Microalgal Phycoremediation of Landfill Leachate

by

Andrea Paskuliakova

Ph.D.

Institute of Technology, Sligo

Supervisors: Dr. Nicolas Touzet, Steve Tonry

Submitted to the Institute of Technology, Sligo, May 2017

Declaration

I declare that this thesis is my own work, and that it has not been previously submitted to any other Institute or University.

I give permission to the Yeats library, Institute of Technology, Sligo to lend this thesis on request.

_____

Andrea Paskuliakova

May 2017

Acknowledgements

I would like to say thank you for all the help and support throughout the years of work on this project to the following people: my Mum, my friend Gerry, Lorraine, Rory and everybody else past and present from the best postgraduate office G2015, my supervisors at IT Sligo and the administrators of the President's Bursary Scheme, Laboratory Technicians and all the other postgraduates and staff with whom I was in contact. County Councils and landfill site operators who supplied me with the research materials, all the reviewers whose constructive criticism helped improve the final scripts and many other unnamed people who helped me in many different ways particularly through their presence and their small acts of kindness.

Sincere thank you, I could not have done it without you all.

Abstract

The aim of this project was to explore the use of microalgae in remediation of landfill leachate.

The isolation of microalgae strains tolerant to a combination of high dissolved salts and ammonia concentrations (typical for landfill leachates) was undertaken. The experiments were set up with low temperature and light intensity which makes microalgae-based phycoremediation relevant to conditions in Ireland.

The growth of several microalgal strains and the resultant nutrient depletion was evaluated in laboratory batch culture experiments. The *Chlamydomonas* sp. strain SW15aRL achieved the highest pollutant reduction whereby a decrease of 90.7% of ammonia-nitrogen within 24 days was observed in 10% raw leachate (~100 $mg \cdot l^{-1}$ NH_4^+-N) supplemented with phosphate. Further assessment of growth and nutrient reduction of strain SW15aRL was carried out across a number of different leachate samples to determine the effects that variable leachate composition can have on the sustainable growth of microalgae, when using leachate as the sole source of nutrients. Dilutions were applied to obtain 30 to 220 $mg \cdot l^{-1}$ NH_4^+-N concentrations. The strain SW15aRL was capable of growth in a variety of leachates but depended on the overall composition profile rather than just dilution. Phosphate addition appeared to be essential even though precipitation occurred in some instances. Both inhibitory and limiting factors were identified, highlighting that dilutions were needed to maintain the solubility of specific constituents and to keep the toxicity of others in check, yet the dilutions also reduced the concentrations of key nutrients and minerals. Finally, a toxicological evaluation showed microalgae treatment contributed to the reduction of pollutant levels and ecotoxicity. While the microalgae activity causes major macronutrient reduction, there are several other physicochemical processes which contribute to reduction such as precipitation and volatilisation while the contribution from coexisting bacterial communities is still poorly understood.

Table of contents

Chapter 1
Introduction

1.1 Overview

The integration of manufacturing, recycling and transformation of waste products into resources of renewable energy or other added value by-products constitute industrial ideals for independent, self-sufficient and ecologically aware economies. There are many goals to be yet achieved but there is considerable evidence that biotechnologies using microalgae have the potential to be used in such integrated processes.

Microalgae comprise a large number of species with various metabolisms. Wastewaters, effluents, leachates and various agricultural, industrial, municipal or household discharges can provide a rich source of nutrients on which the microalgae can grow. These waste streams are otherwise regarded just as sources of pollution that can cause environmental imbalances (i.e. algal blooms, harmful effects to some organisms) and require appropriate management and treatment (Carty et al., 1997; Chowdhury et al., 2012; Olguín, 2012).

Phycoremediation is a process in which wastewater constituents such as nutrients or other pollutants are removed from water or biotransformed by algae. Carbon dioxide (CO_2) sequestration from flue gasses can also be incorporated into this process (Olguín, 2003). An obvious advantage is the utilisation of natural resources such as light, the biodegradability of biomass and its potential utilisation for other purposes such as biofuels or biofertilisers.

Microalgae can be found in a wide range of aquatic environments, including extreme habitats, giving them predispositions to adapt to various substrates. Although microalgae have been shown to be able to grow in and remove pollutants from a wide range of wastewaters, the viability of such technology is still questionable and depends on:

- the selection of appropriate species for a particular application;
- optimising growth conditions;
- the genetic improvement of promising strains;
- optimised engineering design for culturing;
- technologies for downstream removal and processing of biomass.

One of the driving forces for establishing microalgae for wastewater treatment systems is that microalgae have been the subject of interest for 3rd generation biofuels. While microalgae-derived products such as nutraceuticals represent high value products with high production costs, the success of biofuel production is highly dependent on low production costs. Microalgae are well known to be present in biological treatment systems, especially end-line steps in the treatment of municipal wastewater (Carty et al., 1997). It has been indicated that employing integrated systems for wastewater treatment and biomass production for biofuel utilisation could alleviate costs whilst reducing environmental burden and these are the main drivers for establishing stand alone microalgae treatment systems (Olguín, 2012). While it has been shown that microalgae can grow on a number of wastewaters and reduce certain pollutants, it would appear that designing viable microalgae treatment systems is challenging. The most feasible options thus far appear to be those that can be situated in geographical areas with an abundance of natural sunlight employing High Rate Algal Ponds (Rawat et al., 2011). Inorganic anions such as orthophosphate, nitrate, ammonia as well as heavy metals can be readily removed together with simple organic compounds. The type of wastewater with acceptable levels of the aforementioned pollutants, as well as other environmental conditions, need to be assessed to frame where microalgae could be used and to determine the extent to which they can fulfil the treatment process. Experiments conducted to date show the ability to reduce pollutant levels. However, incomplete pollutant removal has also been reported after microalgae treatment. Nutrient limitations such as organic carbon are hypothesised and discussed but insufficiently quantified (Zhou et al., 2011). An optimal or suitable ratio of different nutrients might be the issue, resulting in limited growth, which prevents complete removal of excess nutrients. Other factors may play a role, such as pH variation during microalgal growth.

Throughout the literature, most studies seemed to take two main approaches in investigating microalgae in regard to their potential growth in wastewaters.

1) A strain of commercial interest (i.e. known to produce high levels of lipids intended for biofuel production; strain suitable for feed purposes) was investigated to determine if it could grow on wastewater with a composition

profile thought suitable for growth (Kumar et al., 2010; Mandal and Mallick, 2011).

2) A type of wastewater known to contain pollutants used by microalgae has been selected and a number of strains isolated and purified from the wastewater itself or a number of different environments were scanned for their ability to grow in that particular wastewater (Lin and Chan, 2007; Zhou et al., 2011).

While microalgal lipids are one type of potential biofuel resource, other technologies such as anaerobic digestion can alleviate the problem of strain selection based on particular characteristics (i.e. lipid accumulation) as this process can utilise the entire microalgae biomass. However certain fractions are more favourable than others, such as saccharides and lipids rather than proteins (Ras et al., 2011).

1.2 Microalgal biological diversity and applications

Microalgae inhabit a wide range of environments, freshwater to marine, terrestrial and some are able to live under extreme conditions (i.e. very low pH or low temperature). Their ability to adapt to such diverse conditions suggests the existence of a wide range of metabolic capabilities in this diverse group of organisms. Although most microalgae are photosynthetic or mixotrophic, there are species that seem to have lost the ability to photosynthesise during evolution or seem never to have acquired it. In spite of their relations to other microalgae these are often referred to as protists. However the terms protists and microalgae are not clearly distinguished in some instances as microalgae are often viewed as a subgroup of protists. Taxonomically there are 15 divisions (phyla) of microalgae (John et al., 2002):

1) Cyanophyta,
2) Rhodopyta,
3) Euglenophyta,
4) Cryptophyta,
5) Pyrrophyta,
6) Raphidophyta,
7) Haptophyta,
8) Chrysophyta,
9) Xanthopyta,
10) Eustigmatophyta,
11) Bacillariphyta,
12) Phaeophyta,
13) Prasinophyta,
14) Chlorophyta,
15) Glaucophyta.

The classical taxonomic classification has been based especially on morphological features, reproductive cycles and later with the advances of science on biochemical analysis (in case of microorganisms i.e. cell wall composition, energy storage compounds, types of pigments present). Taxonomical groupings are to an extent artificial as from a biological point of view they do not reflect phylogenetic relations between different species and the degree of evolutionary change or complexity of organism.

With the advance of molecular biology, the relationships in this versatile group of organisms have become clearer (Figure 1.1). Phylogenetic analyses are probability tests that form a hypothesis of how much different species relate to each other based on the comparison of genetic and biochemical markers. Small subunit rRNA gene, large subunit rRNA gene and mitochondrial markers are used commonly and have also been suggested as barcoding tools of organisms to aid with their identification. However, other genes are also used to reveal information about the origins of plastids or the acquisition of toxic properties (Sonnenberg et al., 2007). Microalgae are polyphyletic groups of organisms, meaning that the evidence suggests that different groups have risen independently of each other.

Microalgae have been long known to be present in polluted waters due to the widespread issue of eutrophication and harmful algal blooms (Granéli et al., 2008; Abdel-Raouf et al., 2012). This has been used to the advantage of the development of treatment processes where microalgae can be employed. Microalgae have also been cultivated for their biomass, especially for aquaculture purposes but also for human nutrition and diet supplements (Table 1.1). Microalgal production associated with human consumption is complicated by the necessity for biomass screening for other toxic compounds that can be present and cause harm. With technological advances, it is the many microalgal metabolites that raise interest in current research efforts. Their exploitation for various fine chemicals, bioactive substances with therapeutical importance such as antiviral compounds, immunomodulators, inhibitors or cytostatics is on the increase (Pulz and Gross, 2004 and references within; Spolaore et al., 2006). Much work is also invested into productivity maximisation through culturing process optimisation or genetic engineering. Apart from the major commercially interesting compounds, the microalgae metabolomics profiling could help identify biomarkers potentially useful for environmental monitoring. Understanding the mechanisms of detoxification and the biochemical

compounds produced in response to environmental stresses provides information on the state or changes in the environmental systems. This includes for example the presence of certain proteins such as metallothioneins, phytochelatins and heat shock proteins that are produced in response to high concentrations of metals in the environment (Torres et al., 2008).

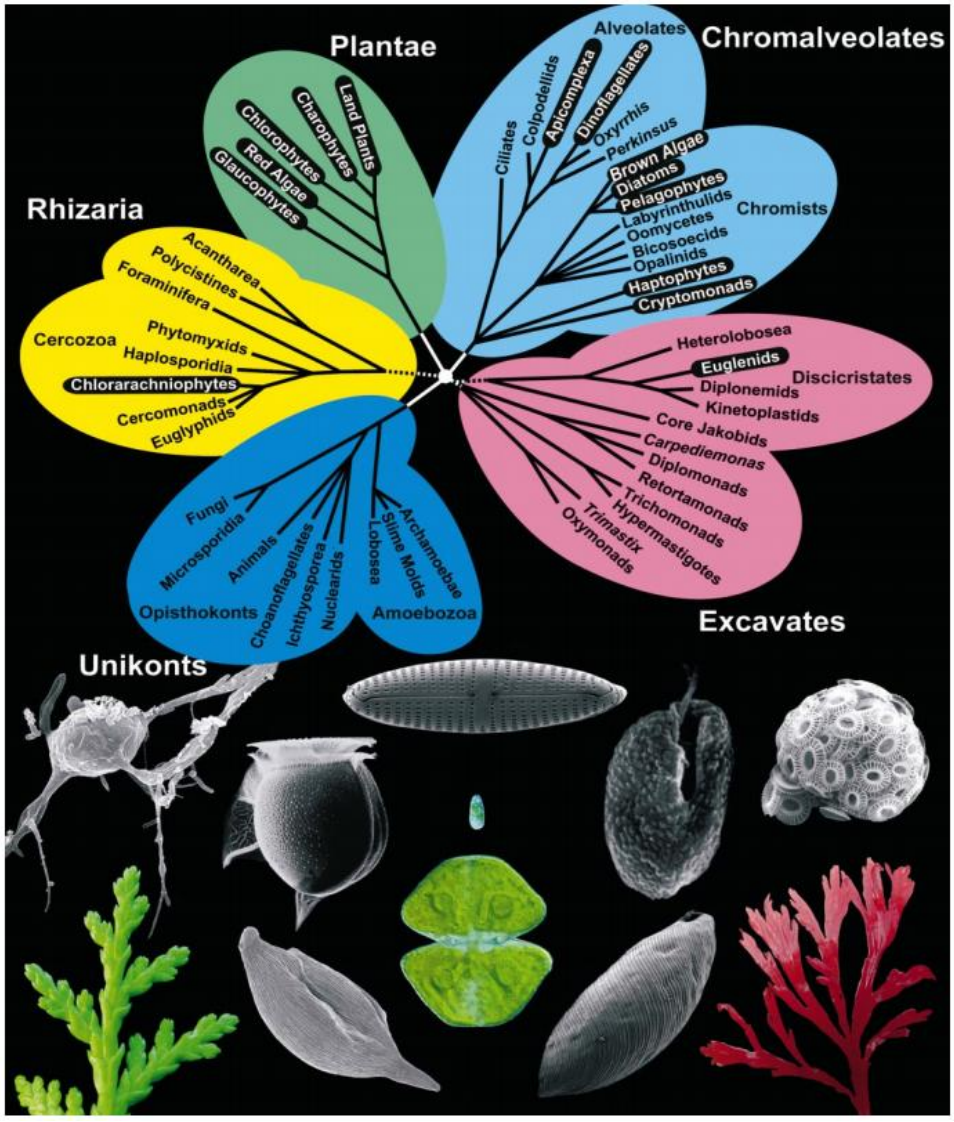


Figure 1.1. Tree of eukaryotes and diversity of plastid-bearing eukaryotes. Top: an unrooted hypothetical phylogeny of eukaryotes based on a synthesis of many gene trees, protein insertions and deletions, and cellular and biochemical characters. Bottom: a small sample of the diversity of plastid-bearing eukaryotes can be seen from representatives of each of the major "algal" lineages (taken from Keeling, 2004).

Table 1.1. Microalgae species used and researched for various technological applications

Area of application	**Product**	**Microalgae species**	**Reference**
Aquaculture	biomass	*Tetraselmis* spp., *Pavlova lutheri*, *Isochrysis* spp. (Prasinophyta)	(Pulz and Gross and references within, 2004; Spolaore et al., 2006)
		Pyramimonas spp., *Micromonas* spp. (Chlorophyta)	
		Skeletonema spp., *Chaetoceros* spp., *Nitzschia* spp., *Thalassiosira* spp. (Bacillariophyta)	
		Gymnodinium spp. (Dinoflagellata)	
Human nutrition	biomass	*Spirulina* (Cyanobacteria)	
		Chlorella (Chlorophyta)	
		Dunaliella (Chlorophyta)	
Diet supplements · unsaturated fatty acids · pigments · antioxidants	arachidonic acid	*Porphyridium* sp. (Rhodophyta)	
	docosahexaenoic acid	*Crypthecodinium cohnii* (Dinoflagellata)	
	ß-carotene	*Dunaliella salina* (Chlorophyta)	(Pulz and Gross and references within, 2004)
	astaxantin	*Haematococcus pluvialis* (Chlorophyta)	
	phycocyanin, phycoerythrin	*Porphyridium* spp. (Rhodophyta)	
	phycocyanin	Galdieria sulphuraria (Rhodophyta) option of heterotrophic/mixotrophic production	(Sloth et al., 2006)
Nanotechnology (area in research)	diatom silica frustules	Bacillariophyta	(Gordon et al., 2009)
Biofuels	microalgal oil	*Nannochloropsis* spp. (Heterokontophyta), *Tetraselmis* spp. (Prasinophyta)	(Bondioli et al., 2012;Van Vooren et al., 2012)
	biomass for anaerobic digestion	*Chlorella* sp. (Chlorophyta)	(Ras et al., 2011)
Phycoremediation	nutrient removal	*Scenedesmus* spp., *Chlamydomonas* spp., *Chlorella* spp. (Chlorophyta)	(Mustafa et al., 2012; Kothari et al., 2013)

1.3 Bioremediation potential of microalgae

Numerous studies have shown that different algal species can efficiently reduce levels of pollutants in different wastewater discharges. Phycoremediation of a wide range of wastewaters has been investigated, including:

- municipal wastewater (Zhou et al., 2011),
- animal wastewaters such as piggery (Kumar et al., 2010), fish (Seng et al., 2012), dairy (Wilkie and Mulbry, 2002; Levine et al., 2010),
- landfill leachates (Lin et al., 2007),
- industrial effluents (Rao et al., 2011).

Most commonly, the phycoremediation of macronutrients such as nitrogen and phosphorus is the end point targeted. A number of examples from the literature are documented in Table 1.2.

Table 1.2. Overview of microalgae phycoremediation efficiency in various wastewaters.

