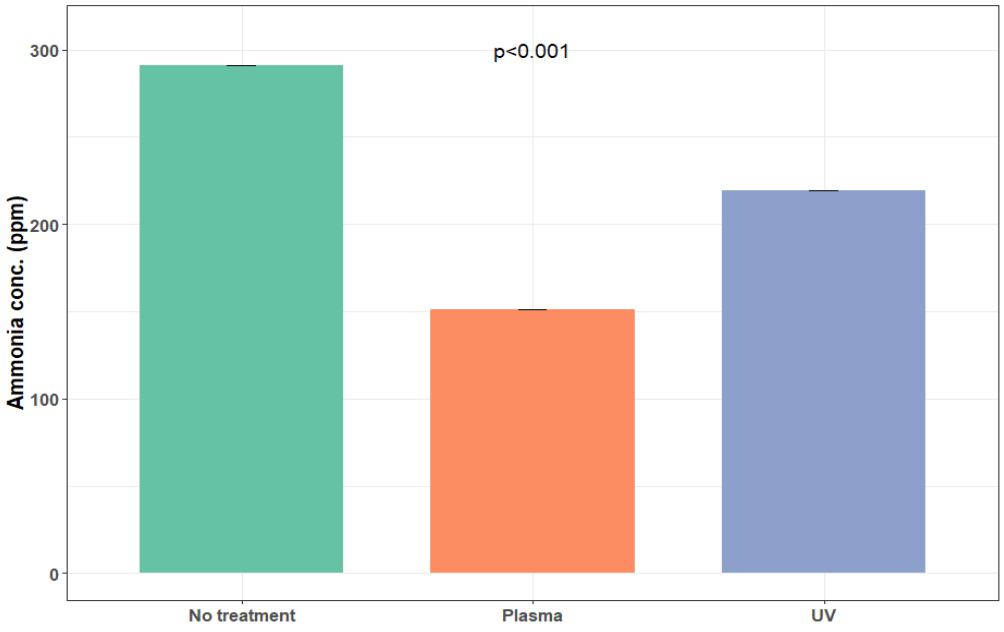

Figure 6-11 Difference in ammonia-N concentrations in different LL treatments at the start of the experiment (Day 0) with a significant difference between all of them (p<0.001). Mean and standard deviation values of two replicates are plotted.

The Ammonia-N uptake by the two tested strains of *Chlorella vulgaris* (C.V.M* and C.V.N) in different LL treatments was monitored continuously every five days

throughout the time interval of the experiment (30 days). The results shown in Figure 6-12 indicate that the highest decrease in the ammonia-N content was achieved by C.V.M* grown in plasma pre-treated LL at day 25. The strain C.V.M* exhibited significant reduction (p<0.05) in ammonia-N in all the tested LL treatments from Day 0 to Day 30, on the contrary, no significant decrease in ammonia-N concentration was recorded for the strain C.V.N at any of the tested treatments of LL.

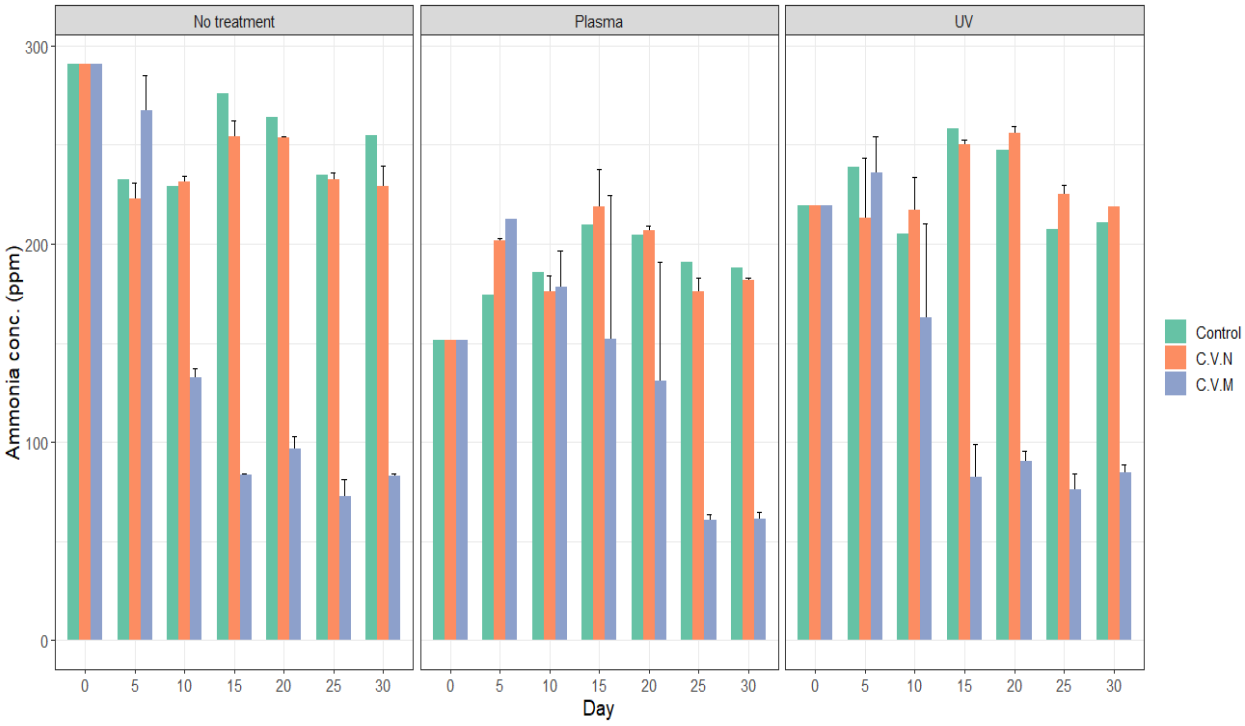

Figure 6-12 Difference in ammonia-N concentrations throughout the period of the experiment in different LL treatments with the two tested strains of *Chlorella vulgaris* (C.V.M* and C.V.N). Mean and standard deviation values of two biological replicates are plotted.

The highest reduction in ammonia content was observed at Day 25 of the experiment for the strain C.V.M* in all LL treatments. Therefore, the removal percentage of ammonia-N at Day 25 was shown in Figure 6-13, for each treatment coupled with the microalgal strain/control effect. The highest removal percentage was achieved by C.V.M* in plasma pre-treated LL (79%) which was significantly higher ($p < 0.01$) than its peer C.V.N in plasma pre-treated LL as well as the plasma control. The ammonia removal percentage for the strain C.V.N in plasma pre-treated LL at Day 25 was 39.5% which is half the amount removed by C.V.M* in plasma pre-treated LL (79%).

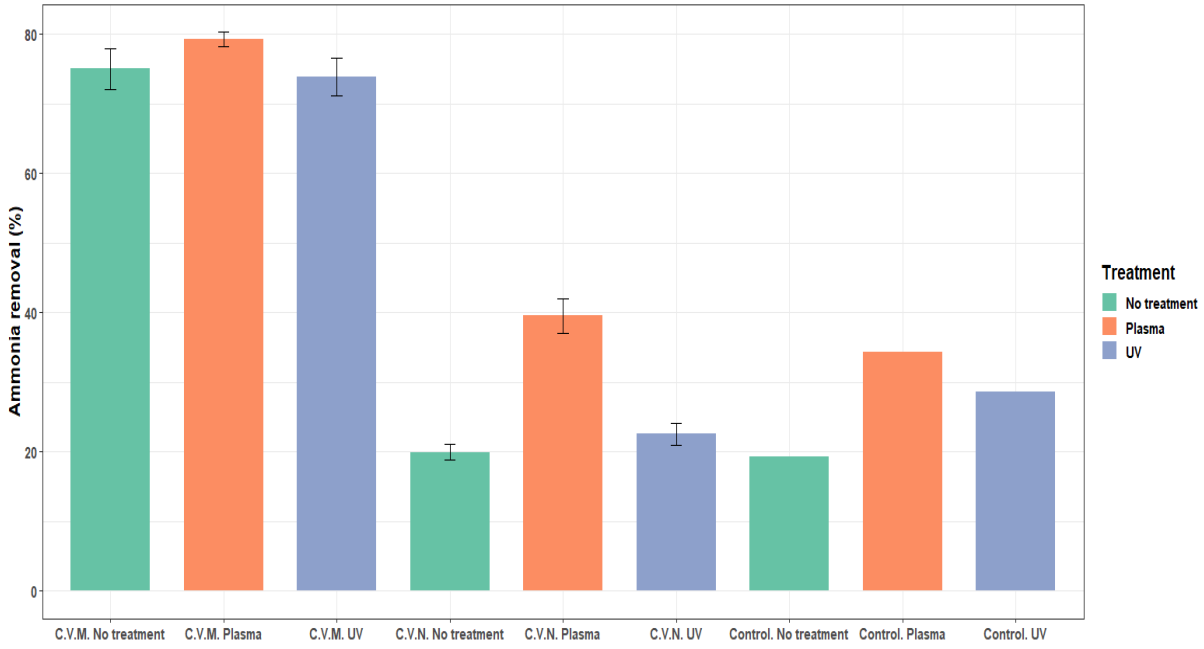

Figure 6-13 Comparing the percentage of ammonia-N removal in LL treatments solely (control) and with the microalgal strains tested (C.V.M* and C.V.N) at Day 25 of the experiment. Mean and standard deviation values of two biological replicates are plotted.