Type of wastewater	Species	Strain source	Pollution level*	Remediation effectiveness/ Reduced by: "%"	Reference
Leather processing wastewater	*Chlorella vulgaris*	Leather processing effluent	nitrate (as NO_3^-) 7 mg l^{-1} free ammonia (as NH_3) 56.3 mg l^{-1} phosphate (as PO_4^{3-}) 78.09 mg l^{-1} BOD 230 mg l^{-1} COD 582 mg l^{-1}	Laboratory trials; reduction in 7 days: free ammonia 80%, nitrates 29%, phosphates 94%, BOD 22% and COD 38%; Field trials: higher removal rates thought to be a result of improved mixing.	(Rao et al., 2011)
Synthetic wastewater (secondary oxidation ponds)	*Chlorella vulgaris, Planktothrix isothrix*	Wastewater: secondary treatment oxidation ponds	NH_4^+-N 79.3 mg l^{-1} PO_4^{3-} -P 7.47 mg l^{-1}	In 9 days: 80 % of nitrogen (expressed as NH_4^+) 100% phosphorus (as PO_4^{3-})	(Silva-Benavides and Torzillo, 2012)
Landfill leachate	*Scenedesmus* sp.	Pond environ. sample	10% leachate: TN 109.7 mg l^{-1} NH_4^+-N 90.5 mg l^{-1} TP 0.69 mg l^{-1} COD 1440 mg l^{-1}	In 10% leachate; reduction in 20 days: 70% TN 72% NH_4^+-N 91% TP COD 16%	(Cheng and Tian, 2013)
Treated landfill leachate	Algal consortium: Chlorophytes & *Euglena gracilis*	Culture collection UMACC, Malaysia	COD 784 mg l^{-1} NH_4^+-N 0.21 mg l^{-1} PO_4^{3-} 0.06 mg l^{-1}	Loading rate 4% for 42 days: 69.4% COD 98.7 % NH_4^+-N 76.4 % PO_4^{3-}-P	(Mustafa et al., 2012)
Household wastewater	*Chroococcus* sp. (strain 1 & 2)	Drain wastewater & surrounding soil	TDP 26.9 mg l^{-1} COD 310.5 mg l^{-1} NO_3^--N 9.8 mg l^{-1} NH_4^+-N 10.0 mg l^{-1}	Reduciton in 12 days: TDP 91 -92 % COD 55 – 70 % NO_3^--N 94 – 97 % NH_4^+-N 100 %	(Prajapati et al., 2013)
Agricultural run-off mixed with household wastewater	*Chroococcus* sp. (strain 1 & 2)	Drain wastewater & surrounding soil	TDP 5.0 mg l^{-1} COD 250 mg l^{-1} NO_3^--N 10.0 mg l^{-1} NH_4^+-N 5.0 mg l^{-1}	Reduction in 12 days: TDP 70 -78 % COD 67 - 92 % NO_3^--N 96 - 98 % NH_4^+-N 80 - 100 %	(Prajapati et al., 2013)
Municipal secondary settling tank discharge	*Scenedesmus obliquus*	Culture collection	orthophosphate 16.3 mg l^{-1} ammonium 131.4 mg l^{-1} nitrate 85.2 mg l^{-1} nitrite 0.5 mg l^{-1} TOC 11.8 mg l^{-1}; BOD 86.7 mg l^{-1}; COD 160.2 mg l^{-1}	Reduction in 7 days: orthophosphate 69.9 %, ammonium 88.5 %, nitrate 64.2 %, nitrite to not detectable levels, TOC 40 %, BOD 53.7 %, COD 48.9 %	(Mandal and Mallick, 2011)
Fish pond discharge	*Scenedesmus obliquus*	Culture collection	orthophosphate 8.5 mg l^{-1} ammonium 8.3 mg l^{-1} nitrate 4.2 mg l^{-1} nitrite 0.6 mg l^{-1} TOC 17.2 mg l^{-1}, BOD 118.7 mg l^{-1}, COD 237.1 mg l^{-1}	Reduction in 7 days: orthophosphate 77.6 %, ammonium 92.8%, nitrate 45.2 %, nitrite to not detectable levels, TOC 21.5 %, BOD 48.3 %,COD 39.9 %	(Mandal and Mallick, 2011)
Poultry litter	*Scenedesmus obliquus*	Culture collection	Not listed	Reduction after 21 days: nitrate 100 %, nitrite 100%, ammonium 97 – 99 %, orthophosphate 92 – 98 %, TOC 41 – 52 %.	(Mandal and Mallick, 2011)
Concentrated municipal wastewater (centrate after sludge removal)	*Chlorella* spp., *Heynigia* spp., *Micractinium* spp., *Scenedesmus* spp.	Variety of environ. samples	Orthophosphate 212 mg(P) l^{-1} ammonia 91 mg(N) l^{-1} nitrate 0.34 mg(N) l^{-1} TKN 134 mg l^{-1} COD 2324 mg l^{-1}, TOC 960 mg l^{-1}	Reduction within 3 days when strain reached stationary phase (data derived from graphical display): nitrogen reduced to 30 – 60 mg l^{-1}, orthophosphate to 50 – 60 mg l^{-1}, COD and TOC reduced to range 500 – 1000 mg.l^{-1} and < 200 mg l^{-1} respectively	(Zhou et al., 2011)
Dairy wastewater	*Chlamydomonas polypyrenoideum*	Department of Phycology, NBRI, India	75 % wastewater: phosphate 5.6 mg l^{-1} ammonia 18.5 mg l^{-1} nitrate 78.3 mg l^{-1} nitrite 5.0 mg l^{-1} COD 6000 mg l^{-1}	Reduction after 15 days: nitrate 96.6% nitrite 99.4 % phosphate 98.0 % ammonia 99.0 % COD 82.4 %	(Kothari et al., 2013)
Primary sewage effluent diluted with seawater 1:1	*Phaeodactylum tricornutum* & m *Oscillatoria* spconsortium.	Sewage outfall on a bay in Scotland	Highest observed conc. throughout the duration of the experiment: NH_4^+-N 498 mmol m^{-3} PO_4^{3-}-P 76 mmol m^{-3}	Field study /Immobilisation /continuous system: 100 % removal of ammonium and phosphorus (0.5 day retention time)	(Craggs et al., 1997)

* Individual pollutant names are denoted as listed in the original articles (i.e. either nitrate of NO_3^--N, as in some cases it is not clear if expressed concentration is meant to be mg l^{-1} of nitrate of nitrogen contained within nitrate; consequently the phycoremediation efficiency is expressed as a percentage for ease of comparison).

1.4 Nutrient-linked Limitations in Phycoremediation Processes

Nutrient limitations are rarely discussed in phycoremediation applications. The issue seems to appear indirectly in articles with wastewaters that are more heavily polluted or more specifically contain very high concentrations of a particular pollutant. Heavily polluted wastewaters have complex chemistries. In these cases the inhibitory effect of pollution is often considered and probably more obvious and easily measured. It might be impossible to determine all the effects of the various components in the wastewater on the growth of a microalgal culture due to the complex nature of the matrix. Indeed, thorough studies are highly demanding on analytical techniques and their capabilities.

One such example, when growth of microalgae reached the stationary phase without any of the main nutrients being depleted was in the work of Zhou et al. (2011). Concentrated municipal wastewater (centrate after sludge removal), high in COD 2324 mg l^{-1}, TOC 960 mg l^{-1}, orthophosphate 212 mg(P) l^{-1}, ammonia 91 mg(N) l^{-1} and TKN 134 mg l^{-1} was used as substrate. Species isolated from a number of environmental samples and including *Chlorella* sp., *Heynigia* sp., *Micractinium* sp. and *Scenedesmus* sp. were able to adapt and grow on the centrate and to reduce pollutant levels. Within 3 days when the strains approached the maximal biomass observed, the TOC was reduced to below 200 mg l^{-1}, COD was reduced to range 500 – 1000 mg l^{-1}, nitrogen was reduced to 30 – 60 mg l^{-1} and orthophosphate to 50 – 60 mg l^{-1}. The biomass, monitored after day 4 was reported to begin to decline. Although none of the main macronutrients appeared to be limiting, the authors hypothesised a shortage of carbon (Zhou et al., 2011). The micronutrient levels were also not monitored. Similarly, Hu et al. (2012) studied the use of 20-fold diluted digested liquid swine manure for the growth of *Chlorella* sp., which showed both increased growth rate and pollutant removal when supplemented with 0.1% (v/v) of low molecular weight organic acids as a carbon source, highlighting its potential shortage (Figure 1.2 and 1.3).

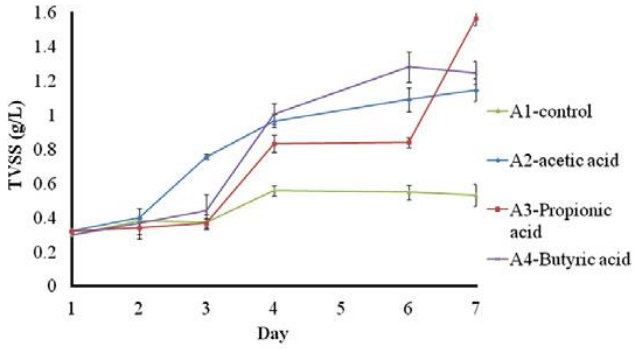


Figure 1.2. Comparison of biomass growth on diluted digested liquid swine manure and same substrate supplemented with organic acids as carbon source (taken from Hu et al., 2012).

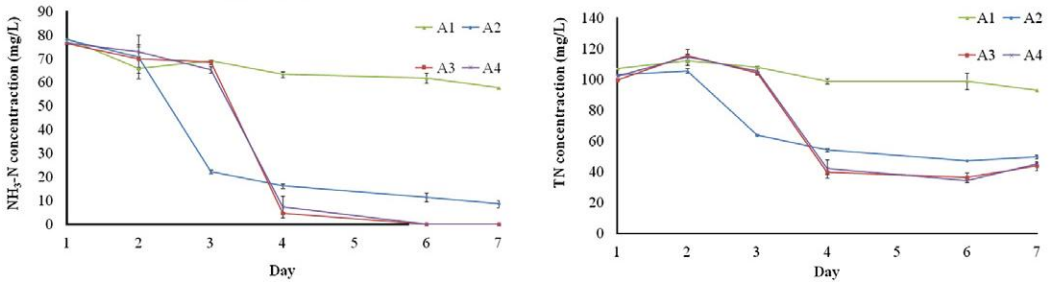


Figure 1.3. Comparison in depletion of ammoniacal and total nitrogen depletion in diluted digested liquid swine manure and same substrate supplemented with organic acids as carbon source (taken from Hu et al., 2012).

1.5 Special applications: remediation of heavy metals and xenobiotics

Some algal species have shown the ability to degrade low molecular weight phenols (which have antibacterial and phytotoxic effects) from olive-mill wastewater (Pinto et al., 2003) or other aromatic compounds such as p-chlorophenol and nitrophenols (Lima et al., 2004). *Nannochloropsis oculata* ST-3 strain showed tolerance to formaldehyde at concentrations up to 19.9 ppm when gradually increased, and was able to degrade 99.3% of it in the medium for 22 days (Yoshida et al., 2009).

Heavy metal removal has been studied with regard to both microalgae (Perez-Rama et al., 2002; Ajayan et al., 2011) and macroalgae (Kumar et al., 2007; Sooksawat et al., 2013) in their live form as well as dead biomass. Although some microalgae

species are tolerant to increased heavy metal concentrations, their growth and pigment levels can be negatively affected (Shanab et al., 2012).

Mechanisms of removal have to be also considered:

- adsorption on surface (passive – bound by surface charge of cell wall such as carboxyl groups, i.e. in polysaccharides, that are believed to play a role)
- bioaccumulation and speciation within cells (active transport into the cells – various mechanisms of accumulation: detoxification of metals in apoplasts and chelation of metals in cytoplasm with various ligands, such as phytochelatins, metallothioneins, metal-binding proteins; sequestration of metals into the vacuole of tonoplast located transporters) (Olguín and Sanchez-Galvan, 2012).

The fate of metals within the biomass also requires consideration in regard to future biomass use and processing. Surface adsorption offers opportunities for selective recovery by targeted desorption.

1.6 Landfill leachate: formation and treatment

Understanding the polluted environment in which the microalgae are proposed for growth and treatment is essential. A short introduction into landfill leachate formation, composition and the changes it undergoes is outlined in the following sections. Throughout the literature, the composition of landfill leachate shows a wide range of variation for individual parameters. The higher reported values can be 10 or even 100 fold of the lower reported values. This provides a significant challenge to biological systems such as phycoremediation. A landfill site is a dynamic system. Leachate changes occur as a result of the age of the landfill as well as with seasonal and climatic variations. Understanding these changes can help appreciate how they affect the biology of the treatment system.

1.6.1 Formation and composition of leachate

Leachate is liquid extracted from the bottom of the landfill. It is formed by water originating from the waste itself and water entering the landfill (such as rain or

surface water) and percolating through the waste. It is enriched by various soluble compounds and suspended matter. The composition usually depends on the type of waste deposited in the landfill, the age of landfill, construction of the site and climatic conditions (Johannessen, 1999).

Landfill leachate usually contains high concentrations of organic compounds, nitrogenous molecules (mainly ammonia), salts and metals which could affect environmental and human health if released uncontrollably into watercourses.

The composition of leachate changes with the age of the landfill. Typically, it undergoes 5 phases (Heyer and Stegmann no date) as also illustrated in Figure 1.4 and 1.5.

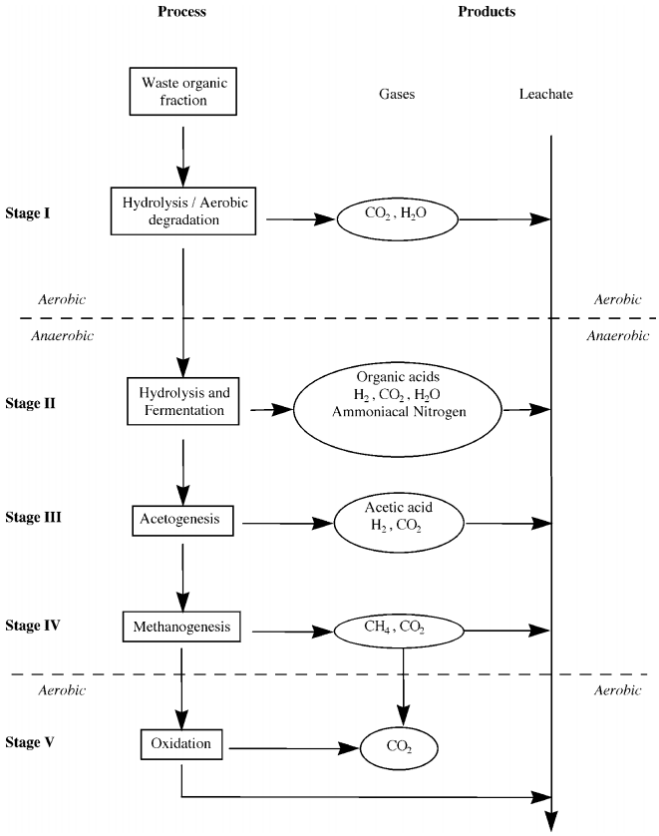


Figure 1.4. Summary of different stages of decomposition the waste undergoes within a landfill (taken from Carey et al., 2000).

- Phase 1: in the first stage the waste undergoes short term aerobic degradation until oxygen trapped in the upper layers is depleted. Complex organic molecules, proteins, saccharides and lipids are degraded into simpler compounds and eventually into CO_2, water and oxidised salts of sulphur and nitrogen.

- Phase 2: is the anaerobic phase, which is characterised by acid fermentation and high increase in volatile acids and considerable concentrations of inorganic ions (chlorides, sulphates, calcium, magnesium and sodium). The changes in the chemical environment promote the formation of sulphites of heavy metals such as iron and manganese which are not soluble and hence precipitate out. As a result of the production of volatile acids and the increase in CO_2, pH drops. High BOD_5/COD ratios (>0.7) indicate a high level of biodegradable organic matter.

- Phase 3: the second intermediate anaerobic phase is characterised by the increase in methanogenic bacteria population and a decrease in the production of CO_2, hydrogen and volatile fatty acids. Sulphates are biologically reduced to sulphites. Increase in pH and alkalinity decreases the solubility of metals, such as calcium, iron, manganese and other heavy metals. Ammonia is released but not converted into nitrate.

- Phase 4: anaerobic, methanogenic fermentation. The pH is stabilised at around neutral values. Most of the organic components in the waste have decreased although this stage can last for several decades. BOD_5/COD ratios would be in the range of 0.4 – 0.2, indicating a decrease in biodegradable organic matter while ammonia continues to be produced during degradation.

- Phase 5: A final aerobic phase can appear in some landfills.

Differences in composition between acid phase and methanogenic phase are illustrated and highlighted in Table 1.3. More recently, lower COD and BOD values can be found throughout the literature, which is probably a reflection of improved waste management practices (Heyer & Stegmann, no date).

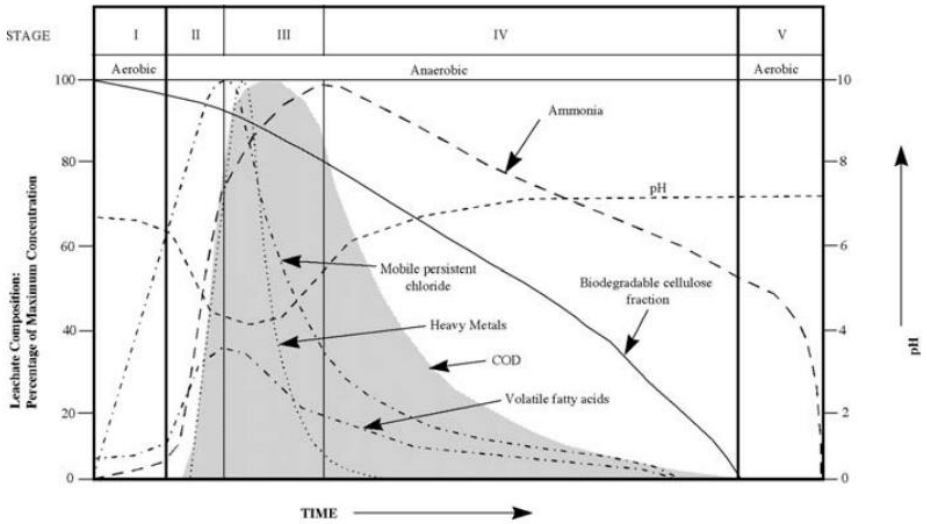


Figure 1.5. Physico-chemical changes in leachate composition during the lifetime of a landfill outlining different stages (taken from Carey et al., 2000).

Table 1.3. Composition from MSW landfill modified from Ehrig (1990) cited in (Heyer & Stegmann, no date)

	Acid phase		Methanogenic phase	
	Average	Range	Average	Range
	mg l^{-1}	mg l^{-1}	mg l^{-1}	mg l^{-1}
pH	6	4.5 - 7	8	7.5-9
COD	22000	6000-60000	3000	500-4500
BOD_5	13000	4000-40000	180	20-550
TOC	7000	1500-25000	1300	200-5000
NH_4^+-N	750	30-3000	750	30-3000
TON	3.5	0.1-75	3.5	0.1-75
tot P	6	0.1-30	6	0.1-30
SO_4^{2-}	500	70-1750	80	10-420
Cl	2100	100-5000	2100	100-5000
Na	1350	50-4000	1350	50-4000
K	1100	10-2,500	1100	10-2,500
Mg	470	50-1150	180	40-350
Ca	1200	219421	60	20-600
Cr	0.3	0.03-1.6	0.3	0.03-1.6
Fe	780	20-2100	15	3-280
Ni	0.2	0.02-2.05	0.2	0.02-2.05
Cu	0.08	0.004-1.4	0.08	0.004-1.4
Zn	5	0.1-120	0.6	0.03-4
As	0.16	0.005-1.6	0.16	0.005-1.6
Cd	0.006	0.0005-0.14	0.006	0.0005-0.14
Hg	0.01	0.0002-0.01	0.01	0.0002-0.01
Pb	0.09	0.008-1.02	0.09	0.008-1.02

Leachates are very complex in composition. Parameters such as COD or TOC can reflect a range of chemical compounds whose profile varies over time. A portion of organic content in landfill leachates is formed by compounds such as volatile organic acids or humic substances that form in the process of decomposition of organic matter such as degrading plant and animal material. Humic substances can be divided into three groups based on their solubility at different pH. Humic acids constitute the fraction soluble in alkaline conditions. Fulvic acids are soluble in aqueous solutions regardless of pH and humin is insoluble (Badis et al., 2009 and references within). Humic substances are a group of highly heterogeneous substances with no exact defined chemical structure and high molecular weight. Generally, they contain condensed aromatic and aliphatic structures with a large amount of hydroxyl and carboxyl groups that give them high chelating ability. This chelating property is responsible for binding minerals and even low molecular weight compounds, influencing thus their bioavailability. They give the leachate its typical brown colour. These compounds are often found in soils and marshes where they are part of the humus fraction. They are relatively stable. As mentioned earlier the BOD/COD ratio does stabilise with the age of produced leachate, indicating the decrease in biodegradable portion of organic matter. There have been a number of bacterial species, actinomycetes and fungi that have been shown to be able to degrade or adsorb humic substances (Badis et al., 2009).

Hazardous compounds are known to be present in leachate. A Swedish study from 2007 examined leachates from 12 landfills and screened them for 400 parameters (Oman and Junestedt, 2008). More than 90 organic and metal organic compounds and 50 inorganic elements were identified. These included halogenated aliphatic compounds, benzene and alkylated benzenes, phenol and alkylated phenols, ethoxylates, polycyclic aromatic compounds, phthalic esters, chlorinated benzenes, chlorinated phenols, PCB, chlorinated dioxins and chlorinated furans, bromated flame-retardants, pesticides, organic tin, methyl mercury and heavy metals. This study showed the presence of a large range of substances at very low concentration levels often difficult to detect by standard analytical procedures and also suggested the presence of more compounds that have not yet been identified. It also highlighted that leachate sediments accumulate certain substances in higher concentrations than the leachate itself, especially hydrophobic compounds (Oman and Junestedt, 2008).

Many of the ‘priority’ or ‘priority hazardous’ substances listed in Directive 2008/105/EC (Daughter Directive to the Water Framework Directive) do not exceed the limit values but the leachates still exhibit high toxicity as shown by ecotoxicological testing. It is thought that the combined effect of many compounds at levels under detection is causing these effects (Brito-Pelegrini, 2007; Matejczyk et al., 2011). Due to the complex composition of some wastewater samples such as landfill leachate, biological testing has been used as an indicatory means of evaluating ecotoxicological impact. Usually, multispecies assays are recommended that cover a number of trophic levels. In this way, different groups of pollutants can be detected by species sensitive to them. Two types of toxicity are recognised: acute and chronic. A number of standardised and/or commercially available bioassays currently exist, encompassing for example bacterial, microalgal and micro crustacean organisms. Assays with dicot and monocot seed germination are also recommended (Persoone and Gillett, 1990; Brito-Pelegrini, 2007).

1.6.2 Landfill leachate treatment technologies

Landfill leachate treatment is extremely difficult. A wide range of treatment technologies reflects the range of challenges that landfill leachate treatment presents. Either biological or physico-chemical processes achieve only partial treatment and a combination of the two may be required. Some municipal landfills have treatment plants present on site however most of the landfill leachate eventually ends up in the local authority waste water treatment plant (WWTP) eventually. In fact, co-treatment of landfill leachate with sewage is one of the most common ways of dealing with leachates. However, landfill leachate has been known to negatively impact this treatment process when added above a certain concentration (Carey et al., 2000; Quant et al., 2009). It has been suggested that the addition of landfill leachate influences the waste stream composition in the way not favoured by microbial communities. It unsettles the optimal nutrient ratio (BOD:N:P) and/or presence of many toxic compounds including high concentrations of ammonia and also high levels of hard COD (recalcitrant organic matter) also appears to be undesirable. These composition alterations can put the microbial community within WWTPs under stress (Quant et al., 2009) and result in decreased treatment efficiencies and

reduced nutrient removal. Process optimisation can be achieved but usually this leads to overall higher operational demands, costs and increased throughput time (Arundel, 1995; Renou et al., 2008; Spellman, 2009; Ahmed and Lan, 2012).

Activated sludge treatments such as sequencing batch reactors (SBR) and membrane bioreactor (MBR) are commonly used at landfill sites and are efficient at reducing ammonia-nitrogen by biological transformation into nitrate and subsequent denitrification. COD decrease can be quite effective as well especially in younger leachates. Success of these sludge processes employed in landfill leachate treatment in comparison to traditional WWTP arrangements is based on different engineering design, more efficient biosolids separation and conditions allowing for operation at higher liquor concentrations. As these are biological treatment technologies, high ammonia-nitrogen (over 1000 mg l^{-1} NH_4^+-N) can also have a negative impact. Even though activated sludge processes can be very effective, residual ammonia-nitrogen of 100 mg l^{-1} can result (Ahn et al., 2002; Ahmed and Lan, 2012). Other technologies can be employed as complementary (pre-treatment and/or post treatment processes) or stand-alone treatment procedures. These include physico-chemical methods (coagulation-flocculation, chemical precipitation, ammonia stripping, reverse osmosis/membrane filtration, activated carbon adsorption) (Kurniawan et al., 2006), oxidation processes (i.e. Fenton process) (Deng and Englehardt, 2006) and constructed wetlands (Justin and Zupančič, 2009; Lavrova and Koumanova, 2010).

1.7 Microalgae metabolism and considerations regarding phycoremediation

Wastewater composition is variable. Even one single wastewater source stream does vary throughout the year, depending on factors such as seasonal/weather changes or rainfall.

It is important during phycoremediation to select microalgal species that are adaptable to the composition of wastewater and that can tolerate the changes in composition that are experienced throughout the year without major negative effect to their growth. Strains that do not have particular nutrient demands (such as vitamins) and can alternate their metabolisms in response to changes in medium

composition depending on nutrient source (such as nitrate/ammonia) are more likely to succeed. Tolerance to toxic compounds (heavy metals, xenobiotics) or levels of nutrients that can become toxic at higher concentration (i.e. ammonia) are also desirable.

Wastewaters that are heavily polluted, such as landfill leachate, have complex composition matrices. In cases where it is possible to identify which factors within a wastewater source have inhibiting effects or which are limiting for the growth of selected strains (if any), it can help to optimise the phycoremediation process. At a research level, it has been shown that dilution or nutrient supplementation (Hu et al., 2012; Cheng and Tian, 2013) might be required to increase the effectiveness of treatment and biomass production. Consequently, a detailed understanding of microalgae metabolisms, nutrient requirements and toxicology is required.

1.7.1 Effect of ammonia on microalgal growth

Ammonia is one possible nitrogen source for microalgae. The energy cost for ammonium assimilation is lower than for nitrate (Fernandez and Galvan, 2008 and references within). Nitrate has to undergo double reduction, first into nitrite and then eventually into ammonium ion within the cell (Figure 1.6) (Fernandez and Galvan, 2008).

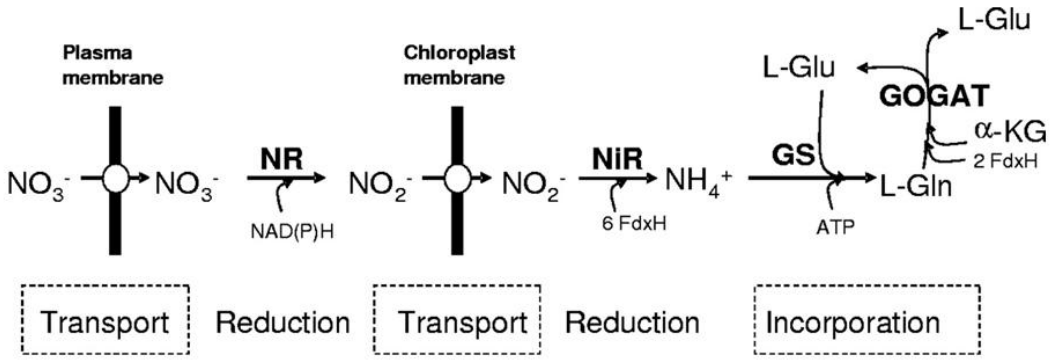


Figure 1.6. Nitrate transport into a microalgal cell displaying processes and enzymes involved (taken from Fernandez and Galvan, 2008).

However ammonia is also known to negatively influence cell growth or can even be toxic at high concentrations. The concentration ranges are species specific.

Ammonium has been known to become toxic to some algae at higher concentrations and at higher pH because with the increase of pH (above pH 8) the equilibrium between ionised and free ammonia moves toward free ammonia, which is more toxic. It can diffuse into cells without the need of cell transporters and interfere with electron transfer in photosynthesis or increase intracellular pH with the same consequences. *Spirulina platensis* is an example of alkalophilic cyanobacterium, growing at high pH (~10) and high ammonia concentrations (Belkin and Boussiba, 1991). *Scenedesmus obliquus* growth was inhibited by ammonia at concentrations of 2 mM and did not grow at concentrations over 3 mM at high pH (Abeliovich and Azov, 1976).

Although ammonia can be assimilated faster and can support higher growth rate as shown in the work of Xin et al. (2010), the growth of *Scenedesmus* sp. with ammonia as the only nitrogen source reached lower cell density than when grown on nitrate of equal nitrogen concentration. Also, nitrogen and phosphorus removal from the media was higher in the experiments with nitrate as the nitrogen source (90% N, 100% P) rather than with ammonia (31% N, 76% P).

Some microalgae species can grow at relatively high ammonia concentration. For example, Tam and Wong (1996) investigated growth of *Chlorella vulgaris* within the concentration range of 10 – 1000 mg l^{-1} NH_4^+-N in Bold Basal media (BBM) with nitrate substituted by ammonia in batch experiments (Figure 1.7). They found that growth rate and maximum cell number achieved did not significantly differ in the range of 20 – 250 mg l^{-1} NH_4^+-N and were comparable to those in BBM. However, at higher concentrations, the maximum cell density achieved was lower with resulting residual ammonia. Several other studies report similar growth and nutrient removal trends with other microalgae species in media with comparable ammonia profiles (Lin et al., 2007; Zhao et al., 2014; Sforza et al., 2015). Tam and Wong (1996) pointed out that the removal at concentrations up to 20 mg l^{-1} NH_4^+-N was effective and complete by day 7 but the residual ammonia can be detected in cultures which contained more than 80 mg l^{-1} NH_4^+-N even after 20 days of treatment and could be higher than 50%. At 1000 mg l^{-1} NH_4^+-N, the resulting ammonia reduction was less than 20% (corresponding to 180 mg l^{-1} NH_4^+-N). As the experiments were conducted with modified BBM, the growth was also likely to be affected by changes in nutrient proportions (e.g. molecular N:P ratio in BBM is 1.7

and changes to 41.5 at 1000 mg l^{-1} NH_4^+-N). The same study reported that cultures grown in media with higher nitrogen concentrations yield biomass with increased protein content (Tam and Wong, 1996).

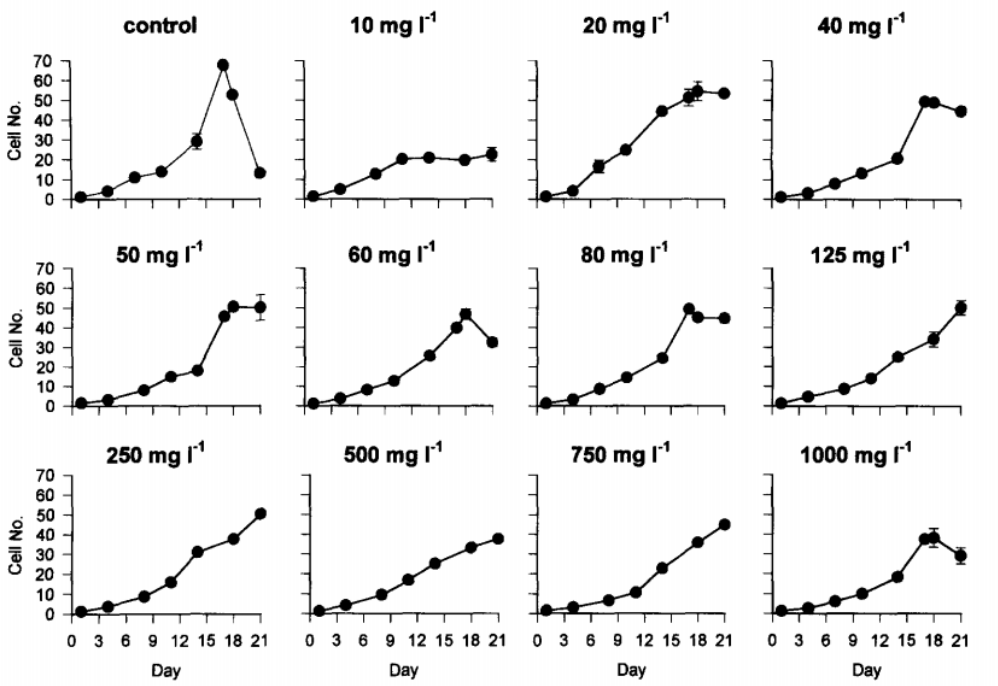


Figure 1.7. Cell number (× 10^6 cells.ml^{-1}) of *Chlorella vulgaris* cultivated at different ammonia concentrations (0-1000 mg(N) l^{-1}). Mean and standard deviation values of four replicates are shown (taken from Tam and Wong, 1996).