II. pH measurements:

The change in pH values in the experiment was recorded every five days and presented in Figure 6-14. A significant decrease (p<0.05) in the pH values from Day 0 to Day 30 was observed for the strain C.V.M* growing in all LL treatments. However, the pH values in case of the strain C.V.N as well as the control of all the treatments increased from Day 0 to Day 30.

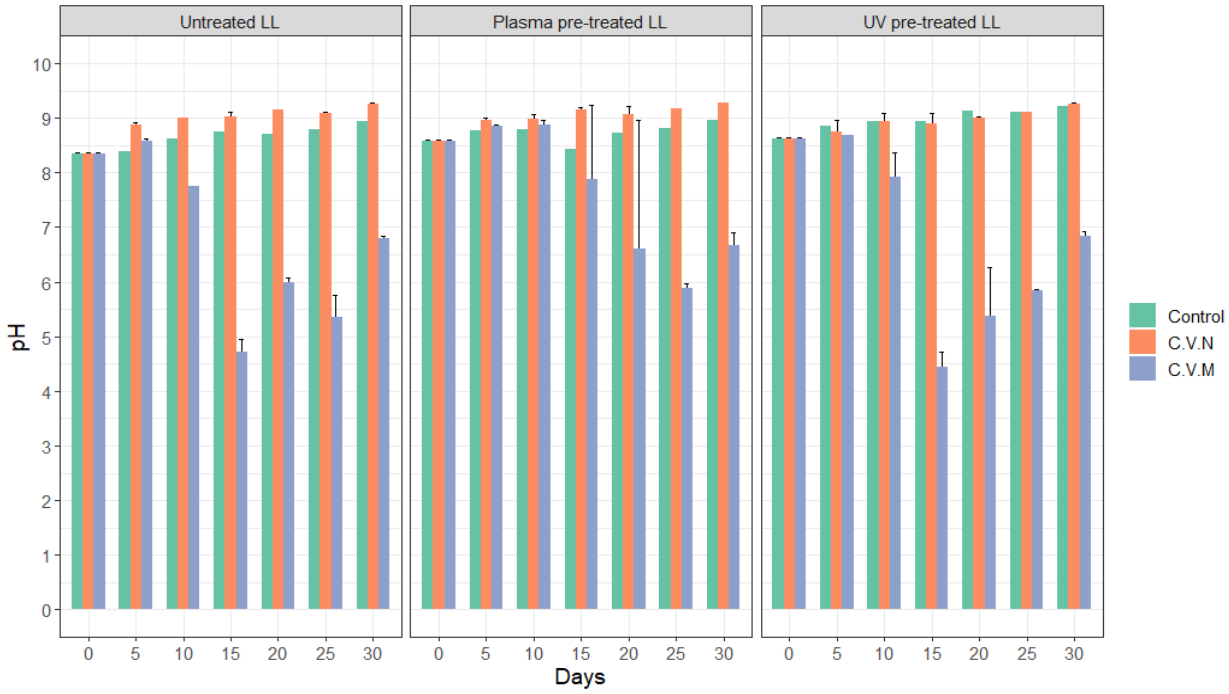

Figure 6-14 pH dynamics change throughout the experimental time interval (30 days) as measured every five days for the tested algal strains (C.V.M* and C.V.N) in the different LL pre-treatments. Mean and standard deviation values of two biological replicates are plotted.

I. COD measurements:

Change in the chemical oxygen demand (COD) values throughout the time interval of the experiment (30 days) for the two tested strains of *Chlorella vulgaris* in the different LL treatments was monitored every five days and plotted in Figure **6-15**. A significant increase (p<0.05) in values of the COD was observed, from Day 0 to Day 30, in case of the strain C.V.M* grown in all different LL treatments (Figure **6-15**). On the other hand, the strain C.V.N did not exhibit any significant change (p<0.05) in COD values from Day 0 to Day 30 of the experiment. Similar to the strain C.V.N, the control also did not show any significant change (p<0.05) from Day 0 to Day 30 in any of the tested LL treatments.

The values of COD, in case of C.V.M* in different treatments, continue rising until the end of the experiment (Day 30), the highest COD value was recorded for C.V.M* grown in untreated LL, which was significantly higher than all other treatments except for C.V.M* grown in UV pre-treated LL (p<0.01).

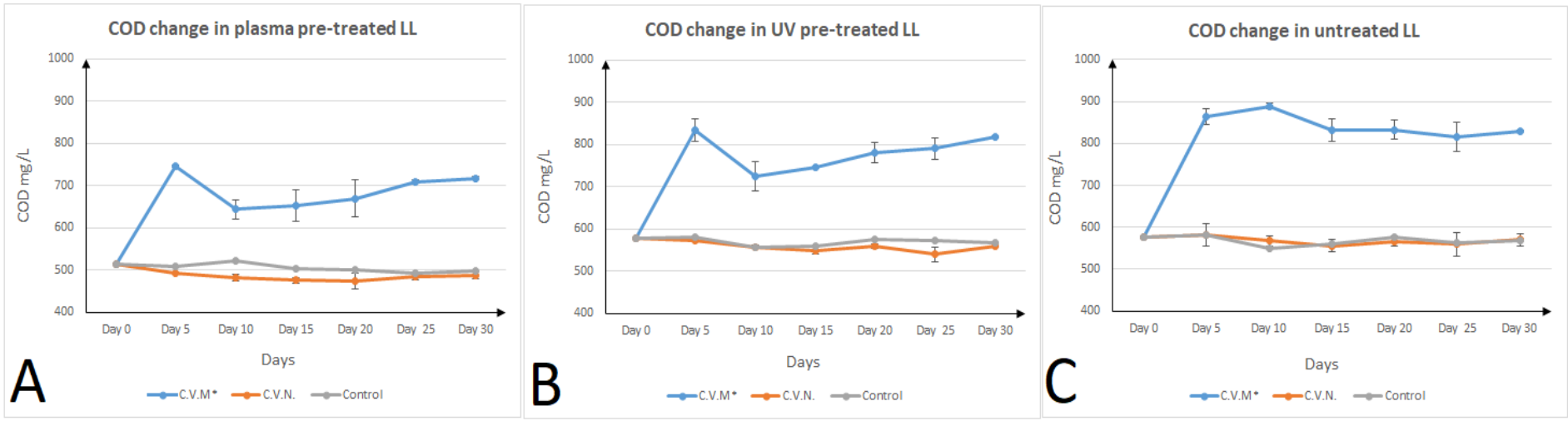

Figure 6-15 Change in COD throughout the time period of the experiment (30 days) as recorded every five days for the two tested microalgae (C.V.M* & C.V.N) in the different treatments: A) plasma pre-treatment LL; B) UV pre-treatment LL; C) Untreated LL. Mean and standard deviation values of two biological replicates are plotted.

II. Heavy metals content

Assessing the concentrations of four heavy metals before and after the experiment was carried out as mentioned previously.

The concentration of Arsenic (As) at Day 0 and Day 30 for the tested microalgal strains in all different treatments was recorded and presented in Figure 6-16. The strain C.V.M* didn't exhibit any significant removal (p<0.05) for Arsenic in case of the plasma pre-treated LL whilst it didn't show any removal at all in the UV pre-treated and untreated LL. On the other hand, the strain C.V.N also didn't show any significant *As* removal (p<0.05) when compared to the control in case of the plasma pre-treated and the UV pre-treated LL. However, a removal percentage of 19% was recorded in the case of C.V.N in the untreated LL which was significantly higher than that of the control (p<0.05).

In terms of Copper, neither C.V.M* nor C.V.N showed any significant Copper removal in any of the tested treatments (p<0.05) as shown in Figure 6-17.

No removal was recorded for the other tested heavy metals (*Pb* and *Cr*) for any of the tested microalgal strains in the different treatments.

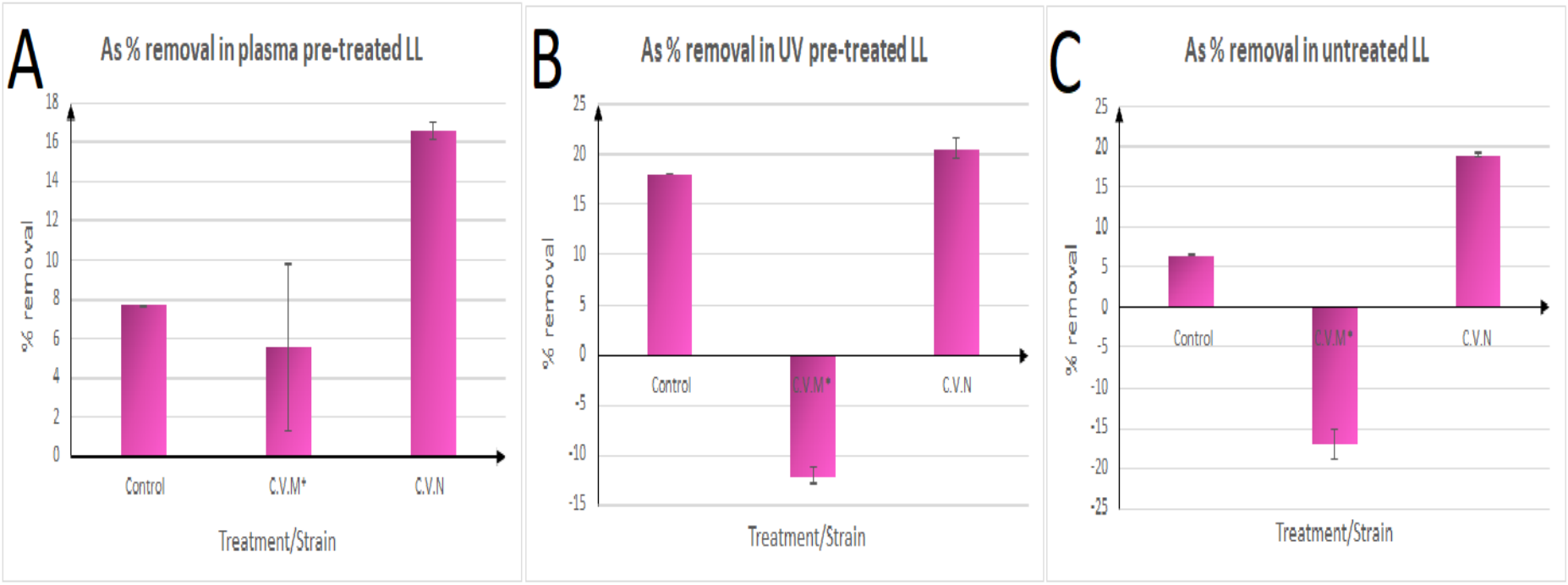

Figure 6-16 Percentage of Arsenic (*As*) removal of the two tested microalgal strains (C.V.M* & C.V.N) Vs control in the different treatments: A)plasma pre-tretament LL; B) UV pre-treatment LL; C) Untreated LL. Mean and standard deviation values of two biological replicates are plotted.

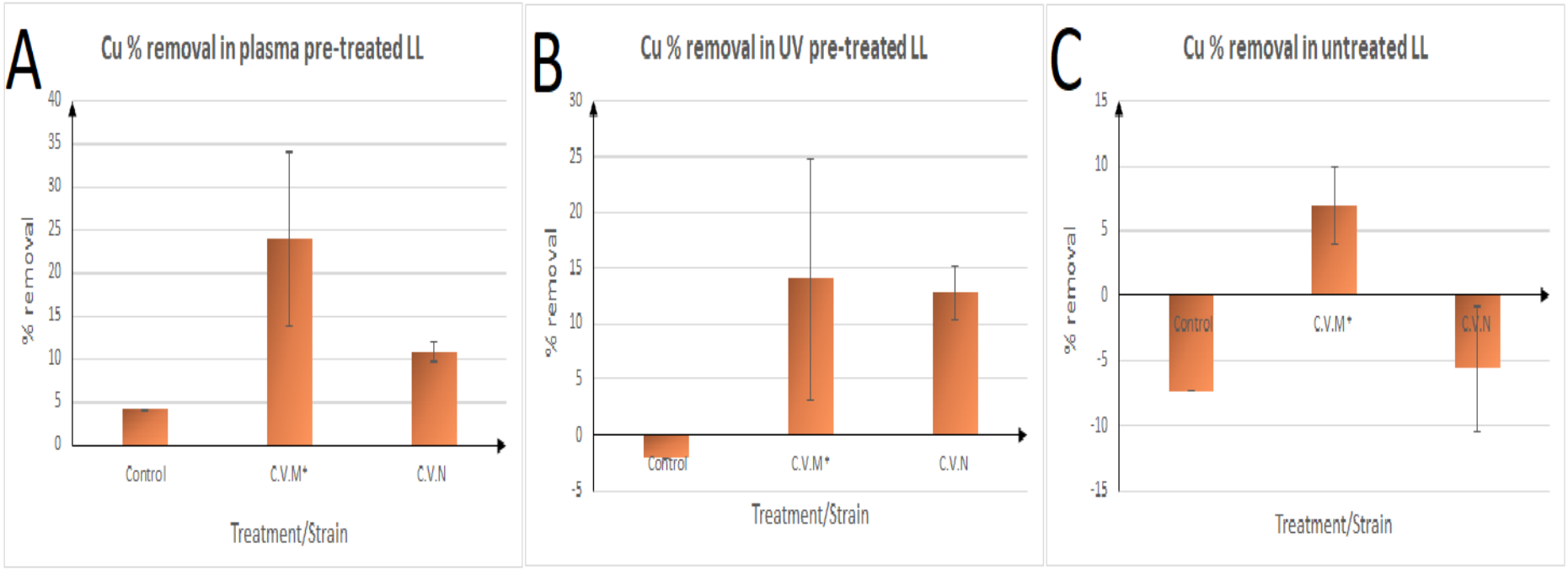

Figure 6-17 Percentage of Copper (*Cu*) removal of the two tested microalgal strains (C.V.M* & C.V.N) Vs control in the different treatments: A)plasma pre-treatment LL; B) UV pre-treatment LL; C) Untreated LL. Mean and standard deviation values of two biological replicates are plotted.

6.4.4. Genome sequencing for C.v.1.1 (C.V.M*) & C.v.1.2 (C.V.N):

The two tested *Chlorella vulgaris* strains did not show significant differences when examined microscopically, except that cells of C.V.N might be slightly bigger than cells of C.V.M* (Figure 6-18), that is why a meticulous differentiation tool on the genomic level was essential.

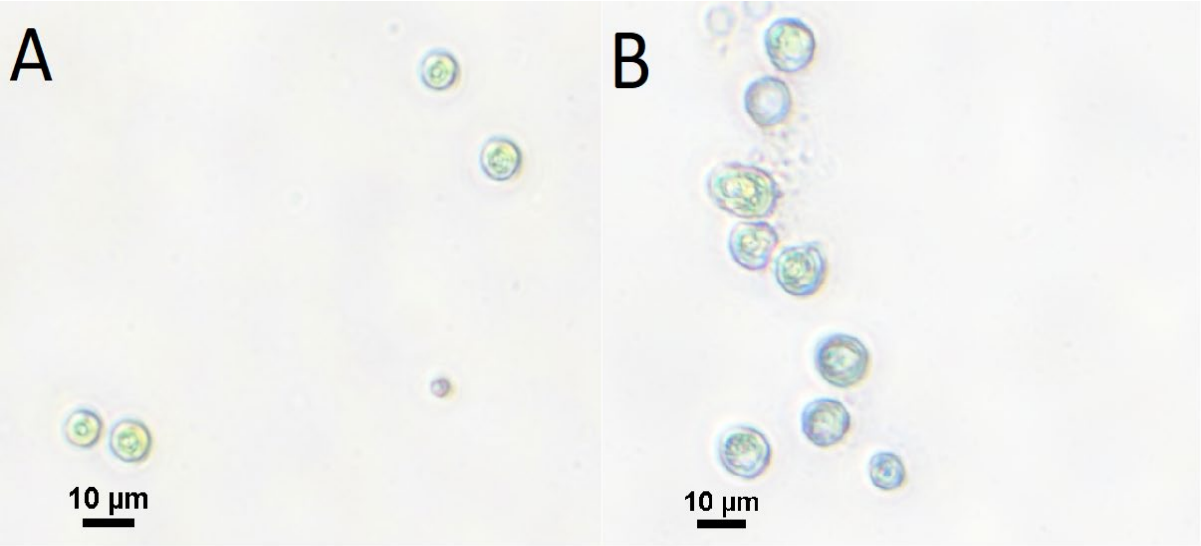

Figure 6-18 Light microscope pictures for the two tested *Chlorella vulgaris* strains at the end of the experiment A) The strain C.V.M* B) The strain C.V.N.

Comparing all the SNP (single nucleotide polymorphism) loci of C.V.N and C.V.M*, the genotypes of 347218 SNP loci were found to be the same in both types. In the same context, when comparing the indels of C.V.N and C.V.M*, 39509 indel loci were similar between the two. However, the analysis also revealed that there are 15169 SNPs and 2046 indels with varying genotypes between C.V.N and C.V.M* (excluding the deletion loci (./.).