The tolerance to ammonia and other components of substrate is species specific which was illustrated in the study by Lin et al. (2007). *Chlorella pyrenoidosa* and *Chlamydomonas snowiae* were grown in 10, 30, 50, 80% dilutions of landfill leachate or undiluted leachate (ammonia 1345 mg(N) l^{-1}). The 10% leachate (approx. 135 mg l^{-1} NH_4^+-N) supported growth of both *C. pyrenoidosa* and *C. snowiae*. While *C. pyrenoidosa* still grew at 30 % (approx. 405 mg l^{-1} NH_4^+-N), *C. snowiae* growth was inhibited. Higher concentrations suppressed growth in both (Figure 1.8) which was also reflected in nutrient depletion (Figure 1.9). Further, although at higher ammonia concentrations, it may be possible to achieve apparently higher amount of biomass, it does not necessarily results in better productivities. Kumar et al. (2010) grew *Chlorella vulgaris* at different dilutions of digested piggery waste corresponding to concentrations 10, 20, 30, 40, 50 and 60 mg l^{-1} NH_4^+-N (Figure 1.10). The highest cell density of 10 million cells ml^{-1} was achieved with the highest

ammonia concentration; however the concentration of 7.17 million cells ml^{-1} was achieved with 20 mg l^{-1} ammonia on day 4, at which point the cell density was only 2.42 million cells ml^{-1} in the experiments with the highest ammonia concentration and thus faster growth was achieved at lower concentrations. Also, the ammonia reduction in media 20 mg l^{-1} ammonia-nitrogen was 61.8% while it was only 41.3% at the highest concentration. Overall, the biomass production and remediation effectiveness in the shortest time was achieved at 20 mg l^{-1} ammonia (Kumar et al., 2010).

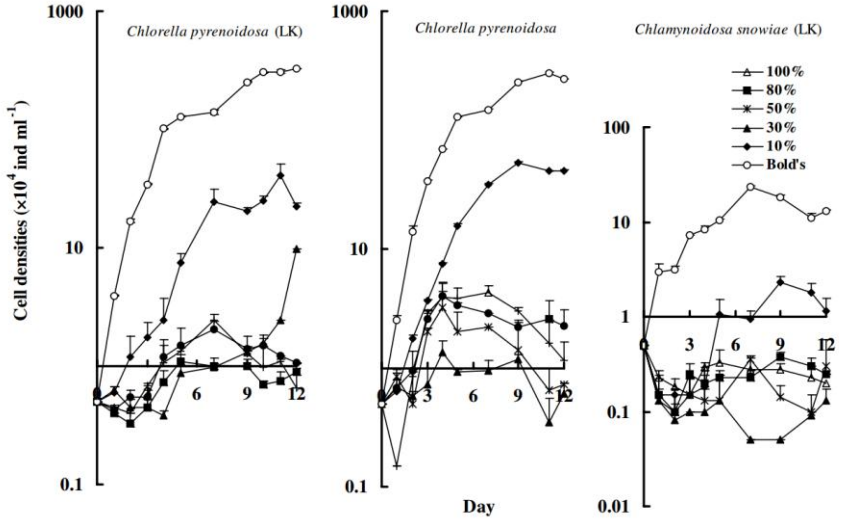


Figure 1.8. Algal growth curves in different leachate concentrations. LK denotes a strain isolated from the Li Keng Landfill (taken from Lin et al., 2007).

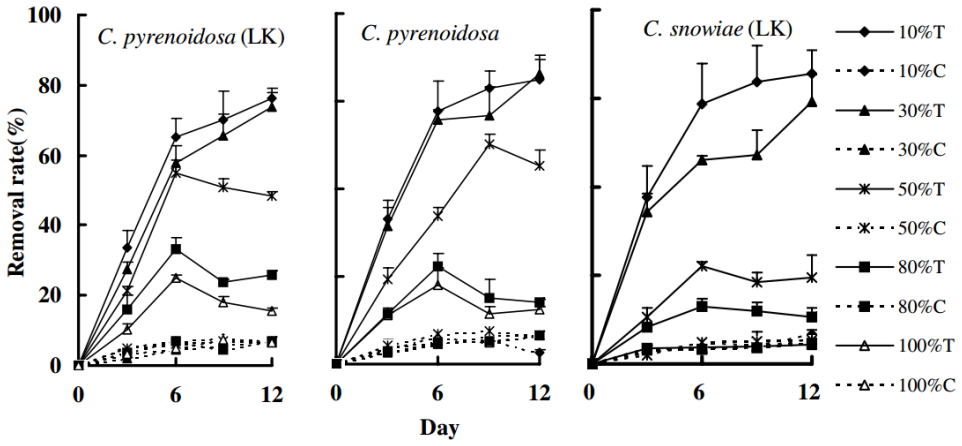


Figure 1.9. Ammonia-N removal rates in leachate at different dilutions, with and without algae. LK denotes strain isolated from the Li Keng Landfill (taken from Lin et al., 2007).

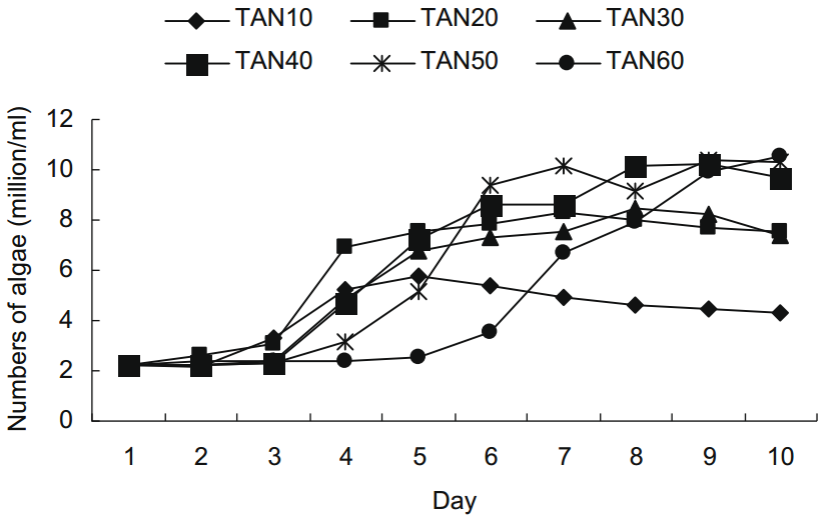


Figure 1.10. Growth of *Chlorella vulgaris* in diluted digested piggery waste at different total ammonia concentrations (taken from Kumar et al., 2010).

1.7.2 Availability of vitamins

In spite of being photosynthetic, a wide range of microalgae are vitamin auxotrophs and require them or at least their precursors in their environment. These are usually not present at high levels in nature and it is presumed that the algae obtain them by interaction with bacteria (Croft et al., 2006).

In the case of microalgae, three vitamins usually form part of artificial media recipes: cobalamin, thiamine and biotin. Vitamin auxotrophs can be found within various phyla and even different species within the same genera can differ by their vitamin requirements, as an example *Hematococcus* (chlorophyta), *Nitzschia* (heterokontophyta) and *Peridinium* (dinophyta) have representatives that are cobalamin auxotrophs and some that are not (Croft et al., 2006). One example of microalgae that is known to be capable of living without vitamins in growth media is *Chlamydomonas reinhardtii*. It is able to synthesise thiamine, does not require cobalamin although it can utilise this vitamin if it is available, as it possesses alternative enzymatic routes. As this algae does have biotin depended carboxylases and does not depend on it to be present in the media, it is assumed it must have biosynthetic pathways for this vitamin (Croft et al., 2006).

1.7.3 Mineral limitation and toxicity

Minerals are cofactors of many enzymes and their presence is essential. However, too high concentrations of many can have inhibitory or even toxic effects.

In the case of the commonly used *Chlamydomonas reinhardtii*, a study by Kropat et al. (2011) compared its four strains studied in laboratory experiments. The four most abundant (micro)metals in the biomass appeared to be iron, manganese, zinc and copper, respectively. However the concentrations within the four strains differed although the general profile seemed to be the same. Interestingly, copper and iron were accumulated at higher concentrations in cells grown photoheterotrophically, illustrating that mineral requirements differ depending on macronutrient availability (Kropat et al., 2011).

Mineral deficiencies induce metabolic and various morphological and size changes. In the case of the studied *Chlamydomonas* spp. strains, the nitrogen, iron and zinc deficiencies induced lipid accumulation which coincided with growth inhibition. Cell size and morphology is affected by a lack of zinc, manganese and nitrogen. Iron deficiency was reported to result in chlorotic (decreased levels of chlorophyll) phenotype (Kropat et al., 2011).

Pallmeloid formation was observed in some microalgae such as *Chlamydomonas* sp. It is a formation of cell clusters as a result of abnormal cell wall formation and/or incomplete development. Instead, cells remain attached to each other within multiple layers of membranes surrounding them. Dividing cells thus are unable to liberate (Nakamura et al., 1975). In the case of *Chlamydomonas* spp., it has been observed that calcium deficiency and certain organic compounds can cause pallmeloid formation or it can also be induced by chemical stress such as the presence of chloroplatinic acid (Nakamura et al., 1975).

High salt concentrations can cause stress in microalgae, affecting their photosynthetic apparatus. Studies in *C. reinhardtii* showed damage to photosynthetic systems at concentration of 100 mM NaCl (equivalent of 3.5 g(Cl) l^{-1}; equivalent of 2.3 g(Na) l^{-1}). For the same species, salt stress was observed to cause reduction or loss of motility, formation of palmelloids and slower cell division at concentration of 50 mM salt concentration but was more severe at 100 and 150 mM (1.7, 3.5 and 7 g(Cl) l^{-1}) (Neelam and Subramanyam, 2013). Apart from growth inhibition, salt

stress can also result in triacyglycerides and starch accumulation in *Chlamydomonas* sp. (Siaut et al., 2011). In this study, cell stress was related to the concentration of sodium chloride as salt content, which changes the osmotic pressure of the surrounding environment. Osmotic pressure can also be related to conductivity as a property reflecting the amount of dissolved salt and other ionic substances in the media.

Cobalt was found to be toxic to *Chlamydomonas* spp. at concentrations of 10 ppm cobalt nitrate and the toxicity was significant at concentrations of 20 ppm (Kropat et al., 2011 and references within). It has been also reported previously that manganese deficiency results in secondary iron and phosphorus deficiency (Allen et al., 2007; Kropat et al., 2011 and references within).

1.7.4 Storage compounds and reserve accumulation

It has been widely reported that many microalgae accumulate lipids in response to nutrient deficiency/depletion or in response to a stress. The most researched of these is nitrogen deficiency but phosphorus, iron and zinc are also mentioned (Kropat et al., 2011). Accumulation under particular conditions is also species-specific and depends on the overall composition of growth media. This was well demonstrated in a study by Deng et al. (2011) who compared lipid accumulation of two species (*Chlorella vulgaris* and *Chlamydomonas reinhardtii* CC124) in four different media (BG11, SE, TAP, HSM) and under a number of deficiencies (N, P, S, K, Fe, Mg, Ca). Several deficiencies caused increased lipid accumulation, such as nitrogen, sulphur and phosphorus. However, the overall composition profile was important to consider; some mineral deficiencies caused triacylglicerides (TAG) accumulation in one media while the same mineral deficiency did not have the same effect in the other, thus suggesting complex mechanisms involved in lipid accumulation and various interactions amongst the nutrients.

As lipids accumulate in response to a particular nutrient deficiency it has been a subject of interest to identify specific periods at which maximum accumulation occurs after depletion of identified triggers in media. Studies by Siaut et al. (2011) in several *Chlamydomonas reinhardtii* strains showed that starch accumulation starts immediately after nitrogen depletion while lipids start accumulating after 2 days.

There is also a change in the fraction of lipids. While neutral/non polar lipids (which act as a carbon and energy storage and are present in form of lipid droplets) increase, there is a decrease in intracellular membrane lipids (polar lipids such as glycolipids and phospholipids) and also a change in the composition of the fatty acids profile from polyunsaturated to saturated and monounsaturated. According to Siaut et al. (2011), *Chlamydomonas reinhardtii* strain *cw15* accumulated the highest concentration of lipids (40 μg per million cells) on day 5 after N depletion, which corroborates the study of Deng et al. (2011) in which lipid accumulation was maximal between 4 and 8 days in *Chlamydomonas reinhardtii* and the 5 day N-starvation in *Scenedesmus obliquus* (Ho et al., 2012).

1.8 Microalgae biomass as a valuable resource

Naturally occurring species with lipid content in the range of 30 % dry weight (DW) or higher have been found and are attracting attention as biofuel feedstock, for example:

- *Nannohloropsis* strains with absolute lipid yield ranging from 39.4 – 44.9 % (Doan et al., 2011), 52 % (Moazami et al., 2011), 39.1 % (Bondioli et al., 2012),
- *Neochloris* sp. 46 % (Moazami et al., 2011),
- *Botryococcus braunii* 30-40% (Sakthivel et al., 2011 and references within).

A number of issues hinder the viability of wider commercialisation. To increase the viability of such production one of the options is to couple biomass culturing with remediation of wastewaters (Chowdhury, 2012; Olguín, 2012).

The growth rate and composition of biomass, apart from being species-specific, has also been shown to be influenced by the composition of the substrate on which they are grown as well as by other environmental conditions (Chen et al., 2011; Van Vooren et al., 2012). These factors are quantifiable by mathematical modelling and this knowledge can be used to the advantage of predicting the composition of biomass, selecting optimal culturing conditions and process control (Mandal and Mallick, 2009; Quinn et al., 2011). It has also been found that different starvation modes initiate differential accumulation of lipids (Mandal and Mallick, 2009; Chen et al., 2011; Ho et al., 2012; Tan and Lin, 2011), or in certain species (i.e.

Chlamydomonas reinhardtii) it can induce the production of hydrogen (He et al., 2011; Tamburic et al., 2012). For example, Mandal and Mallick (2009) achieved lipid content of 58.3% DW in *Scenedesmus obliquus* by optimising substrate composition for lipid accumulation in comparison to a control of 12.7%. There have been listed lipid contents as high as 68.5% DW in *Nannochloropsis* sp. (Bondioli et al., 2012), and 73% DW for *Scenedesmus rubescens* (Matsunaga et al., 2009) throughout the literature under controlled conditions.

Depending on the composition of the algae biomass and its processability various options are possible for deriving biofuels, for example, lipid extraction for biodiesel production (Schlagermann et al., 2012), anaerobic digestion to produce methane (Lakaniemi et al., 2011; Zamalloa et al., 2012; Hernandez et al., 2013), alcohol fermentation (Brunton et al., 2009), hydrogen production (He et al., 2011; Jones and Mayfield, 2012) or even pyrolysis (Yang et al., 2012).

There are different cultivation systems based on opened and closed designs. The choice of their use is dependent on the purpose of the application. Whereas open systems are cheaper to set up and operate, they have smaller productivity, limited control over cultivation conditions and are not suitable in certain geographical locations, possibly such as Ireland due to lack of sunshine. While closed systems offer continual operation, better control over culturing conditions and low contamination, the capital and operational costs are higher. At the present time, these higher costs could be justified only for the production of biomass that is a source of high value products such as pharmaceutical compounds (Brunton et al., 2009).

The coupling of biomass production (especially with high lipid content) for biofuels and phycoremediation is another way of increasing viability of microalgal technologies. Zhou et al. (2011) attempted to isolate species suitable for growing on concentrated municipal wastewater with high lipid production potential. After screening 60 species, 17 strains were tolerant to this substrate and five strains with lipid content around 30% DW were found.

Also Mandal and Mallick (2011) showed that algal biomass can be grown on effluents as a cheap and easily available growing medium with the dual benefit of remediation and of biomass generation as an added value by-product. As the conditions inducing high lipid content in microalgae coincide with reduced biomass production, in their work they adopted a two-stage batch process. The first step

aimed at producing large amount of biomass on an effluent. In the second stage, the cultivated biomass was transferred into nutrient deficient medium to induce starvation and lipid accumulation. This additional step increased the lipid accumulation 9-fold in comparison with the control. In another study Jiang et al. (2011) grew the marine algae *Nannochloropsis* sp. on a 50:50 mixture of municipal wastewater and seawater, with the growth further enhanced by aeration with 15% CO_2. Cells transferred from the first growth phase to the second lipid accumulation phase under the combination of nitrogen starvation and high light were able to increase lipid content from 33.8 to 59.9%.

Hernandez et al. (2013) employed a similar idea of culturing microalgae (*Chlorella sorokiniana*) and a bacterial consortium on wastewater. They used a potato processing industry wastewater and a treated liquid fraction of pig manure. In this case however a semicontinuous flow photobioreactor was employed and coupled with anaerobic digestion of the obtained biomass. Biomass grown on the wastewater from the potato processing industry had a lipid content of 30.2% while the treated liquid fraction of pig manure produced biomass with 4.3% lipids. Similarly, Chinnasamy et al. (2010) used untreated carpet industry medium as substrate in outdoor small scale photobioreactors while monitoring the influence of environmental conditions in different setups on the composition of the biomass.

1.9 Photobioreactors and harvesting

There are various designs for mass algae cultivation. Design factors are dependent on the application at hand, the algal cultures used and geographical position with regards to availability of sunlight, temperature and water access and quality. Various designs exist, including High Rate Algal Ponds, Raceways, Vertical-column photobioreactors, Flat-plate photobioreactors, Tubular photobioreactors and others (Ugwu et al., 2008). One of the main issues in microalgae technology is biomass harvesting and dewatering, which are estimated to represent as much as 69% of the input energy (Jones and Mayfield, 2012 and references within).

From the point of view of microalgal bioremediation, the concept of cell immobilisation is interesting and promising. Microalgae cells are usually freely dispersed throughout the liquid volume. Immobilisation binds the cells either to a

solid surface or entraps them within a material. It offers a number of advantages. These include a reduction in cell washout, increase in biocatalyst concentration with optimum contact with the substrate and ease of separation of algal biomass after waste water treatment (Thakur and Kumar, 1999). Craggs et al. (1997) used two species that showed adherence to surface (diatom *Phaeodactylum tricornutum* and cyanobacterium *Oscillatoria* sp.) for a corrugated raceway in an outdoor experiment in the treatment of sewage effluent (Craggs et al., 1997). Another case of cell immobilisation was the entrapment of *Dunaliella salina* in alginate beads where immobilised cells showed increased growth rate after 25 days by 71% and increased nutrient uptake rates in comparison to non immobilised cells (Thakur and Kumar, 1999). Ozkan et al. (2012) also successfully cultured *Botryococcus braunii* as a biofilm on a concrete surface while reporting savings on dewatering energy requirements by 99.7% in comparison to open ponds.

An alternative idea to immobilisation is the formation of microalgal-bacterial flocks which could, according to Van Den Hende et al. (2014) offer an advantage of biomass separation by filter press (with a large pore size - 200 μm) in which between 79 – 99% biomass could be recovered with 12 – 21% dry weight content.

1.10 Methodologies and analytical techniques

The following section outlines the principles behind some of the techniques and analytical methods that may be used in this project and are relevant in evaluation of phycoremediation and produced microalgal biomass.

1.10.1 Strain isolation and culturing

Monocultures can be obtained by a number of isolation techniques. Environmental samples collected can be preconcentrated by filtration or enriched by artificial media and incubated for a number of days to increase the number of cells in the sample (He et al., 2011). Subsequently, individual cells can be isolated by microcapillarity or devices such as micromanipulator (Lim et al., 2012) or serial dilutions. More sophisticated techniques exist such as flow cytometry cell sorting or Fluorescence Activated Cell Sorting (Doan et al., 2011; Pereira et al., 2011 and references within)

that use fluorescence and light scatter to analyse cell size prior to separation. Another approach is plate streaking (applying sample to an agar surface enriched by artificial media) and work with isolation of individual colonies (He et al., 2011). Cultures can be further propagated in well plates of increasing well volume up to a desired volume in flasks or other vessels. An important factor is the selection of media for culturing and the incubation conditions, such as light cycle and temperature, as they can selectively favour growth of certain species while making growth of others impossible.

1.10.2 Culture enumeration techniques

Cell enumeration involves expressing the number of individual cells per unit volume (Andersen, 2005). Alternative methods exist and from these most commonly used are dry weight, volatile solids or chlorophyll content which might be particularly useful when algae grow in colonies, form clumps or attach to surfaces. In these cases it is difficult to obtained individual cells homogenously dispersed throught the media. The aim is to make correct estimate for a known volume. This usually involves couting cells in one plane of known area and known depth. Cell immobilisation and preservation may be required to facilitate the counting. Counting chambers, such as hemacytometers, are often employed as they have precisely calibrated dimensions for this purpose. Otherwise, if cells are counted in a field of view with no impressed rulings of known measurements, the area/field of view must be measured with a calibrated stage micrometer and ocular graticule for each magnification and different microscope. Epifluorescence microscopy can offer advantage to quantifying amount of picoalgae where it would be difficult to distinguish small algae cells from bacteria. Pigment autofluorescence in this technique can provide clear destinction of microalgae as photothophic organisms (Andersen, 2005).

1.10.3 Lipid content analysis

A number of methods exist for the determination of lipids and their composition. The oldest one is Bligh and Dyer gravimetric determination after extraction of lipids

by organic solvents. Modifications of the method exist, usually involving different mixtures of organic solvents (Manirakiza et al., 2001). This method is viewed as inconvenient due to amount of biomaterial required to obtain reliable results, and also due to lack of control over possible coextraction of other substances soluble in organic solvents other than lipids. Chromatographic methods are commonly used. These methods, apart for quantification, allow for profiling of lipid composition. However, these techniques also require solvent extraction, they are time consuming and instrumentation demanding.

Due to the demand for rapid screening, several methods have been proposed recently as potentially suitable for quantification of lipids. These include fluorescence techniques employing probes such as Red nile or BODIPY (Brennan et al., 2012; Kou et al., 2013) or colorimetric spectroscopic methods (Mishra et al., 2014). Red nile fluorescence, which has been used for many years for visualisation or as a semi quantitative technique, is becoming more commonly used. It requires a relatively small amount of cells and minimal sample preparation before analysis. With the use of well plate readers, this method appears promising for quantitative analyses (Bertozzini et al., 2011). The principle of the method lies in the ability of Red nile (9-diethylamino-5H-benzo[alpha]phenoxazine-5-one) to dissolve in a variety of solvents but it fluoresces only in non-polar environments. The excitation wavelength of Red nile has a maximum at 549 nm and the emission wavelength varies with the polarity of the solvent used, shifting from lower wavelengths for nonpolar (580 nm) to higher wavelengths for more polar (640 nm) solvents. It does not fluoresce in water at all (Greenspan and Fowler, 1985). Alternative to Red nile is BODIPY (4,4-Difluoro-1,3,5,7-Tetramethyl-4-Bora-3a-Diaza-s-Indacene), which has excitation and emission wavelengths of 488 and 515 nm and has advantages in that these wavelengths are not affected by chlorophyll fluorescence (Brennan et al., 2012). However, in the case of both methods there are inconsistencies with regards to different species, and especially the ones with thick cell walls (Chen et al., 2009; Brennan et al., 2012).

The colorimetric spectroscopic method (sulpho-phospho-vanillin reaction) has been recently applied to analysis of lipids in microalgae. This method has been used routinely for many years in medical diagnostics for analysis of lipid levels in human serum. The method does not require lipid extraction and deals with relatively small

amount of sample in comparison to methods requiring extraction. In comparison to fluorescence detection methods that require well plate readers with specific filters; this method uses spectrophotometer that is commonly found in most laboratories. Although the method has a digestion step it is generally simple. The principal requirement is a presence of unsaturated bonds within lipids which is absolutely necessary for the sulpho-phospho-vanillin based reaction. This should be satisfied with the type of lipids found in microalgae. However, the signal varies to a certain degree depending on the level of lipid saturation. As the principle of the method is based on the reaction of phosphor-vanillin reagent with carbonium ion, formed during the digestion step from the aldehyde group and unsaturated bonds in the fatty acid chain, this reaction has possible interference from other organic substances containing these structures, such as tannins. Absorbance is measured at 530 nm (Mishra et al., 2014).

1.10.4 Nutrient analyses in media

As the landfill leachate contains high levels of interfering substances, the choice of analytical techniques is based on the suitability of the methods' chemistry to the substrate analysed, analysis turnaround time, volume of sample required, availability of equipment or costs involved.

The most commonly used techniques for the nutrient analysis in wastewaters are spectrophotometric. Others can be used such as chromatographic or electrophoresis (APHA, 2005). However, these are more equipment demanding, more time consuming and quite sensitive to interfering substances and thus require sample pretreatment. Spectrophotometric methods have been extensively automated in recent years and a number of autoanalysers or test kits compliant with international standardts (e.g. ISO 15923, ISO 15705) exist to facilitate the use of small amounts of samples and rapid analysis time. The Aquakem autoanalyser is often employed for water quality analysis and allows for the processing of a large number of samples of small volumes, which would be otherwise very impractical with manual methods (Rastetter et al., 2015). Also certain analyses requiring digestion steps are commercially available in the form of test kits such as COD analyses, or total nitrogen and phosphorus, which are adapted from Standard Methods for the

Examination of Water and Wastewater. The choice of technique for mineral profiling is dependent on expected concentration, sample matrix and interferences. Alkali metals such as sodium, potassium and the alkali earth metal calcium, which would be usually present in landfill leachate in high concentration, can be determined by flame photometry. Metals requiring lower concentration levels for determination can be measured by Atomic Absorption Spectrometry (AAS). Inductively coupled plasma mass spectrometry (ICP-MS), which can measure even lower concentration levels, has also the advantage of measuring multiple elements in a single analytical run. However this technique is technically demanding and quite expensive. As these techniques for determining minerals require sample digestion, they allow only for the determination of the total concentration of a particular element in a sample. Further chemical speciation or their bioavailability cannot be ascertained unless additional procedures are employed or different analytical methods are used (Baysal et al., 2014).

1.11 Summary of research objectives

This project aims to:

- Isolate microalgae strains suitable for the phycoremediation of wastewaters with high ammonia nitrogen concentration and high salt content which are suitable for application in temperate climates.
- Characterise these species and evaluate their effectiveness for nutrient depletion and biomass composition.
- Pre and post phycoremediation, evaluate the toxicity of the landfill leachate.

1.12 References

Abdel-Raouf, N., Al-Homaidan, A.A., Ibraheem, I.B.M. (2012). 'Microalgae and wastewater treatment', Saudi Journal of Biological Sciences, 19 (3), 257–75. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4052567&tool=pmcentrez&rendertype=abstract [Accessed 14 July 2014].

Abeliovich, A., Azov, Y. (1976). 'Toxicity of Ammonia to Algae in Sewage Oxidation Ponds', Applied and Environmental Microbiology, 31 (6), 801–806.

Ahmed, F.N., Lan, C.Q., (2012). 'Treatment of landfill leachate using membrane bioreactors: A review', Desalination, 287, 41–54. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0011916411010320 [Accessed 1 July 2015].

Ahn, W.-Y., Kang, M.-S., Yim, S.-K., Choi, K.-H. (2002). 'Advanced landfill leachate treatment using an integrated membrane process', Desalination, 149 (1-3), 109–114.
Available at: http://linkinghub.elsevier.com/retrieve/pii/S0011916402007403.

Ajayan, K. V., Selvaraju, M., Thirugnanamoorthy, K. (2011). 'Growth and Heavy Metal Accumulation Potential of Microalgae Grown in Sewage Wastewater and Petrochemical Effluents', Pakistan Journal of Biological Sciences, 14 (16), 805-811.

Allen, M.D., Kropat, J., Tottey, S., Del Campo, J.A., Merchant, S.S. (2007). 'Manganese Deficiency in *Chlamydomonas* Results in Loss of Photosystem II and MnSOD Function, Sensitivity to Peroxides, and Secondary Phosphorus and Iron Deficiency', Plant Physiology, 143, 263-277.

Andersen, R. A. (2005). Algal Culturing Techniques, Elsevier Inc., ISBN: 0-12-088426-7.

APHA (American Public Health Association), (2005). Standard methods for the examination of water and wastewater, 21st ed. Washington, D.C.

Arundel J. (1995). Sewage and Industrial Effluent Treatment, Blackwell Science, ISBN 0–632–03898–5.

Badis, A., Ferradji, F. Z., Boucherit, A., Fodil, D., Boutoumi, H. (2009). 'Characterization and biodegradation of soil humic acids and preliminary identification of decolorizing actinomycetes at Mitidja plain soils (Algeria)', African Journal of Microbiology Research, 3 (13), 997–1007.

Baysal, A., Ozbek, N., Akman, S., (2013). 'Chapter 7: Determination of Trace Metals in Waste Water and Their Removal Processes', Waste Water - Treatment Technologies and Recent Analytical Developments, INTECH, 145-171. Available at: http://dx.doi.org/10.5772/52025 [Accessed 12 August 2017].

Belkin, S., Boussiba, S., (1991). 'Resistance of *Spirulina platensis* to Ammonia at High pH Values', Plant Cell Physiology, 32 (7), 953–958.

Bertozzini, E., Galluzzi, L., Penna, A., Magnani, M. (2011). 'Application of the standard addition method for the absolute quantification of neutral lipids in microalgae using Nile red', Journal of Microbiological Methods, 87 (1), 17–23. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21767582 [Accessed 7 May 2014].

Bondioli, P., Bella, L. D., Rivolta, G., Zittelli, G. C., Bassi, N., Rodolfi, L., Casini, D., Prussi, M., Chiaramonti, D., Tredici, M.R. (2012). 'Bioresource Technology Oil production by the marine microalgae *Nannochloropsis* sp . F & M-M24 and *Tetraselmis suecica* F & M-M33', Bioresource Technology, 114, 567–572. Available at: http://dx.doi.org/10.1016/j.biortech.2012.02.123.

Brennan, L., Fernández, A. B., Mostaert, A. S., Owende, P. (2012). 'Enhancement of BODIPY 505/515 lipid fluorescence method for applications in biofuel-directed microalgae production', Journal of Microbiological Methods, 90 (2), 137–143. Available at: http://dx.doi.org/10.1016/j.mimet.2012.03.020.

Brito-Pelegrini, N.N., Pelegrini, R.T., Paterniani, J.E.S. (2007). 'Ecotoxicological evaluation of leachate from the Limeira sanitary landfill with a view to identifying acute toxicity', An Interdisciplinary Journal of Applied Science, 2 (3), 34-43.

Brunton, T., Lyons, H., Lerat, Y., Stanley, M. Bo Rasmussen, M. (2009). 'A Review of the Potential of Marine Algae as a Source of Biofuel in Ireland', [online], available: https://docs.google.com/viewer?a=v&q=cache:V01Gec2TTXkJ:www.sei.ie/algaereport+www.sei.ie/algaereport&hl=en&gl=ie&pid=bl&srcid=ADGEESjLdMieWlNfCaeL0x9bzNfdfLjzsHteJLJtxBqvEp4AUm7cE5NNezKhz16D-3ucKwSjWtoGbMxSmJ5if4CpjeRrzej3TJl-dS8I88bOlrkNuyZBVUP8_fKCIKYUJYrTCEr8keXC&sig=AHIEtbTlte158aAOV05ED0ouE-iTmUKTtg&safe=vss [Accessed 19 Dec 2012].

Carey, P., Carty, G., Donlon, B., Howley, D., Nealon, T. (2000). 'Landfill Manuals; Landfill Site Design', EPA, ISBN 1 84095 026 9.

Carty, G., O'Leary, G., Meaney, B. (1997). 'Wastewater Treatment Manuals: Primary, Secondary and Tertiary Treatment', EPA, ISBN 1899965467.

Chen, M., Tang, H., Maa, H., Holland, T.C., Simon Ng, K.Y., Salley, S.O. (2011). 'Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*', Bioresource Technology, 102 (2), 1649–1655. Available at: http://dx.doi.org/10.1016/j.biortech.2010.09.062.

Chen, W., Zhang, C., Song, L., Sommerfeld, M., Hu, Q. (2009). 'A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae', Journal of Microbiological Methods, 77 (1), 41–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19162091 [Accessed 30 April 2014].

Cheng, H.-X., Tian, G.-M. (2013). 'Preliminary Evaluation of a Newly Isolated Microalga *Scenedesmus* sp. CHX1 for Treating Landfill Leachate', 2013 Third International Conference on Intelligent System Design and Engineering Applications, 1057–1060. Available at: http://ieeexplore.ieee.org/lpdocs/epic03/wrapper.htm?arnumber=6456120 [Accessed 13 August 2014].

Chinnasamy, S., Bhatnagar, A., Claxton, R., Das, K.C. (2010). 'Biomass and bioenergy production potential of microalgae consortium in open and closed bioreactors using untreated carpet industry effluent as growth medium', Bioresource Technology, 101, 6751-6760.

Chowdhury, R., Viamajala, S., Gerlach, R. (2012). 'Reduction of environmental and energy footprint of microalgal biodiesel production through material and energy integration', Bioresource Technology, 108, 102–111. Available at: http://dx.doi.org/10.1016/j.biortech.2011.12.099.

Craggs, R.J., McAuley, P.J., Smith, V.J. (1997). 'Wastewater Nutrient Removal by Marine Microalgae Grown on Corrugated Raceway, Water Research, 31 (7), 1701–1707.

Croft, M.T., Warren, M.J., Smith, A.G. (2006). 'MINIREVIEWS: Algae Need Their Vitamins', Eukaryotic Cell, 5 (8), 1175–1183.

Deng, X., Fei, X., Li, Y. (2011). 'The effects of nutritional restriction on neutral lipid accumulation in *Chlamydomonas* and *Chlorella*', African Journal of Microbiology Research, 5 (3), 260–270.

Deng, Y., Englehardt, J.D. (2006). 'Treatment of landfill leachate by the Fenton process', Water research, 40 (20), 3683–94. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17045628 [Accessed 1 July 2015].