6.5. Discussion

Efficient removal of contaminants from LL (especially ammonia-N and COD) might require a combination of both physical-chemical and biological treatments approaches (Luo *et al.*, 2020). In this study, two different physical pre-treatments approaches were tested; plasma pre-treatment as well as UV pre-treatment and were compared against no treatment for 20% diluted raw LL. The physical pre-treatment steps were followed

by adding the green microalgal strains (C.V.M* and C.V.N) to the pre-treated/untreated LL as the subsequent biological treatment step while assessing the algal growth profiles and the LL treatment efficiency during a 30-day experiment.

Comparing the chemical characters of the 20% LL after plasma/UV pre-treatment as well as untreated LL prior to the microalgae inoculation revealed that the pH didn't show a significant change in any of the treatments, on the other hand, the COD exhibited a significant change in the different treatments with the highest reduction in COD levels recorded for the plasma pre-treatment which was 10.6% less than that of the untreated LL. The COD levels in the UV pre-treated LL and the untreated LL did not show a marginal difference in their values yet they showed a statistically significant difference. Our results were in agreement with those reported by Silva, Dezotti and Sant'Anna Jr (2004) who found that the COD removal percentages were in a moderate range of 2%-12% with 0.1-1.5 ozone dose i.e., the amount of ozone absorbed per litre of the effluent (g O_3/l), and the highest dose of ozone used (3 g/l) only induced the removal percentage of 48% of COD of the tested LL whilst the TOC (Total Organic Carbon) actually showed an increase rather than decrease in content with different doses of ozone used. This might be attributed to the incomplete oxidation of the compounds found in LL. Landfill leachates contain high molecular mass molecules that could undergo partial break down (by ozonation) to yield smaller molecules which might in turn be completely or partially oxidised thus still be detected by the analysis equipment (Silva, Dezotti and Sant'Anna Jr, 2004). The ammonia-N content also showed significant difference prior to the different pre-treatments, with the lowest ammonia-N content recorded for the plasma pre-treated LL (151.38 mg/L) while the UV pre-treated LL was significantly higher in its ammonia-N content (219.43 mg/L), and the untreated LL recorded the highest ammonia-N level (290.73 mg/L) which was significantly higher than the former treatments. Zhao *et al.* (2011) also reported that plasma treatment for industrial LL using atmospheric pressure dielectric barrier discharge (DBD) system affected the ammonia-N removal significantly. Different activated species (including ozone) produced by plasma/UV pre-treatments might be responsible for the ammonia-N removal by the oxidation of the ammonia to form ammonium nitrates (Zhao *et al.*,

2011), which might be stronger in case of the plasma treatment rather than the UV treatment. Another possible reason for the ammonia-N removal from both the plasma and UV pre-treated LL might be the bubbling effect (of the bubbled air) in case of the plasma treated LL and the stirring effect (of magnetic stirrers) in case of the UV treated LL, which might induce ammonia stripping and thus account for some of the ammonia-N loss compared to the untreated LL.

The bench-scale plasma reactor used in this study induced a colour change in the treated diluted LL, as the colour of LL changed from dark brown to yellow after three hours of plasma pre-treatment. On the contrary, Zhao *et al.* (2011) reported a change in LL colour from deep grey-black to deep yellow after one hour of plasma treatment and a further change to light yellow after two hours, whilst almost a complete decolourisation happened after six hours. Moreover, Singh *et al.* (2021) reported a colour change in the LL after 30 minutes of plasma treatment. This may be explained by the ability of the activated species produced as a result of plasma treatment to oxidise and cleave the chromophores thus resulting in a colour change/removal (Silva, Dezotti and Sant'Anna Jr, 2004; Zhao *et al.*, 2011). Different time intervals required for the colour change to occur after plasma treatment might be a result of difference in the variable conditions of the plasma bioreactor and/or the produced plasma used in each study.

After three hours of UV-lamp treatment (with ozone produced), no change in the leachate colour was observed which indicates that the chromophores were not destroyed to the level that induces a colour change, this might imply that the used UV treatment did not induce the production of sufficient activated species to perform significant oxidation in the tested time interval (three hours) as compared to the plasma treatment. Shu *et al.* (2006) on the other side, found that 91% of the colour of 20% diluted LL could be removed using four 36-W UV lamps and 232.7 mM of H_2O_2 in 120 minutes of treatment. Shu *et al.* (2006) explained these results by the increase in the formation of free radicals (e.g., $OH^{\bullet}$) with a faster rate as a result of the higher UV lamp power used in their study thus achieving a better colour removal rate.

After applying the different physical pre-treatments to the tested diluted 20% LL, the two tested strains of *Chlorella vulgaris* (C.V.M* & C.V.N) were inoculated in duplicates into each treatment. The experiment was run for 30 days, at the end of the experiment it was observed that the green colour indicating algal growth was more observed in the strain C.V.M* growing in all treatments as well as the untreated LL, on the other hand the strain C.V.N did not develop such a dense growth. All the control treatments did not show any green growth nor turbidity in colour to indicate a contamination. During the time interval of the experiment, algal growth and LL characters were monitored every five days.

Growth of the two strains of the green microalga *Chlorella vulgaris* (C.V.M* and C.V.N) in the different LL pre-treatments was estimated by optical density measurements every five days as well as dry weight measurements at the end of the experiment after thirty days. Results indicated that the highest growth was achieved by the strain C.V.M* grown in plasma treated LL and it was significantly higher than the other strain C.V.N (p<0.05) in all the tested treatments in case of OD measurements, similar results were obtained by the dry weight measurements. However, there was no significant difference in the growth of the strain C.V.M* (as indicated by OD and dry weight measurements) in case of the different treatments ($p < 0.05$). These results indicates that different pre-treatments applied to the tested LL didn't induce a significant growth increase in the highly growing strain C.V.M*, however the dramatic increase in growth exhibited by the strain C.V.M* over the strain C.V.N could be directly related to the robustness of this strain and its tolerance to the tested LL. This conclusion is further supported by the findings of Quan *et al.* (2020) who reported an increase in growth rate in case of the green microalga *Scenedesmus* sp. grown in LL pre-treated with ozone compared to that in untreated LL. Quan *et al.* (2020) mentioned a higher biofilm density (18.9 g/m^2) in case of the oxidised (ozone treated) LL compared to that in untreated LL (12.7 g/m^2) and it was concluded that this might be a result of the improvement achieved by ozonisation which helped reducing the macromolecular organics content in LL as well as reducing its chromaticity, as organic macromolecules content and the dark colour of leachate might be possible reasons for hindering microalgal growth by being toxic and

preventing light transfer, respectively. Interestingly, our results did not show a significant enhancement in growth in case of plasma or UV treatments compared to the untreated LL. However, the strain C.V.M* outperformed its peer C.V.N by 18.7 times and 7.5 times as estimated by OD measurements and dry weight measurements in untreated LL, respectively, which implies that strain C.V.M* is capable of a better tolerance for the harsh environment of the raw diluted untreated LL (20%) with all its contents of toxic organic and inorganic compounds as well as its dark colour with lower light transmittance.

Ammonia-N (especially in high concentrations) is one of the most important toxicants in LL and in turn one of the key parameters during LL treatment. Removal of ammonia-N throughout the experiment was detected every five days, for the two tested strains in the different LL pre-treatments. It was evident from the present study that ammonia-N removal (expressed as percentage) was significantly higher in case of the strain C.V.M* than the other tested strain C.V.N for all the tested pre-treatments. The highest removal percentage for C.V.M* was recorded at the twenty fifth day of the experiment, nonetheless the decrease in the ammonia-N content was still significant from the beginning of the experiment (Day 0) until the end (Day 30) for C.V.M* in case of the plasma/UV LL as well as the untreated LL. These results are very interesting compared to the results presented in a recent study conducted by Zheng *et al.* (2019), who highlighted the significant effect (p<0.05) induced by the ammonia concentration in wastewater on the cell viability of *Chlorella vulgaris*. In their study, Zheng *et al.* (2019) reported the toxic effect of high ammonia concentration (> 110 mg/L) on the cell viability, biomass concentration and biomass productivity of the green alga *Chlorella vulgaris* and they stated that both cell viability and biomass concentration of *Chlorella vulgaris* decreased significantly (p<0.05) with increasing ammonium concentration above 110 mg L^{-1}. They further concluded that the threshold for the ammonia toxicity in their study is 110 mg/L and concentrations exceeding this value might induce microalgal growth inhibition by ammonia toxicity. In view of such results, it could be concluded that the strain C.V.M*, which showed positive correlation between growth and significant ammonia removal (p<0.05) in LL with ammonia-N concertation; 151.38 mg/L, 219.43 mg/L and 290.73 mg/L in case of