Doan, T.T.Y., Sivaloganathan, B., Obbard, J.P. (2011). 'Screening of marine microalgae for biodiesel feedstock', Biomass and Bioenergy, 35 (7), 2534–2544. Available at: http://dx.doi.org/10.1016/j.biombioe.2011.02.021.

Edgar R.C. (2004). 'MUSCLE: multiple sequence alignment with high accuracy and high throughput', Nucleic Acids Research, 32 (5), 1792-1797.

Fernandez, E., Galvan, A. (2008). 'Minireview: Nitrate Assimilation in *Chlamydomonas*', Eukaryotic Cell, 7 (4), 555-559.

Gordon, R., Losic, D., Tiffany, M.A., Nagy, S.S., Sterrenburg, F.A.S. (2009). 'The Glass Menagerie: Diatoms for Novel Applications in Nanotechnology', Trends in Biotechnology, 27 (2), 116–27. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19167770 [Accessed 27 October 2014].

Granéli, E., Weberg, M., Salomon, P.S. (2008). 'Harmful algal blooms of allelopathic microalgal species: The role of eutrophication', Harmful Algae, 8 (1), 94–102. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1568988308001054 [Accessed 10 November 2014].

Greenspan, P., Fowler, S.D. (1985). 'Spectrofluorometric studies of the lipid probe, nile red', Journal of Lipid Research, 26 (7), 781–789. Available at: http://www.ncbi.nlm.nih.gov/pubmed/4031658.

He, M., Li, L., Liu, J. (2011). 'Isolation of wild microalgae from natural water bodies for high hydrogen producing strains', International Journal of Hydrogen Energy, 37 (5), 4046–4056. Available at: http://dx.doi.org/10.1016/j.ijhydene.2011.11.089.

Heyer, K.U., Stegmann, R. (no date). Leachate management: leachate generation , collection , treatment and costs, Ingenieurbüro für Abfallwirtschaft, Prof. R. Stegmann und Partner, 1–23.

Hernández, D., Riaño, B., Coca, M., García-González, M.C. (2013). 'Treatment of agro-industrial wastewater using microalgae–bacteria consortium combined with anaerobic digestion of the produced biomass', Bioresource Technology, 135, 598-603.

Ho, S., Chen, C.Y., Chang, J.S. (2012). 'Effect of light intensity and nitrogen starvation on CO_2 fixation and lipid/carbohydrate production of an indigenous microalga *Scenedesmus obliquus* CNW–N', Bioresource Technology, 113, 244–252. Available at: http://dx.doi.org/10.1016/j.biortech.2011.11.133.

Hu, B., Min, M. Zhou, W., Du, Z., Hohr, M. Chen, P. Zhu, J. Cheng, Y. Liu, Y., Ruan, R. (2012). 'Enhanced mixotrophic growth of microalga *Chlorella sp.* on pretreated swine manure for simultaneous biofuel feedstock production and nutrient removal', Bioresource Technology, 126, 71–79. Available at: http://dx.doi.org/10.1016/j.biortech.2012.09.031.

Jiang, L., Luo, S., Fan, X., Yang, Z., Guo, R. (2011). 'Biomass and lipid production of marine microalgae using municipal wastewater and high concentration of CO_2',Applied Energy, 88, 3336–3341.

Johannessen, L. M. (1999). 'Guidance Note on Leachate Management for Municipal Solid Waste Landfills', The International Bank for Reconstruction and Development/The World Bank, USA.

John, D. M., Whitton, B. A., Brook, A. J. (2002). 'The Freshwater Algal Flora of the British Isles: An Identification Guide to Freshwater and Terrestrial Algae', The Natural History Museum and the British Phycological Society, UK, ISBN 0 521 77051 3.

Jones, C.S., Mayfield, S.P. (2012). ‘Algae biofuels: versatility for the future of bioenergy’, Current Opinion in Biotechnology, 23, 346–351. Available at: http://dx.doi.org/10.1016/j.copbio.2011.10.013.

Justin, M.Z., Zupančič, M.. (2009). ‘Combined purification and reuse of landfill leachate by constructed wetland and irrigation of grass and willows’, Desalination, 246 (1-3), 157–168. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0011916409004305 [Accessed 4 August 2015].

Keeling, P.J. (2004). ‘Diversity and Evolutionary History of Plastids and Their Hosts’, American Journal of Botany, 91 (10), 1481–1493.

Kothari, R., Prasad, R., Kumar, V., Singh, D.P. (2013). ‘Production of biodiesel from microalgae *Chlamydomonas polypyrenoideum* grown on dairy industry wastewater’, Bioresource Technology, 144, 499-503. Available at: http://dx.doi.org/10.1016/j.biortech.2013.06.116.

Kou, Z., Bei, S., Sun, J., Pan, J. (2013). ‘Fluorescent measurement of lipid content in the model organism *Chlamydomonas reinhardtii*’, Journal of Applied Phycology, 25 (6), 1633-1641.

Kropat, J., Hong-Hermesdorf, A., Casero, D., Ent, P., Castruita, M., Pellegrini, M., Merchant, S. S., Malasarn, D. (2011). ‘A revised mineral nutrient supplement increases biomass and growth rate in *Chlamydomonas reinhardtii*’, The Plant Journal 66 (5), 770–780.

Kumar, K.S., Ganesan, K., Rao, P.V.S. (2007). ‘Phycoremediation of heavy metals by the three-color forms of *Kappaphycus alvarezii*’, Journal of Hazardous Materials, 143, 590–592.

Kumar, M.S., Miao, Z.H., Wyatt, S.K. (2010). 'Influence of nutrient loads, feeding frequency and inoculum source on growth of *Chlorella vulgaris* in digested piggery effluent culture medium', Bioresource Technology, 101 (15), 6012–6018. Available at: http://dx.doi.org/10.1016/j.biortech.2010.02.080.

Kurniawan, T.A., Lo, W.-H., Chan, G.Y.S. (2006). 'Physico-chemical treatments for removal of recalcitrant contaminants from landfill leachate', Journal of hazardous materials, 129 (1-3), 80–100. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16314043 [Accessed 19 May 2015].

Lavrova, S., Koumanova, B. (2010). 'Influence of recirculation in a lab-scale vertical flow constructed wetland on the treatment efficiency of landfill leachate', Bioresource technology, 101 (6), 1756–61. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19932959 [Accessed 20 June 2015].

Lakaniemi, A., Hulatt, C.J., Thomas, D.N., Tuovinen, O.H., Puhakka, J.A. (2011). 'Biogenic hydrogen and methane production from *Chlorella vulgaris* and *Dunaliella tertiolecta* biomass', Biotechnology for Biofuels, 4 (34), 1-12. Available at: http://www.biotechnologyforbiofuels.com/content/4/1/34.

Levine, R.B., Costanza-Robinson, M.S., Spatafora, G.A. (2010). '*Neochloris oleoabundans* grown on anaerobically digested dairy manure for concomitant nutrient removal and biodiesel feedstock production', Biomass and Bioenergy, 35 (1), 40–49. Available at: http://dx.doi.org/10.1016/j.biombioe.2010.08.035.

Lim, D.K.Y., Garg, S., Timmins, M., Zhang, E.S.B., Thomas-Hall, S.R., Schuhmann, H., Li, Y., Schenk, P.M. (2012). 'Isolation and Evaluation of Oil-Producing Microalgae from Subtropical Coastal and Brackish Waters', PLoS ONE, 7 (7), 1-13.

Lima, S.A.C., Raposo, M.F.J., Castro, P.M.L., Morais, R.M. (2004). 'Biodegradation of p -chlorophenol by a microalgae consortium', Water Research, 38, 97–102.

Lin, L., Chan, G.Y.S., Jiang, B.L., Lan, C.Y. (2007). 'Use of ammoniacal nitrogen tolerant microalgae in landfill leachate treatment', Waste Management, 27, 1376–1382.

Mandal, S., Mallick, N. (2009). 'Microalga *Scenedesmus obliquus* as a potential source for biodiesel production', Applied Microbiology and Biotechnology, 84 (2), 281–291.

Mandal, S., Mallick, N. (2011). 'Waste Utilization and Biodiesel Production by the Green Microalga *Scenedesmus obliquus*', Applied and Environmental Microbiology, 77 (1), 374–377.

Manirakiza, P., Covaci, A., Schepens, P. (2001). 'Comparative Study on Total Lipid Determination using Soxhlet, Roese-Gottlieb, Bligh & Dyer, and Modified Bligh & Dyer Extraction Methods', Journal of Food Composition and Analysis, 14 (1), 93–100.

Matejczyk, M., Płaza, G.A., Nałecz-Jawecki, G., Ulfig, K., Markowska-Szczupak, A. (2011). 'Chemosphere Estimation of the environmental risk posed by landfills using chemical, microbiological and ecotoxicological testing of leachates', Chemosphere, 82, 1017–1023.

Matsunaga, T., Matsumoto, M., Maeda, Y., Sugiyama, H., Sato, R., Tanaka, T. (2009). 'Characterization of marine microalga, *Scenedesmus* sp. strain JPCC GA0024 toward biofuel production', Biotechnology Letters, 31 (9), 1367–1372.

Mishra, S.K., Suh, W.I., Farooq, W., Moon, M., Shrivastav, A., Park, M. S., Yang, J.W. (2014). 'Rapid quantification of microalgal lipids in aqueous medium by a simple colorimetric method', Bioresource Technology, 155, 330–333. Available at: http://dx.doi.org/10.1016/j.biortech.2013.12.077.

Moazami, N., Ranjbar, R., Ashori, A., Tangestani, M., Nejad, A.S. (2011). 'Biomass and lipid productivities of marine microalgae isolated from the Persian Gulf and the

Qeshm Island', Biomass and Bioenergy, 35 (5), 1935–1939. Available at: http://dx.doi.org/10.1016/j.biombioe.2011.01.039.

Mustafa, E.M., Phang, S.M., Chu, W.L. (2012). 'Use of an algal consortium of five algae in the treatment of landfill leachate using the high-rate algal pond system', Journal of Applied Phycology, 24 (4), 953–963.

Nakamura, K., Bray, D.F., Wagenaar, E.B. (1975). 'Ultrastructure of *Chlamydomonas eugametos* Palmelloids Induced by Chloroplatinic Acid Treatment', Journal of Bacteriology, 121 (1), 338-343.

Neelam, S., Subramanyam, R. (2013). 'Biology Alteration of photochemistry and protein degradation of photosystem II from *Chlamydomonas reinhardtii* under high salt grown cells', Journal of Photochemistry and Photobiology B: Biology, 124, 63–70. Available at: http://dx.doi.org/10.1016/j.jphotobiol.2013.04.007.

Olguín, E.J. (2003). 'Phycoremediation: key issues for cost-effective nutrient removal processes', Biotechnology Advances, 22, 81–91.

Olguín, E.J., Sanchez-Galvan, G. (2012). 'Heavy metal removal in phytofiltration and phycoremediation: the need to differentiate between bioadsorption and bioaccumulation', New Biotechnology, 30 (1), 3–8.

Olguín, E.J. (2012). 'Dual purpose microalgae – bacteria-based systems that treat wastewater and produce biodiesel and chemical products within a Biorefinery', Biotechnology Advances, 30 (5), 1031–1046. Available at: doi:10.1016/j.biotechadv.2012.05.001.

Oman, C.B., Junestedt, C. (2008). 'Chemical characterization of landfill leachates – 400 parameters and compounds', Waste Management, 28, 1876–1891.

Ozkan, A., Kinney, K., Katz, L., Berberoglu, H. (2012). 'Reduction of water and energy requirement of algae cultivation using an algae biofilm photobioreactor', Bioresource Technology, 114, 542-548.

Pereira, H., Barreira, L., Mozes, A., Florindo, C., Polo, C., Duarte, C.V., Custódio, L., Varela, J. (2011). 'Microplate-based high throughput screening procedure for the isolation of lipid-rich marine microalgae', Biotechnology for Biofuels, 4, 1-12. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3287250&tool=pmcentrez&rendertype=abstract [Accessed 2 May 2014].

Perez-Rama, M., Alonso, J. A., Lopez, C. H., Vaamonde, E. T. (2002). 'Cadmium removal by living cells of the marine microalga *Tetraselmis suecica*', Bioresource Technology, 84, 265–270.

Persoone, G., Gillett, J. (1990). 'Toxicological versus Ecotoxicological Testing', Short-term Toxicity Tests for Non-genotoxic Effects, John Wiley & Sons Ltd, Chapter 17, 287-299.

Pinto, G., Antonino Pollio, A., Previtera, L., Stanzione, M., Temussi, F. (2003). 'Removal of low molecular weight phenols from olive oil mill wastewater using microalgae', Biotechnology Letters, 25, 1657–1659.

Prajapati, S.K., Kaushik, P., Malik, A., Vijay, V.K. (2013). 'Phycoremediation and biogas potential of native algal isolates from soil and wastewater', Bioresource Technology, 135, 232–238. Available at: http://dx.doi.org/10.1016/j.biortech.2012.08.069.

Pulz, O., Gross, W. (2004). 'Valuable products from biotechnology of microalgae', Applied Microbiology and Biotechnology, 65 (6), 635–48. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15300417 [Accessed 9 July 2014].

Quant, B., Fudala-Ksiazek, S., Luczkiewicz, A., Kulbat, E., Jankowska, K., Czerwionka, K., Olanczuk-Neyman K. (2009). 'Influence of landfill leachate on municipal wastewater treatment -model plant (WWTP- MP)', Conference: IWA 2nd Specialized Conference Nutrient Management In Wastewater Treatment Processes Kraków.

Rastetter, M., Gawler, J., Kiviluoma, M., Suoniemi-Kähärä, A. (2015). 'Determination of Water Pollutants Using Photometric Analysis', Application Note 71728, Thermo Fisher Scientific, AN71728-EN 1015S. Available at: https://tools.thermofisher.com/content/sfs/brochures/AN-71728-DA-ISO-Water-Pollutants-AN71728-EN.pdf [Accessed 12 August 2017]

Quinn, J., Winter, L., Bradley, T. (2011). 'Microalgae bulk growth model with application to industrial scale systems', Bioresource Technology, 102, 5083–5092. Available at: http://dx.doi.org/10.1016/j.biortech.2011.01.019.

Rao, P.H., Kumar, R.R., Raghavan, B.G., Subramanian, V.V., Sivasubramanian, V. (2011). 'Application of phycoremediation technology in the treatment of wastewater from a leather-processing chemical manufacturing facility', Water SA, 37 (1), 7–14.

Ras, M., Lardon, L., Bruno, S., Bernet, N., Steyer, J.P. (2011). 'Experimental study on a coupled process of production and anaerobic digestion of *Chlorella vulgaris*', Bioresource Technology, 102, 200–206. Available at: http://dx.doi.org/10.1016/j.biortech.2010.06.146.

Rawat, I., Kumar, R.R., Mutanda, T., Bux, F. (2011). 'Dual role of microalgae: Phycoremediation of domestic wastewater and biomass production for sustainable biofuels production', Applied Energy, 88, 3411–3424. Available at: http://dx.doi.org/10.1016/j.apenergy.2010.11.025.

Renou, S., Givaudan, J.G., Poulain, S., Dirassouyan, F., Moulin, P. (2008). 'Landfill leachate treatment: Review and opportunity', Journal of hazardous materials, 150 (3), 468–93.

Available at: http://www.ncbi.nlm.nih.gov/pubmed/17997033 [Accessed 22 April 2015].

Sakthivel, R., Elumalai, S., Mohommad, M. (2011). 'Microalgae lipid research, past, present: A critical review for biodiesel production, in the future', Journal of Experimental Sciences, 2 (10), 29–49.

Schlagermann, P., Gottlicher, G., Dillschneider, R., Rosello-Sastre, R., Posten, C. (2012). 'Composition of Algal Oil and Its Potential as Biofuel', Journal of Combustion, 2012, 1-14.

Seng, L.K., Wah, N.B., Chieh, D.C.J. (2012). 'Biotreatment of Fish Farm Wastewater with Microalgae', Proceedings of International Conference on Engineering and Information Technology "ICEIT2012", ISBN: 978-1-77136-064-7.

Sforza, E., Al, M.K., Sharif, A. (2015). 'Exploitation of Urban Landfill Leachate as Nutrient Source for Microalgal Biomass Production', Chemical Engineering Transaction, 43, ISBN 978-88-95608-34-1, ISSN 2283-9216.

Shanab, S., Essa, A., Shalaby, E. (2012). 'Bioremoval capacity of three heavy metals by some microalgae species (Egyptian Isolates)', Plant Signaling and Behavior 7 (3), 1–8.

Siaut, M., Cuiné, S., Cagnon, C., Fessler, B., Nguyen, M., Carrier, P., Beyly, A., Beisson, F., Triantaphylidès, C., Li-Beisson, Y., Peltier, G. (2011). 'Oil accumulation in the model green alga *Chlamydomonas reinhardtii*: characterization, variability between common laboratory strains and relationship with starch reserves', BMC Biotechnology, 11 (7), 1-15. Available at: http://www.biomedcentral.com/1472-6750/11/7.

Silva-Benavides, A.M., Torzillo, G. (2012). 'Nitrogen and phosphorus removal through laboratory batch cultures of microalga *Chlorella vulgaris* and

cyanobacterium *Planktothrix isothrix* grown as monoalgal and as co-cultures', Journal of Applied Phycology, 24, 267–276.

Sloth, J.K., Wiebe, M.G., Eriksen, N.T. (2006). 'Accumulation of phycocyanin in heterotrophic and mixotrophic cultures of the acidophilic red alga *Galdieria sulphuraria*', Enzyme and Microbial Technology, 38, 168–175. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0141022905002309 [Accessed 7 October 2014].

Sonnenberg, R., Nolte, A.W., Tautz, D. (2007). 'An evaluation of LSU rDNA D1-D2 sequences for their use in species identification', Frontiers in Zoology, 4 (6), 1-12. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1805435&tool=pmcentrez&rendertype=abstract [Accessed 11 June 2014].

Sooksawat, N., Meetam, M., Kruatrachue, M., Pokethitiyook, P., Nathalang, K. (2013). 'Phytoremediation potential of charophytes: Bioaccumulation and toxicity studies of cadmium, lead and zinc', Journal of Environmental Sciences, 25 (3), 596–604. Available at: http://dx.doi.org/10.1016/S1001-0742(12)60036-9.

Spellman F. R. (2009). Water and Wastewater Treatment Plant Operations, CRC Press Taylor & Francis Group, Second Edition, ISBN 978–1–4200–7530–4.

Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A. (2006). 'Commercial applications of microalgae', Journal of Bioscience and Bioengineering, 101 (2), 87–96. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16569602 [Accessed 10 July 2014].

Tam, N. F. Y., Wong, Y. S. (1996). 'Effect of ammonia concentrations on growth of *Chlorella vulgaris* and nitrogen removal from media', Bioresource Technology, 57 (1), 45-50.

Tamburic, B., Zemichael, F.W., Maitland, G.C., Hellgardt, K. (2012). 'A novel nutrient control method to deprive green algae of sulphur and initiate spontaneous hydrogen production', International Journal of Hydrogen Energy, 37, 8988–9001. Available at: http://dx.doi.org/10.1016/j.ijhydene.2012.02.043.

Tan, Y., Lin, J. (2011). 'Biomass production and fatty acid profile of a *Scenedesmus rubescens*-like microalga', Bioresource Technology, 102 (21), 10131–10135. Available at: http://dx.doi.org/10.1016/j.biortech.2011.07.091.

Thakur, A., Kumar, H.D. (1999). 'Nitrate , Ammonium , and Phosphate Uptake by the Immobilized Cells of *Dunaliella salina*', Environmental Contamination and Toxicology, 62, 70–78.

Torres, M.A., Barros, M.P., Campos, S.C.G., Pinto, E., Rajamani, S., Sayre, R.T., Colepicolo, P. (2008). 'Biochemical biomarkers in algae and marine pollution: A review', Ecotoxicology and Environmental Safety, 71 (1), 1–15. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18599121 [Accessed 19 September 2014].

Ugwu, C.U., Aoyagi, H., Uchiyama, H. (2008). 'Photobioreactors for mass cultivation of algae', Bioresource Technology, 99, 4021–4028.

Van Vooren, G., Le Grand, F., Legrand, J., Cuiné, S., Peltier, G., Pruvost, J. (2012). 'Investigation of fatty acids accumulation in *Nannochloropsis oculata* for biodiesel application', Bioresource Technology, 124, 421–432. Available at: http://dx.doi.org/10.1016/j.biortech.2012.08.009.

Van Den Hende, S., Carré, E., Cocaud, E., Beelen, V., Boon, N., Vervaeren, H. (2014). 'Treatment of industrial wastewaters by microalgal bacterial flocs in sequencing batch reactors', Bioresource Technology, 161, 245–254. Available at: http://dx.doi.org/10.1016/j.biortech.2014.03.057.

Wilkie, A.C., Mulbry, W.W. (2002). 'Recovery of dairy manure nutrients by benthic freshwater algae', Bioresource Technology, 84, 81–91.

Xin, L., Hong-ying, H., Ke, G., Jia, Y. (2010). 'Growth and nutrient removal properties of a freshwater microalga *Scenedesmus* sp . LX1 under different kinds of nitrogen sources', Ecological Engineering, 36 (4), 379–381. Available at: http://dx.doi.org/10.1016/j.ecoleng.2009.11.003.

Yang, C., Jia, L., Su, S., Tian, Z., Song, Q., Fang, W., Chen, C., Liu, G. (2012). 'Utilization of CO_2 and biomass char derived from pyrolysis of *Dunaliella salina*: The effects of steam and catalyst on CO and H_2 gas production', Bioresource Technology, 110, 676–681. Available at: http://dx.doi.org/10.1016/j.biortech.2012.01.124.

Yoshida, K., Ishii, H., Ishihara, Y., Saito, H., Okada, Y. (2009). 'Bioremediation potential of Formaldehyde by the Marine Microalga *Nannochloropsis oculata* ST-3 Strain', Applied Biochemistry and Biotechnology, 157, 321 – 328.

Zamalloa, C., Vrieze, J.D., Boon, N., Verstraete, W. (2012). 'Anaerobic digestibility of marine microalgae *Phaeodactylum tricornutum* in a lab-scale anaerobic membrane bioreactor', Applied Microbiology and Biotechnology, 93, 859–869.

Zhao, X., Zhou, Y., Huang, S., Qiu, D., Schideman, L., Chai, X., Zhao, Y. (2014). 'Characterization of microalgae-bacteria consortium cultured in landfill leachate for carbon fixation and lipid production', Bioresource Technology 156, 322–328. doi:10.1016/j.biortech.2013.12.112.

Zhou, W., Li, Y., Min, M., Hu, B., Chen, P., Ruan, R. (2011). 'Local bioprospecting for high-lipid producing microalgal strains to be grown on concentrated municipal wastewater for biofuel production', Bioresource Technology, 102 (13), 6909–6919. Available at: http://dx.doi.org/10.1016/j.biortech.2011.04.038.

Chapter 2

Microalgae isolation and selection for the treatment of landfill leachate

This chapter describes and summarises the findings of the microalgae isolation and screening processes conducted for the purpose of this research. It was published as a peer-reviewed article in the Water Pollution 2016 international conference proceedings publication published by WIT press and can be accessessed on doi:10.2495/WP160071.

Authors: A. Paskuliakova, S. Tonry, N. Touzet

School of Science, Centre for Environmental Research Innovation and Sustainability (CERIS), Institute of Technology, Ireland

This paper is part of the Proceedings of the 13th International Conference on Modelling, Monitoring and Management of Water Pollution (WP 2016)
www.witconferences.com

Microalgae isolation and selection for the treatment of landfill leachate

A. Paskuliakova, S. Tonry & N. Touzet
School of Science, Centre for Environmental Research Innovation and Sustainability (CERIS), Institute of Technology, Ireland

Abstract

The use of microalgae in remediation has been researched for a variety of waste effluents, yet algal remediation of landfill leachate is somewhat less explored. Very high levels of pollutants, such as ammonia nitrogen, salts and recalcitrant organic matter are present in landfill leachate and render it toxic to many organisms. Thus the selection of suitably tolerant microalgal strains is crucial for phycoremediation attempts. Other factors such as temperature and light requirements and the variable composition of landfill leachates also need to be incorporated into the remediation strategy.

This study focused on isolating microalgae strains from different environments in the North-West of Ireland, which might have the potential to use leachate pollutants as their source of nutrients. A screening process was applied to select the most promising strains which was followed by a preliminary assessment of nutrient depletion.

Altogether 34 strains were obtained from marine, freshwater and polluted environments. Further screening yielded 16 strains capable of growth in leachate samples to varying degrees. Generally, the strains isolated from landfill leachate itself appeared to perform better, while some freshwater and marine species could adapt if the leachate was appropriately diluted. A preliminary nutrient depletion experiment with the chlorophyte strain *Chlamydomonas* sp. SW13aLS grown on 10% permeate leachate indicated a substantial reduction in nutrients such as ammonia-nitrogen (93%) and nitrate (54%) when supplemented with phosphorus.

The results demonstrate the possible application of microalgae for the treatment of leachate when grown under limited light and relatively low temperature; however nutrient limitation could be a key inhibitory factor requiring optimisation.
Keywords: phycoremediation, landfill leachate, microalgae, nutrient limitation.

WIT Transactions on Ecology and The Environment, Vol 209, © 2016 WIT Press
www.witpress.com, ISSN 1743-3541 (on-line)
doi:10.2495/WP160071

2.0 Abstract

The use of microalgae in remediation has been researched for a variety of waste effluents, yet algal remediation of landfill leachate is somewhat less explored. Very high levels of pollutants, such as ammonia nitrogen, salts and recalcitrant organic matter are present in landfill leachate and render it toxic to many organisms. Thus the selection of suitably tolerant microalgal strains is crucial for phycoremediation attempts. Other factors such as temperature and light requirements and the variable composition of landfill leachates also need to be incorporated into the remediation strategy.

This study focused on isolating microalgae strains from different environments in the North-West of Ireland, which might have the potential to use leachate pollutants as their source of nutrients. A screening process was applied to select the most promising strains which was followed by a preliminary assessment of nutrient depletion.

Altogether 34 strains were obtained from marine, freshwater and polluted environments. Further screening yielded 16 strains capable of growth in leachate samples to varying degrees. Generally, the strains isolated from landfill leachate itself appeared to perform better, while some freshwater and marine species could adapt if the leachate was appropriately diluted. A preliminary nutrient depletion experiment with the chlorophyte strain *Chlamydomonas* sp. SW13aLS grown on 10% permeate leachate indicated a substantial reduction in nutrients such as ammonia-nitrogen (93%) and nitrate (54%) when supplemented with phosphorus.

The results demonstrate the possible application of microalgae for the treatment of leachate when grown under limited light and relatively low temperature; however nutrient limitation could be a key inhibitory factor requiring optimisation.

Keywords

phycoremediation, landfill leachate, microalgae, nutrient limitation

2.1 Introduction

Phycoremediation is a process availing of the ability of microalgae to remove or biotransform inorganic and organic pollutants from wastewater [1]. Leachate, a wastewater originating from landfill sites and saturated with decomposition products from landfill waste, is a complex and challenging substrate to treat. Its composition varies and is generally dependent on a variety of factors [2].

Landfill leachate, in comparison with more researched phycoremediation applications such as municipal wastewater or fishpond discharges has higher concentrations of dissolved salts, especially chlorides and extremely high concentrations of ammonia-nitrogen, which can be toxic to many organisms. Thus far, most raw landfill leachate phycoremediation attempts have required it to be diluted to make any microalgal growth possible [3, 4]. The selection of suitable species and strains is important. The most common in the literature in relation to remediation are freshwater chlorophytes, specifically *Scenedesmus* spp., *Chlorella* spp. and *Chlamydomonas* spp. [3, 4, 5, 6, 7]. A Scottish study that used marine species for the treatment of sewage wastewater was applied to a facility being situated near the shoreline so it could be diluted with sea water [8]. Similarly in China, municipal wastewater diluted with seawater has been successfully used as growth medium for the marine microalgae *Nannochloropsis* sp. [9]. However, Aravantinou *et al.* [10] who evaluated the remediation ability of both marine and freshwater species noted that although the growth rates of marine microalgae were higher, nutrient removal was inferior for the marine species examined.

Throughout the literature the species often selected for phycoremediation studies are: (1) either isolated from the wastewater itself; (2) isolated from various environments and subjected to a screening procedure with criteria relevant to a particular application; or (3) based on prior knowledge of which species tend to be present in a wastewater type and thus can be purchased from culture collections.

In the present study, several different sampling sites were selected in northwest Ireland for species isolation with potential application to treatment of landfill leachate. These included various freshwater, marine and polluted environment (landfill leachate) microalgae. A screening procedure was applied to evaluate the tolerance of these strains to some pollutants expected to have an inhibitory effect on

their growth (high salt content and ammonia-nitrogen). A selection of strains able to grow in landfill leachate was obtained for future studies. A preliminary nutrient depletion experiment was set up with one strain to estimate other possible factors that should be included in future studies (i.e. phosphorus limitation).

2.2 Materials and Methods

2.2.1 Strain Isolation

Microalgae were isolated from different environmental habitats in the North West of Ireland in 2012 and 2013 and from samples of landfill leachate collected in 2013 and 2014 at a municipal landfill site in Northern Ireland. Monocultures were obtained through single cell isolation into f/2 medium. In the case of non marine species, the cultures were isolated into f/2 medium that was appropriately diluted with water of lower conductivity or autoclaved (wastewater) leachate. Cultures were brought up in an incubator at a temperature of 15°C and light cycle of 14:10 hours (light:dark) (Illuminance: 1667 lx, Photosynthesis photon flux density (PPFD): 22 µmol m^{-2} s^{-1}).

2.2.2 Microscopy and counting techniques

Species were characterised via light microscopy observations and with the aid of identification manuals [11, 12]. Cell size was determined after fixing cells with formalin or Lugol's iodine and measuring their dimensions with the aid of a calibrated ocular micrometer scale at ×400 magnification. Cell concentration was estimated either by counts with a Neubauer chamber or counts through focal view in 96-well plates using an inverted microscope.

2.2.3 Molecular and Phylogenetic analyses

Partial PCR amplification of the large subunit (28S) ribosomal gene was conducted according Touzet *et al.* [13]. Total genomic cellular DNA was extracted with the use of the VWR OMEGA BIO-TEK Plant DNA kit D3485-01 according to the manufacturer's instructions "Short protocol".

Partial large subunit ribosomal RNA gene sequences were compared to those present in Genbank's library by BLAST analysis (http://blast.ncbi.nlm.nih.gov//Blast.cgi) to indicate higher level taxonomic group allocation. A number of sequences of typical representatives of major taxonomic groups were also downloaded to visualise the genetic similarity of isolated species within these taxonomic groups. Initial alignments were made and edited with Genedoc and Clustal-X.

A non-rooted phylogenetic tree was generated with the use of "Phylogeny.fr" platform (http://www.phylogeny.fr/version2_cgi/index.cgi). Phylogeny.fr analysis involves alignment (MUSCLE v3.7), curation using Gblocks (v0.91b), reconstruction of phylogenetic tree using maximum likelihood method (PhyML v3.0) and graphical output and edition is provided by TreeDyn v198.3. Default settings were used [14, 15, 16, 17, 18].

2.2.4 Landfill leachate collection

Samples were collected at three points of the leachate treatment process: raw leachate, process leachate (or permeate) which is treated effluent after the anoxic tank in the MBR (Membrane Bioreactor) plant and treated leachate which is the final effluent from the MBR plant. Samples were stored at < 5°C. Leachate samples were collected in March 2013 and April 2014.

2.2.5 Landfill leachate physico-chemical analyses

Results for physico-chemical parameters of raw and treated leachate were obtained from certificates of analyses for environmental monitoring, which were carried out by the landfill operator.

Phosphate (PO_4^{3-}-P), total oxidised nitrogen (TON) and total ammonia nitrogen (TAN) were determined spectrophotometrically based on APHA methods (Standard Methods for the Examination of Water and Wastewater) adapted to the Aquakem 250 autoanalyser. Samples were filtered through 0.45 µm filter prior to the analyses.

2.2.6 Physiological screening for strain selection

Microalgae were subjected to a number of tolerance experiments whereby growth was monitored in media of different conductivity (to account for salt tolerance) and different dilutions of landfill leachate (ammonia and other toxicants tolerance). Light and temperature regimes were as per Section 2.2.1.

2.2.6.1 Stress response to different conductivity levels

The ability of strains to survive and grow in different salinities was observed in 24-well plates. The cell growth was monitored at intervals of several days by cell counts (focal view). Marine species were inoculated into f/2 medium of conductivity (2.0-2.4) and (16.0–17.5) mS cm^{-1} while conductivity (49.0-53.0) mS cm^{-1} was used as control. As most effluents are not expected to have conductivity above 16.0, all but the marine species were studied only at conductivity (2.0-2.4) and (16.0–17.5) mS/cm with conductivity (2.0-2.4) being used as control. Experiments were conducted in duplicates due to logistical constraints and the relatively high numbers of strains to screen.

2.2.6.2 Stress response to different leachate substrates

Ammonia tolerance experiments were set up in 96-well plates in three different substrates: treated leachate, 25 % dilution of permeate and 25 % dilution of raw leachate (Feb 2013) that correspond to a concentration of ~10 NH_4^+-N mg l^{-1}, ~50 NH_4^+-N mg l^{-1} and ~125 NH_4^+-N mg l^{-1}, respectively. Growth was compared to a f/2 medium control. One single count was carried out on cultures that showed survival after a number of days based on previous growth experiments. Experiments were conducted in duplicates.

The strains that showed some growth were further evaluated on 25%, 35% and 50% permeate for growth as a compromise between treated and raw leachate.