plasma pre-treated LL, UV pre-treated LL and untreated LL, respectively, could be deemed an ammoniacal-N tolerant strain with high capability of thriving in 20% raw LL with relatively high ammonia-N concentrations. The threshold of ammonia-N tolerance in wastewater might vary depending on the microalgal species (Zheng *et al.*, 2019). However, in the present study the *Chlorella vulgaris* strain C.V.M* exhibited a substantial ammonia-N removal of 75% when grown in untreated LL with an initial ammonia-N concentration of 290.73 mg/L indicating its tolerance for high ammonia-N concentrations i.e., concentrations above 134 mg L^{-1} (Lin *et al.*, 2007) and above 110 mg L^{-1} (Zheng *et al.*, 2019). However, the highest ammonia-N removal was obtained in case of C.V.M* growing in plasma pre-treated LL that together achieved 79% removal of the total ammonia-N concentration at day 25 of the experiment, plasma pre-treatment reduced the ammonia-N concentration from 290.73 mg $L^{-1}$to 151.38 mg L^{-1}, after which it was further reduced by growth of C.V.M* towards the end of experiment (Day 25) to 60 mg L^{-1}. This result might be explained by three possible reasons; the first is that the activated species produced by the plasma pre-treatment might have caused ammonia removal by oxidising the ammonia to form nitrates (Zhao *et al.*, 2011) which was evident by the reduction of ammonia concentration by 1.9-fold after plasma pre-treatment, the second is the effect of ammonia removal by the growing strain C.V.M* in LL which was discussed above and the third is a small removal percentage by the effect of volatilisation due to continuous stirring throughout the experiment. However, stirring only accounts for an insignificant removal percentage (p<0.05) as indicated by the control treatments results at the end of the experiment. Reduction in ammonia-N removal percentage of control treatments in this experiment compared to the control treatment in the screening experiment (in the previous chapter) might be explained by the higher speed used in the previous experiment as a result of using 1L Duran bottles with 500 ml of tested 20% LL whilst in this experiment smaller volumes were used (100 ml) in smaller Duran bottles (250 ml) thus less speed was used in stirring which might have caused less ammonia-N loss due to volatilisation/air-stripping.

A significant reduction (p<0.05) in the pH values (from day 0 to day 30) in case of the strain C.V.M* in all treatments of LL was recorded, these results agree with the

findings from the ammonia-N removal experiment mentioned earlier. The pH values decrease as the ammonia-N uptake increases and this was explained in detail in the discussion part of the previous chapter.

In contradiction to the pH results, the COD values exhibited a significant increase ($p < 0.05$) in case of C.V.M* in all the tested pre-treatments, however the COD values in case of C.V.N (all treatments) and control (all treatments) did not show any significant change from Day 0 to Day 30 of the experiment. These results are also in agreement with all the above results where the strain C.V.M* showed a significant growth, ammonia-N removal and pH decrease in all the tested LL treatments, reasons explaining similar results were discussed in detail in the previous chapter.

Different pre-treatments (plasma/UV) of LL did not induce any significant increase in the removal efficiency of any of the tested heavy metals in case of both strains C.V.M* and C.V.N. A 19% significant removal ($p < 0.05$) of Arsenic (As) was recorded in case of C.V.N in untreated LL. No clear explanation could be found to explain the observed results regarding the heavy metals uptake and there is hardly enough information in the literature in the same context.

The genus *Chlorella* is one of the most common genera of green algae (Chlorophyta) which has wide applications in several fields e.g., biofuel production (Gao *et al.*, 2014; Guarnieri *et al.*, 2018); wastewater treatment (Wu *et al.*, 2019; Zheng *et al.*, 2019) and as a model in different molecular biology studies (Blanc *et al.*, 2010) as well as several other biotechnological applications. *Chlorella vulgaris* is one of the most promising green microalgae in wastewater treatment in general (Zheng *et al.*, 2019) and landfill leachate treatment in particular (Okurowska *et al.*, 2021). Dramatic increase in the OD measurements, dry weight production and ammonia-N uptake of the strain C.V.M* over the strain C.V.N when grown in 20% LL, despite being originally replicates for each other and having no sharp differences under microscopic examination nor clear sign of a contaminating organism in the culture, suggested the necessity of performing DNA extraction for both strains as well as carrying out whole genome sequencing for both, to be able to differentiate between them on the molecular basis. Results indicated a high degree of similarity between the two strains as observed by the 347218 SNPs

and 39509 indels shared between them, however, the genotypes of 15169 SNP loci and 2046 indel loci were different. The differences in genotypes might have affected expression of cell cycle genes and/or stress response genes. Unfortunately, due to the absence of GFF/GFF3/GTF files for *Chlorella vulgaris* strains in public databases, genome annotation could not be performed which prevented us from the possibility of making biological sense of the sequences of both strains when mapped to reference sequences. We could only hypothesize the possibility of occurrence of random mutation, which could have happened allowing the *Chlorella vulgaris* strain (C.V.M*) (CCAP 211/141) to show better tolerance for the harsher environment of landfill leachate. Another possible explanation could be the possibility of occurrence of sexual reproduction which might have been triggered by stress conditions and resulted in the production of a more resistant zygote. Although, no sexual life cycle was previously described in the genus *Chlorella* and it has long been assumed to be asexual (Blanc *et al.*, 2010) but recent evidence regarding the presence of meiosis genes in *Chlorella variabilis* NC64A and *Chlorella vulgaris* UTEX 395 was reported by Blanc *et al.* (2010) and Guarnieri *et al.* (2018), respectively. Future work is required to perform genome annotation on the available sequences of strain C.V.M* so as to have a better understanding for the genes responsible for tolerating the harsh conditions of LL and those responsible for the high growth and increased ammonia-N uptake as well as optimising conditions for their growth in addition to optimising the physical steps (e.g. plasma/UV) used in LL pre-treatments thus providing better approaches for LL treatment in a simple, sustainable, and cost-effective way.

6.6. Conclusions

A strain of *Chlorella vulgaris* (C.V.M*) (CCAP 211/141) was proven to be tolerant to the harsh environmental conditions of 20% diluted LL (v/v) as it exhibited a dramatic increase in growth by almost 19-fold compared to its peer (C.V.N) when grown in 20% untreated LL. A significant ammonia-N removal percentage was also observed in strain C.V.M* over strain C.V.N by 75%. However, this percentage was further improved when LL was subjected to plasma pre-treatment first which helped reduce the initial amount of ammonia-N LL by 1.9-fold from 290.73 mg $L^{-1}$to 151.38 mg L^{-1},

after which it was further reduced by growth of C.V.M* to 60 mg L^{-1} i.e. (79% removal) with significant decrease in the pH value from 8.6 to 6.67 at the end of the experiment. Complete genome sequencing for both strains revealed different SNPs and Indels which might suggest the possibility of mutation or sexual reproduction that might have possibly conferred the advantages of better adapting to the harsh environment of LL with its high ammonia-N content. The strain *Chlorella vulgaris* (C.V.M*) might provide a robust candidate for LL treatment with the possibility of utilising plasma as a pre-treatment step, prior to the biological treatment of leachate, to allow better algal growth and ammonia-N uptake, due to the ammonia reducing effect of the plasma treatment as well as its decolourising effect which might allow better light transmittance thus better light availability for microalgal growth. Future work is required for optimising the integrated plasma-microalgal approach for LL treatment with better understanding, on the molecular level, for the genes responsible for LL tolerance in the *Chlorella vulgaris* strain C.V.M*.

CHAPTER SEVEN

Concluding Remarks and Recommendations

With a world-wide growing population, a concomitant increasing production of both municipal and industrial solid wastes is seemingly inevitable (Kumari, Ghosh and Thakur, 2016). The generated municipal solid wastes (MSW) are usually landfilled as landfilling represents an affordable method with less environmental insults compared to other methods e.g., compositing and incineration, respectively (Renou *et al.*, 2008; Kurniawan *et al.*, 2010). The amount of landfilled MSW, on a global level, is estimated to be around 1.5 billion tons(Themelis and Ulloa, 2007). Landfilling, either utilising engineered landfills, or non-engineered landfills/open dumps results in the production of a complex, toxic (with varying degrees) and challenging liquid, known as Landfill leachate, which must be treated before being discharged into the environment as it poses severe hazards to the environment and different living organisms as well as human beings (Renou *et al.*, 2008; Paskuliakova, Tonry and Touzet, 2016; Kumari, Ghosh and Thakur, 2016; Viegas *et al.*, 2021). In the same context, it is worth mentioning that engineered landfills have baseliners and leachate collecting systems for leachate treatment before being discharged, however the major threat arises from non-engineered landfills/open dumps where the infiltration and subsequent contamination of LL to the ground and surface water bodies represent an inevitable danger (Kumari, Ghosh and Thakur, 2016; Parvin and Tareq, 2021). Hence landfill leachate treatment is a necessity in an efficient, eco-friendly, and cost-effective way. Treatment methods of landfill leachate (LL) could be classified into four main categories: conventional methods, biological methods, physical-chemical methods and integrated methods, each category per se include several methods with varying

degrees of efficiency, cost-effectiveness, and impacts on the environment (Renou *et al.*, 2008; Dogaris, Ammar and Philippidis, 2020; Luo *et al.*, 2020; Teng *et al.*, 2021; Wijekoon *et al.*, 2022). This study focuses on LL treatment using a novel integrated approach combining a biological method (using green microalgae) with a physical method (using plasma/UV).

Landfill leachate treatment using microalgae is gaining an increased attention as an appealing biological treatment method with the possibility of coupling leachate treatment with the production of valuable algal biomass which can be further utilised in several purposes (e.g., biofuel production) thus representing an addition to the sustainability and possibly the cost-effectiveness of the whole LL treatment process. Microalgae have shown promising results in this context with several successful trials for growing them in diluted/pre-treated LL, on both lab-scale and larger-scale, however strains isolated from raw leachate itself and/or wet surfaces with leachate runoff (with or without laboratory adaptation) have shown even more interesting results regarding tolerating higher LL concentrations with significant growth and/or significant contaminants removal from leachate (Cheung, Chu and Wong, 1993; Paskuliakova, Tonry and Touzet, 2016; Neofotis *et al.*, 2016; Dogaris, Ammar and Philippidis, 2020; Nawaz *et al.*, 2020; Okurowska *et al.*, 2021). This in turn inspired part of the hypothesis proposed for this study regarding exploring the untapped reservoir of indigenous microalgae naturally inhabiting soil surfaces with leachate runoff in a LL treatment site in Chesterfield, UK, where soil samples with apparent algal growth were collected and transferred to the lab after which subsequent steps of isolation, purification and molecular biology-based identification were carried out.

Combining traditional morphological identification (for the isolated microalgal strains) with molecular markers/genetic based identification is considered a reliable method for a powerful identification of the isolated species on a genus and/or species level whilst reflecting their phylogenetic lineages (Smith, 2016; Kunrunmi, Adesalu and Kumar, 2017; Ferro, Gentili and Funk, 2018). Although identification of algal strains was usually conducted based on the partial amplification of the large subunit rDNA (28S) and/or the small subunit rDNA (18S), followed by subsequent

sequencing and NCBI BLAST analysis (Paskuliakova, Tonry and Touzet, 2016; Nordin, Samsudin and Yusof, 2019), however amplification of the 18S, ITS1, 5.8S and ITS2 regions (to get a better idea of the species identity) was also reported (Neofotis *et al.*, 2016). In the current study, identification of the isolated algal strains was carried out through partial PCR amplification of the small subunit rDNA (18S) coupled with PCR amplification of the entire internal transcribed spacer (ITS) region (ITS1, 5.8S and ITS2) followed by contig for the resultant purified PCR products (after the subsequent sequencing was performed) then NCBI BLAST analysis was carried out. This resulted in more confident and accurate results which was further supported by phylogenetic analysis. The results yielded six stains, four of which were revealed to be green microalgal strains as well as two cyanobacterial strains. The four isolated green microalgae included two strains of *Chlorella vulgaris*, one strain of *Chlorococcum* species and one strain of *Scotiellopsis reticulata*. However, the other two strains could not be identified using the abovementioned protocols but were successfully identified by amplifying the 16S rDNA regions yielding sequences that suggested they belonged to the prokaryotic group of cyanobacteria and were identified as two strains of *Phormidium autumnale.* The two cyanobacterial strains of *Phormidium autumnale* were excluded from any further research in this study because of their reported toxin-production activity (McAllister, Wood and Hawes, 2016). To the best of the authors knowledge, this is the first report on isolation of a *Scotiellopsis reticulata* strain from a landfill leachate treatment site in the UK. Further details on the above section could be found in the discussion part of Chapter three.

The isolated green microalgal strains were grown in BG11 medium as well as different LL concentrations to screen the maximum LL concentration threshold tolerated by the tested algae. In addition to the BG11 medium, five different concentrations of LL (v/v) diluted with sterile distilled water (5%, 10%, 15%, 20% and 25%) were used as media for growing different tested algal strains for a period of 30 days, their growth was determined by cell count every three days. The fact that the growth medium (BG11) supported the highest algal growth observed compared to all the other tested LL concentrations for all the tested algal strains, wasn't surprising due to the well reported LL toxicity (especially in higher concentrations) to algae (Cheung, Chu and

Wong, 1993; Lin *et al.*, 2007; Zhao *et al.*, 2014; El Ouaer *et al.*, 2020). As mentioned earlier in the discussion part of chapter four, the obtained results will be divided into three sections based on the tested LL concentrations: ≤15%, 20% and ≥25%. The lowest tested concentrations ≤15% induced significant increase (p<0.05) in cell densities of all the tested strains with the exception of *Chlorococcum* sp. whose growth increase wasn't significant (p<0.05). This could be attributed to the presence of some nutrients (e.g., organic compounds and ammonia-N) in appropriate amounts that could stimulate algal growth in varying degrees and mitigate LL toxicity (Cheung, Chu and Wong, 1993; Lin *et al.*, 2007). However, concentration 20% induced significant increase in growth (p<0.05) of only *Chlorella vulgaris* (both strains), whilst the highest concentration tested (25%) did not enhance the growth of any of the examined strains throughout the time of the experiment. Higher LL concentrations (≥25%) usually induce inhibitory effects on algal growth (Cheung, Chu and Wong, 1993; Lin *et al.*, 2007; El Ouaer *et al.*, 2016, 2020). This is generally due to multiple reasons such as relatively high ammonia content (Lin *et al.*, 2007), toxic levels of various organic compounds present in leachate, relatively high leachate pH (≥ 8) which might further induce the existence of some salts and/or heavy metals in more toxic forms (Cheung, Chu and Wong, 1993) in addition to the opaqueness of LL which represent a major obstacle for photosynthetic organisms like microalgae (Cheung, Chu and Wong, 1993; Okurowska *et al.*, 2021). This in turn encouraged selecting the concentration 20% LL (v/v) as a challenging concentration to be further tested because it represented the highest concentration that could be tolerated by at least two of the four tested strains, moreover it induced a significant increase in their growth which might indicate their possible tolerance to relatively higher leachate concentrations which in turn inspired a following experiment to test both growth and bioremediation ability of the different strains solely and collectively at the chosen concentration 20% for the purpose of selecting the most powerful candidate/s in LL treatment whilst producing the highest biomass. Further discussion on this part could be found in the discussion section of chapter four.

Although several different factors may significantly affect the composition of landfill leachate such as composition of wastes, their degradation stage, amount of rainfall,

hydrogeology, nonetheless landfill age is a key factor in controlling leachate composition (Kjeldsen *et al.*, 2002; Li, Zhou and Hua, 2010). Analysis of chemical characteristics of the landfill leachate used in this study suggests it is relatively old (stabilised) LL with relatively high pH value (> 8), COD value of around 2272±8 mg/L, relatively high ammonia content (1475± 64 mg/L) as well as relatively low Heavy metals content. Screening experiment (as mentioned earlier) was conducted to select the most efficient strain/s in growth and nutrients/pollutants removal in the concentration 20% (v/v) of the tested old/stabilised LL used in this study, the experiment was carried out for 30 days with regular monitoring for growth and nutrients/pollutants removal every five days. Although each strain was tested in duplicate, interestingly one of the two duplicates of the strain *Chlorella vulgaris* ATFG1 started showing a dramatic increase in growth with an evident darker green colour (starting from Day 15) together with a significant ammonia-N removal compared to its other duplicate as well as the rest of the tested strains. No observed contamination was noticed when examining the highly growing duplicate of the *Chlorella vulgaris* strain (C.v.1.1) under light microscope, however a complete genome sequencing was performed eventually to be able to determine any genotype change that might have affected the higher tolerance of this strain towards the tested LL conc. (20%) reflected by its higher growth in it and higher ammonia-N uptake levels. The strain C.v.1.1 exhibited a significant (p<0.01) higher growth (as shown by OD and dry wight measurements) compared to the rest of the treatments, furthermore it outperformed the growth of its duplicate C.v.1.2 by 3.5-fold with a final dry weight yield of 0.28 g/L. The outstanding growth of the strain C.v.1.1 was also accompanied by a significant ammonia-N uptake (86.7%) and a dramatic decrease in the pH value (from 8.3 to 4.8) of the tested culture. Nevertheless, it is worth mentioning that although the highest ammonia-N uptake level was recorded for the strain C.v.1.1 but the ammonia-N uptake level recorded for the control treatment was 76.2% which suggests that ammonia-N uptake in the culture C.v.1.1 couldn't be fully attributed to the biological uptake of the tested microalga but part of the ammonia-N loss could possibly be as a result of the stripping/volatilisation effect due to the continuous stirring throughout the experiment. In the same context, this speculation was supported by Sniffen, Sales

and Olson (2018) who tracked the nitrogen removal in an algae-based LL remediation system and concluded that not all the nitrogen removal was assimilated into a biological algal biomass nor dissolved inorganic nitrogen in the system but some of the gaseous nitrogen species were lost due to volatilisation which accounted for part of the total nitrogen loss from the system. On the contrary the COD levels in the culture of C.v.1.1 showed a significant increase from Day 0-30 of the experiment which could be explained by the production of microalgal biomass as a result of photosynthesis performed by the increasingly growing algal strain (C.v.1.1) which in turn account for the increase in the dissolved organic matter (Zhao *et al.*, 2014) and/or the production of extracellular polymeric substances (EPS) which could also contribute for the soluble COD values (Cuellar-Bermudez *et al.*, 2017). Thus, it may be concluded the recorded significant increase in the COD levels observed in the culture of strain C.v.1.1 comes in line with the significant increase in its growth ($p<0.05$). In an old/stabilised LL the heavy metals concentrations are usually low, only one of the four tested heavy metals (Arsenic) was significantly removed by 31.2% and 32.9%, in the treatments C.v.1.1 and C.v.1.2, respectively, which was significantly higher than the removal percentage recorded for the control treatment ($p<0.01$). More detailed discussion about this part could be found in the discussion section of chapter five.

Although the two replicates of the strain *Chlorella vulgaris* ATFG1 were inoculated from the same culture, however they showed major different behaviour in growth and nutrients/pollutants uptake during the screening experiment, as mentioned earlier. This in turn paved the way for developing two hypotheses in an attempt to understand and explain the different performance exhibited by the two replicates (C.v.1.1 and C.v.1.2), the first hypothesis was the possibility of contamination of one of the cultures with an outsider microorganism which was excluded when microscopic examination was performed showing no clear evidence for any contaminating organism, this led us to the second hypothesis which was the possibility of a developed mutation by strain C.v.1.1 and this was further tested by gDNA (genomic DNA) extraction and subsequent whole genome sequencing for both strains (C.v.1.1 and C.v.1.2) whose results will be discussed further in the chapter.

In order to test the reproducibility of the results discussed above regarding the two different strains (C.v.1.1 and C.v.1.2), another experiment was conducted growing both strains separately in 20% LL (v/v) for 30 days and monitoring their growth and bioremediation efficiencies every five days. This was done simultaneously with comparing the effect of untreated and plasma/UV pre-treated LL for both strains, as explained in detail in chapter six. Strains C.v.1.1 and C.v.1.2 are referred to as C.V.M* (M* denoting mutant) and C.V.N (N denoting Normal), respectively, in the following discussion.

It is widely reported in the literature that combining two or more techniques/methods of LL treatments especially physical-chemical and biological methods would provide better treatment efficiencies in terms of some contaminants removal such as ammonia-N, organic compounds (as indicated by COD levels) and/or colour, in addition to the possibility of counting for the cost-effectiveness of the whole leachate treatment process (Gotvajn, Tišler and Zagorc-Končan, 2009; Dogaris, Ammar and Philippidis, 2020; Luo *et al.*, 2020; Teng *et al.*, 2021; Wijekoon *et al.*, 2022). Accordingly, an integrated approach of combining biological treatment of LL using the two abovementioned strains of *Chlorella vulgaris* (C.V.M* and C.V.N) with a physical pre-treatment of the tested LL using plasma/UV was investigated for its effectiveness in enhancing better algal growth as well as achieving an overall better nutrients/pollutants removal.

Plasma/UV pre-treatments of the 20% LL (v/v) as compared to the respective untreated LL were examined in terms of five different parameters determining LL quality/characteristics to test the effect of physical pre-treatment step on the tested LL prior to the biological treatment using the two strains C.V.M* and C.V.N. The five parameters are NH_3-N, COD, pH, leachate colour, and heavy metals content, it was found that plasma pre-treatment of LL induced a significant decrease in NH_3-N content of LL by 1.9-fold compared to the untreated LL (from 290.73 mg/L to 151.38 mg/L) whilst the NH_3-N removal recorded in case of UV pre-treatment was less than that of the plasma pre-treatment but still significant when compared to the ammonia-N content in untreated LL. COD levels exhibited a significant change in different treatments with the highest reduction in COD (10.6%) recorded in case of plasma pre-

treatment compared to UV pre-treatment and non-treatment. In the same context an evident change in leachate colour from dark brown to yellow was observed after three hours of plasma pre-treatment and no change in colour was recorded after three hours of UV pre-treatment in the current study. No significant change was observed in the pH in different pre-treatments. Therefore, it could be concluded that plasma pre-treatment was superior to UV pre-treatment in terms of colour change, ammonia-N removal, and COD level decrease. These results could be explained based on the ability of different reactive species (including ozone) generated during plasma production to oxidise/degrade ammonia-N and different organics in the leachate as well as the chromophores accounting for landfill leachate dark brown colour (Misra *et al.*, 2011; Zhao *et al.*, 2011; Pankaj and Keener, 2017; Singh *et al.*, 2021).

After applying plasma/UV pre-treatments, the two tested strains (C.V.M* and C.V.N) were further tested for their growth and nutrients/pollutants removal in plasma/UV pre-treated LL as well as untreated LL for 30 days. Results indicated some interesting points in terms of algal growth and ammonia-N removal efficiency, it was shown that the highest growth and ammonia-N removal (79%) was recorded for the strain C.V.M* grown in plasma pre-treated LL compared to the other strain C.V.N in all the tested treatments. However, the growth increase and ammonia-N removal recorded for C.V.M* grown in plasma pre-treated and untreated LL was not significant ($p < 0.05$). These results indicates that different pre-treatments applied to the tested LL didn't induce a significant growth increase in the highly growing strain C.V.M*, however the dramatic increase in growth exhibited by the strain C.V.M* over the strain C.V.N could be directly related to the robustness of this strain and its tolerance to the tested LL. Whereas the strain C.V.M* outperformed its peer C.V.N dramatically by 18.7 times and 7.5 times as estimated by OD measurements and dry weight measurements in untreated LL, respectively. Moreover, strain C.V.M* exhibited a substantial ammonia-N removal of 75% when grown in untreated LL with an initial ammonia-N concentration of 290.73 mg/L indicating its tolerance for high ammonia-N concentrations compared to those reported in the literature to exert an inhibitory/toxic effect to microalgal growth i.e., concentrations ≥ 200 mg L^{-1} (Hernández-García *et al.*, 2019), above 135 mg L^{-1} (Lin *et al.*, 2007) and above 110 mg

L^{-1} (Zheng *et al.*, 2019). Thus, it could be concluded that strain C.V.M* is a highly ammonia-tolerant strain, however applying plasma pre-treatment improved (although not significantly) its growth and ammonia-N removal uptake. As discussed earlier in the results of the screening experiment (and in detail in the discussion section of chapter three), simultaneously with the significant increase in the growth and ammonia-N removal percentage in the case of the strain C.V.M* an observed significant decrease/drop in the pH (probably because of the ammonia-N uptake) was recorded. On the other hand, the COD levels exhibited a significant increase ($p<0.05$) in the highly growing strain of C.V.M* as explained earlier. Although, microalgae especially in a combined population could uptake metals efficiently (Richards and Mullins, 2013) however no significant removal for any of the tested metals in all the tested treatments for the strain C.V.M* could be detected which might possibly be due to insufficient removal or the relatively low concentrations of heavy metals characterising the old/stabilised leachate might not allow significant removal efficiencies of these metals by the growing microalgae.

In an attempt to reveal the genome level differences that might encounter the observed difference in the growth and ammonia tolerance/uptake pattern between the two strains C.V.M* and C.V.N, whole genome sequencing for both strains was carried out revealing a high degree of similarity between the two strains as observed by the 347218 SNPs and 39509 indels shared between them, however, the genotypes of 15169 SNP loci and 2046 indel loci were different. The differences in genotypes might have affected expression of cell cycle genes and/or stress response genes. Two main hypotheses were developed, first: the possibility of occurrence of random mutation, which could have happened allowing the strain C.V.M* to show better tolerance for the harsher environment of landfill leachate, second: the possibility of occurrence of sexual reproduction which might have been triggered by stress conditions and resulted in the production of a more resistant zygote. Although, no sexual life cycle was previously described in the genus *Chlorella* and it has long been assumed to be asexual (Blanc *et al.*, 2010) but recent evidence regarding the presence of meiosis genes in *Chlorella variabilis* NC64A and *Chlorella vulgaris* UTEX 395 was reported by Blanc *et*

al. (2010) and Guarnieri *et al.* (2018), respectively. More detailed discussion on this part could be found in the discussion section of chapter six.

7.1. Conclusion

A group of four green microalgae and two cyanobacteria were isolated from a landfill leachate treatment site in Chesterfield, UK. They were purified and further identified based on molecular biology tools where their genomic DNA was extracted and partial PCR amplification of the small subunit rDNA (18S) region in addition to complete PCR amplification of the ITS region was performed, this was followed by purification of the produced PCR products after which they were sent for sequencing. The contig of the resulting sequences was submitted to the NCBI GenBank and given accession numbers. The identification accuracy was further aided by phylogenetic analysis for the produced sequences which revealed four green microalgae including two strains of *Chlorella vulgaris* (ATFG1 and ATFG2), one strain of *Chlorococcum* sp. and one strain of *Scotiellopsis reticulata*. The other two strains were identified as cyanobacteria *Phormidium autumnale* (ATFG5 and ATFG6) by amplifying the 16S rDNA region. This was (to the best of the author's knowledge) the first report on a microalgal/cyanobacterial biodiversity in a landfill leachate treatment site in the UK, suggesting the possibility of having potential candidates in LL treatment from the natural microbiota inhabiting the local leachate environment.

The growth profiles of the four green microalgae were examined in different LL concentrations (5%,10%,15%,20% and 25%) to determine the threshold concentration tolerated by the examined microalgae. Although usually the concentration 10% is the preferable for algal growth as reported in several studies in the literature but both strains of *Chlorella vulgaris* exhibited significant growth increase in concentration 20% LL (v/v). Thus, concentration 20% (v/v) was further chosen as challenging concentration to screen the most powerful alga/e for growth and nutrients/pollutants removal.

One of the replicates of one of the *Chlorella vulgaris* strains (C.v.1.1/C.V.M*) started showing an outstanding growth with significant ammonia-N removal compared to its

other replicate with no sign of contamination under microscopic examination in the tested 20% LL concentration. Further experiment to detect the results reproducibility was conducted, similar results were obtained where one strain of *Chlorella vulgaris* (C.V.M*) achieved a dramatic increase by 19 times (in terms of optical density measurements) compared to its peer (C.v.1.2/C.V.N) with a significant removal of 75% of total ammonia-N in twenty-five days (starting from 290.73 mg/L) and a significant reduction in the pH value of the culture after ammonia removal and algal growth.

Plasma pre-treatment for three hours induced LL decolourisation which in turn caused LL colour to change from dark brown to yellow indicating possible degradation/breaking down of the chromogenic substances in leachate with a significant reduction in NH_3-N concentration (from 290.73 mg L^{-1} to 151.38 mg L^{-1}) i.e., by 1.9-fold and this percentage further decreased after algal treatment by the strain Chlorella vulgaris (C.V.M*) to 60 mg L^{-1} i.e. (79% removal), a significant reduction in pH level was concomitant. Moreover, integrating plasma pre-treatment before C.V.M* treatment induced a higher algal growth as well as higher ammonia removal compared to untreated LL.

However, a significant ammonia-N removal percentage (75%) was observed in untreated LL whereas the ammonia-N was initially 290.73 mg L^{-1} (a conc. reported to be highly inhibiting for algal growth in several studies). This indicates that strain C.V.M* is a high ammonia tolerant strain with high ability to grow and uptake ammonia from the tested challenging LL conc. (20%).

Whole genome sequencing for the two replicates of *Chlorella vulgaris* (C.V.M* and C.V.N) indicated the difference in genotypes of 15169 SNP loci and 2046 indel loci. These observed differences in genotypes might have affected expression of cell cycle genes and/or stress response genes. A hypothesis was developed speculating the possibility of occurrence of mutation or sexual reproduction which might be responsible for the high tolerance exhibited by the strain C.V.M* (CCAP 211/141)to the harsh LL conditions. This might provide a potent LL treatment candidate with high tolerance to ammonia-N (the main cause of toxicity in LL) which when being

coupled by plasma pre-treatment might provide a landfill leachate treatment approach which is efficient, eco-friendly, possibly sustainable as well as cost-effective.

7.2. Future work

Future work and recommendations proposed based on the obtained results:

1. Once publicly available, perform annotation to be able to make biological sense of the available sequences of C.V.M* to be able to relate its outstanding growth ability and ammonia-N tolerance/uptake in LL (20% v/v) to the corresponding genes thus become able to develop/maintain an efficient microalgal LL treatment system using a potent highly tolerant isolate/strain.

2. Optimise different factors of the *Chlorella vulgaris* strain C.V.M* regarding N:P ratio, different light regimes/intensities, P supplementation in different concentrations for better growth and performance in LL treatment.

3. Optimising plasma production/conditions to enhance a cost-effective integration of plasma pre-treatment with microalgae treatment for a better performing technology in LL treatment.

4. Diluting LL with municipal water rather than sterile distilled water for a better cost-effective system with less environmental footprints.

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Appendix

Appendix 1

PCR mixtures preparation for different primers.

PCR mixture	18S Lim primer	18S Huss primer	5.8S primer	ITS1 primer	ITS2 primer	16S primer	Control
Master Mix	50	50	20	20	20	50	20/50
Forward primer	10	10	4	4	4	10	4/20
Reverse primer	10	10	4	4	4	10	4/20
DNase/RNase free water	17.5	17.5	17	17	17	17.5	30/17
Genomic DNA	12.5	12.5	5	5	5	12.5	-
Total volume	100	100	50	50	50	100	50/100

Appendix 2

Conditions for running PCR using 18S Lim primer.

Step	Temperature	Time	Number of Cycles
Initial denaturation	94^{o}C	5 minutes	1
Denaturation Annealing Elongation	94^{o}C 58 ^{o}C 72 ^{o}C	30 seconds 30 seconds 1 minute	30
Final Elongation	72 ^{o}C	10 minutes	1

Appendix 3

Conditions for running PCR using 18S Huss primer.

Step	Temperature	Time	Number of Cycles
Initial denaturation	94°C	5 minutes	1
Denaturation Annealing Elongation	94°C 55 °C 72 °C	30 Seconds 30 Seconds 2 minutes	30
Final Elongation	72 °C	10 minutes	1

Appendix 4

Conditions for running PCR using 5.8S, ITS1 and ITS2 primers.

Step	Temperature	Time	Number of Cycles
Initial denaturation	94°C	5 minutes	1
Denaturation Annealing Elongation	94°C 55 °C 72 °C	30 seconds 30 seconds 1 minute	30
Final Elongation	72 °C	5 minutes	1

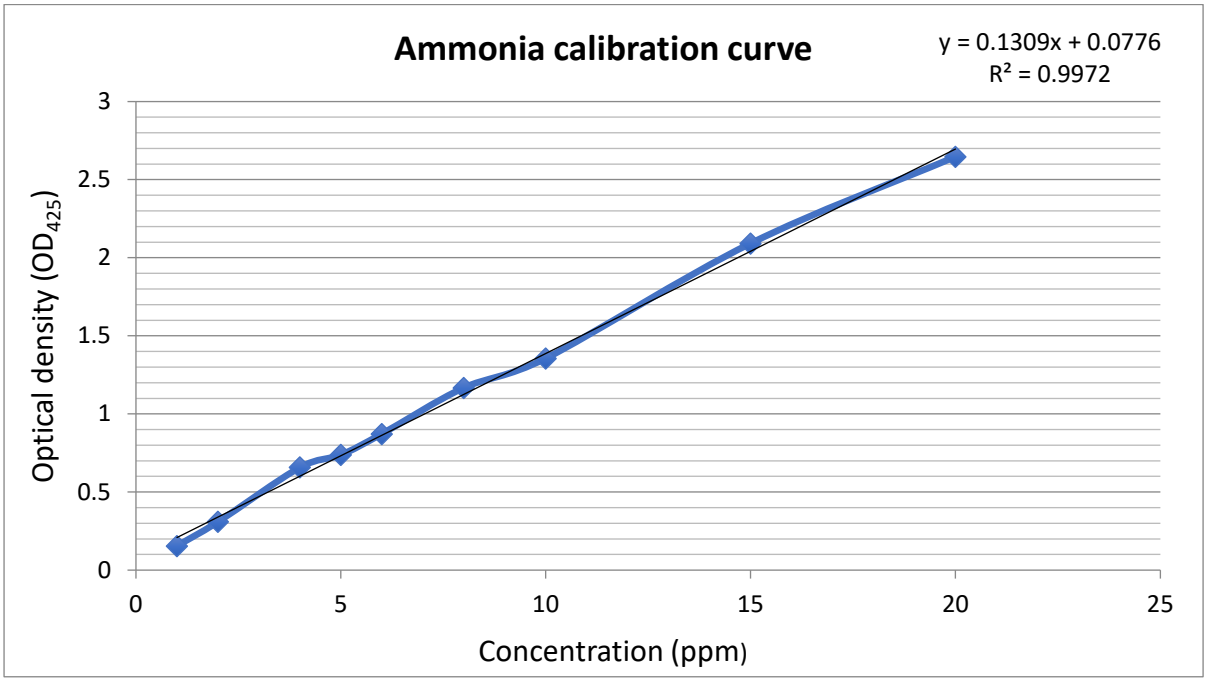
Appendix 5

Conditions for running PCR using 16S primer.

Step	Temperature	Time	Number of Cycles
Initial denaturation	94°C	3 minutes	1
Denaturation Annealing Elongation	95°C 58 °C 72 °C	1 minute 1 minute 1 minute	30
Final Elongation	72 °C	5 minutes	1

Appendix 6

Calibration curve for the ammonia-N analysis



Appendix 7

Calibration curve for the ammonia-N analysis