2.2.7 Selection process after screening

2.2.7.1 Stress response to different conductivity levels

Growth of individual strains in media of different conductivities was expressed through relative increases. These are n-fold increases relative to day 0 count in media of each salinity (equation 1).

$$n\text{-}fold\ increase = \frac{cell\ count\ (\ day\ x)\ [\frac{cells}{ml}]}{cell\ count\ (day\ 0)\ [\frac{cells}{ml}]} \qquad (1)$$

The maximum relative increases were compared to the maximum increase in the control to show the effect of media of different conductivities on the growth of each strain (equation 2).

$$relative\ n\text{–}fold\ increase(\%) = \frac{nf(max\ sample) - 1\ (day\ 0)}{nf(max\ control) - 1\ (day\ 0)}\ .100\ \% \qquad (2)$$

$nf\,(max\ sample)$: *maximum n-fold increase in media of studied conductivity*
$nf\,(max\ sample)$: *maximum n-fold increase in control*

The biovolume increases were also compared to take into account different cell sizes.

2.2.7.2 Stress response to different leachate substrates

The potential to grow in different landfill leachates was evaluated and scores were allocated based on the ability of strains to grow and also on the biovolume increases in the substrate. Based on the results from the screening tests, all microalgae strains were divided into four groups of tolerance: high, medium, low and not suitable.

2.2.8 Preliminary nutrient depletion experiment

One strain from the group of highly tolerant strains was selected at random (SW13aLS) and used in a preliminary nutrient depletion experiment. Three sets of duplicates of 10% permeate leachate diluted with autoclaved deionised water with the same initial cell concentrations were prepared. Two flasks were supplemented with phosphorus (~40 mg l^{-1} PO_4^{3-}-P) and two flasks were supplemented in addition to phosphorus with a mineral stock solution (+ 150 µl of IMR mineral stock solution). Cell concentration was monitored at intervals of several days. Nutrient content reduction was measured after the strains appeared to reach stationary phase.

2.3 Results

2.3.1 Microalgae strain isolation and characterisation

Overall, 34 strains were isolated and successfully brought into culture. Some cultures, although monoalgal, could be observed to undergo morphological changes or be prone to microbial contamination after a certain time. These cultures were not used further. Also, fibrous and pico plankton microalgae were difficult to evaluate for growth and were excluded. 25 strains were evaluated in the tolerance tests.

2.3.2 Phylogenetic analyses

Figure 2.1 displays a phylogenetic tree compiled from the sequences of isolated strains and typical representatives of the major microalgal taxonomic groups. The highest numbers of strains isolated were from the phyla Chlorophytes (16) and Bacillariophyta (7). Two strains of Cryptophyta and only one representative from the Euglenophyta, Rhodophyta and Prasinophyta were successfully brought into culture. While strains from taxa Euglenophyta, Rhodophyta, Cryptophyta and Bacillariophyta formed clearly distinguished clades (bootstrap values ~0.9), the Chlorophyta was not clearly separated from representatives of phylum Prasinophyta.

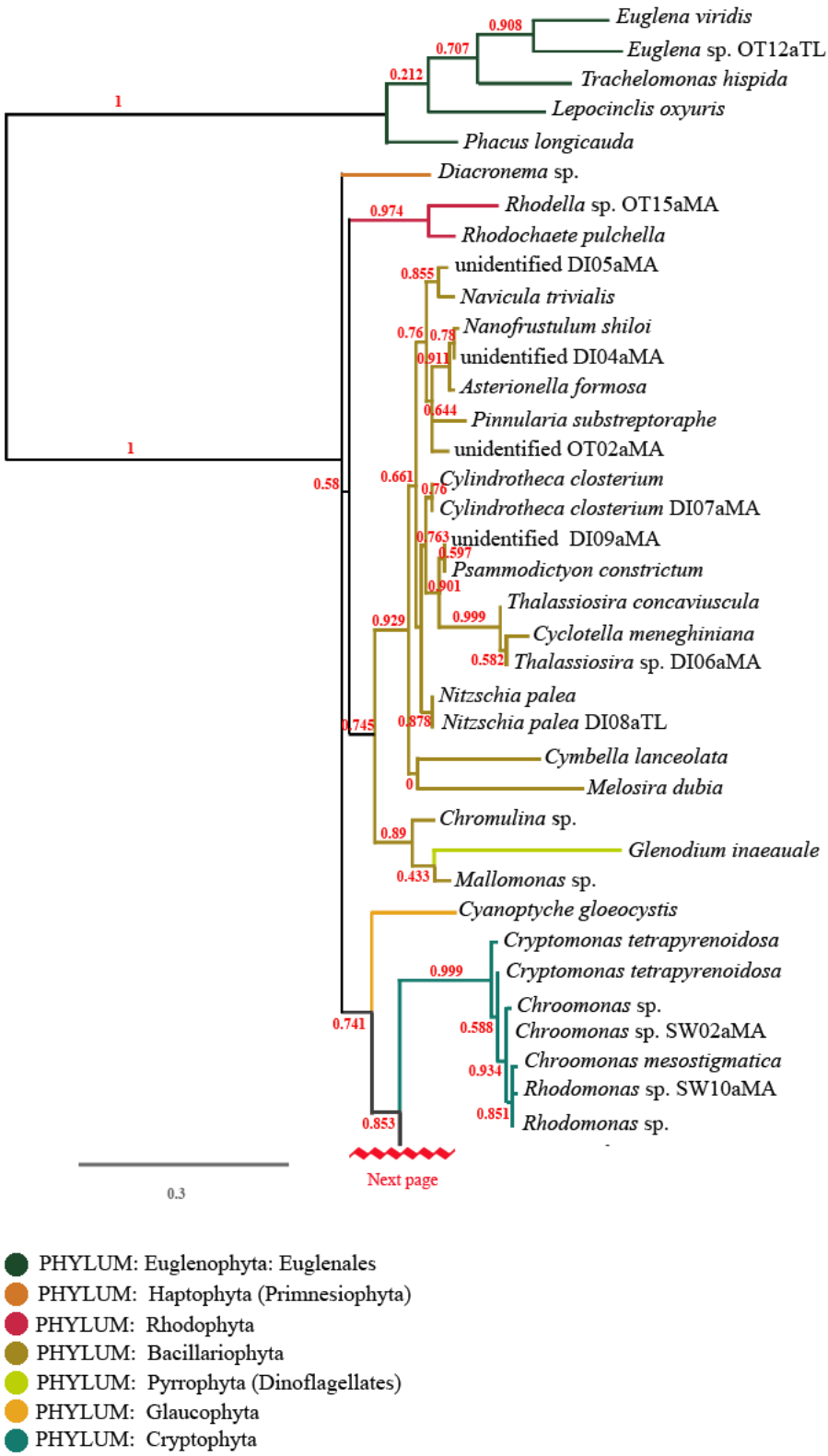


Figure 2.1a. Unrooted phylogenetic tree produced from sequences obtained from isolated strains and sequences obtained from NCBI GeneBank Numbers at nodes represent bootstrap values.

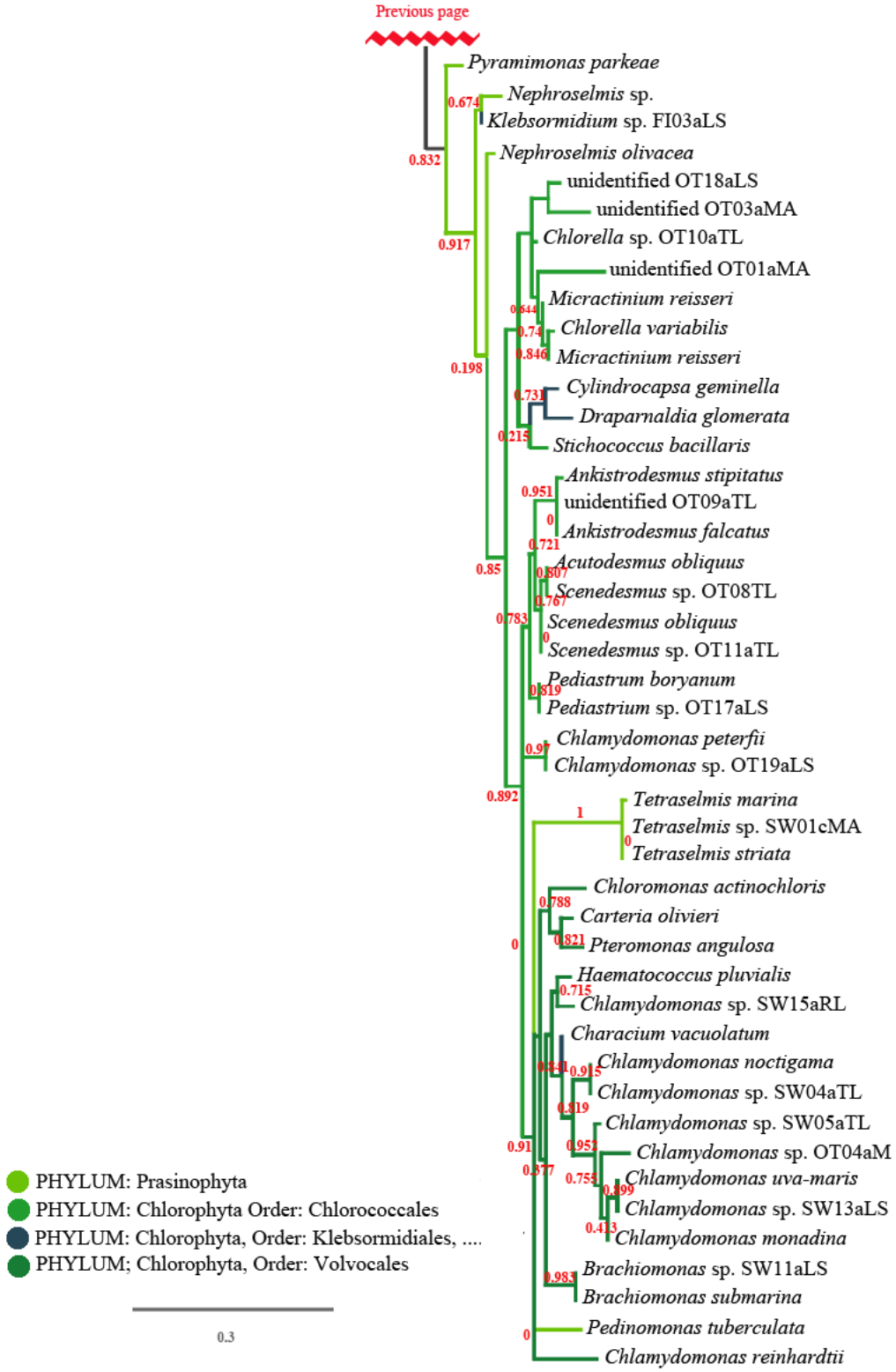


Figure 2.1b. Unrooted phylogenetic tree produced from sequences obtained from isolated strains and sequences obtained from NCBI GeneBank Numbers at nodes represent bootstrap values.

2.3.3 Tolerance studies

2.3.3.1 Stress response to different conductivity levels

Most freshwater species showed growth at the higher conductivity tested. Some freshwater species did not grow at either lower or higher conductivity (i.e. OT12aTL, SW15aRL) and some cultures were highly prone to both bacterial and fungal contamination in the test media (i.e. SW05aTL, DI08aTL), especially the strains originally isolated from the landfill leachate.

Only one of all the marine cultures tested (OT14aMA) was able to tolerate the change of medium conductivity across the whole range and had comparable growth at all three tested conductivities (Figure 2.2).

Two other cultures, OT03aMA and OT04aMA, showed survival at both lower tested conductivities. Strain SW07aMA appeared to be a brackish species as its growth at middle range conductivity exceeded that observed at the highest conductivity. This strain was however not able to adjust to the lowest conductivity medium.

The overview of the adaptability of strains to different conductivities of media is displayed in Figure 2.3.

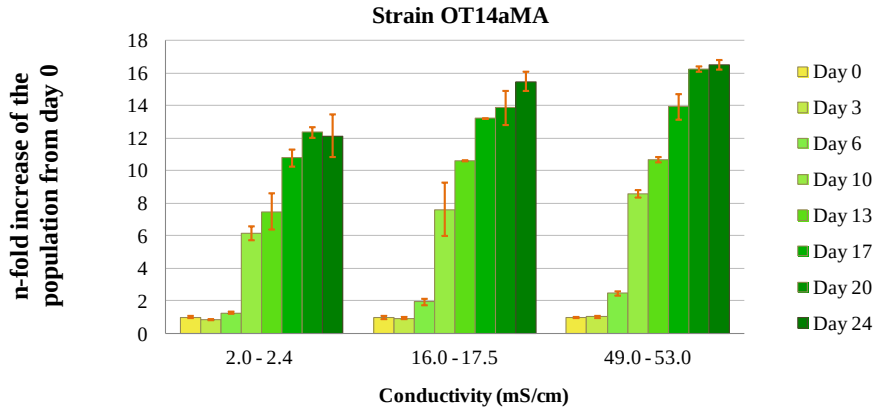


Figure 2.2. Tolerance of marine microalgae strain OT14aMA to media of different conductivities. OT14aMA was the only marine strain that was not affected in a major way by change of media conductivity without acclimatisation.

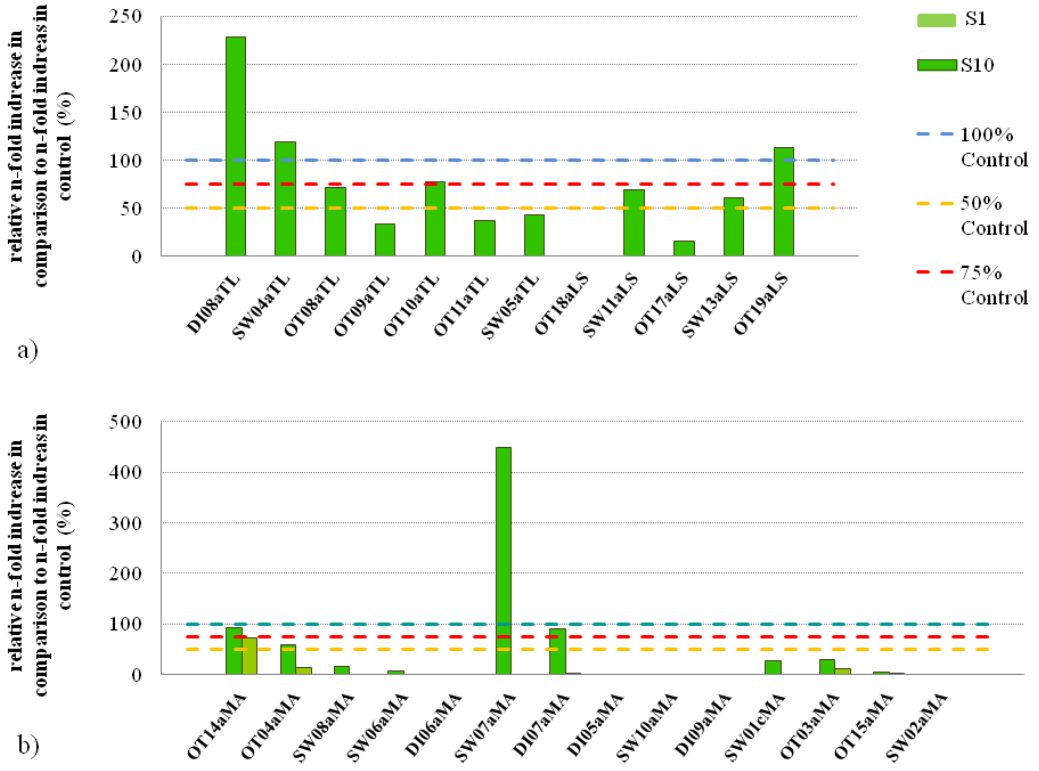


Figure 2.3. Tolerance of a) freshwater and b) marine microalgae to media with lower and higher conductivity (salt strength).

2.3.3.2 Stress response to different leachate substrates

The screening of microalgae strains in landfill leachate stress tests yielded 16 strains able to survive and/or grow. These were mainly freshwater species. Most of the marine species did not grow in any diluted leachate. Even those able to adapt to lower osmotic pressure (as seen from conductivity stress experiments) showed only moderate growth. The marine species that showed some growth were *Tetraselmis* sp. SW01cMA, that was able to grow at 25% dilution (with sea water) of permeate and raw leachate, and the unidentified chlorophyte strain OT03aMA that also showed some growth in treated leachate. Strain OT14aMA was able to tolerate change of conductivity across the whole range and was originally tested during the leachate tolerance experiments using leachate diluted with low salinity dilution water. However, the strain did not appear to be able to adapt to a combination of leachate and change of conductivity within the time period tested. The test was repeated with leachate diluted with seawater and the strain was then able to grow.

Some strains, isolated mostly from landfill leachate, showed promising tolerance to different leachates and dilutions (e.g. SW05aTL, Figure 2.4). The results for the tolerance test were compiled and a table summarising species most likely to suit landfill leachate treatment are listed in Table 2.1.

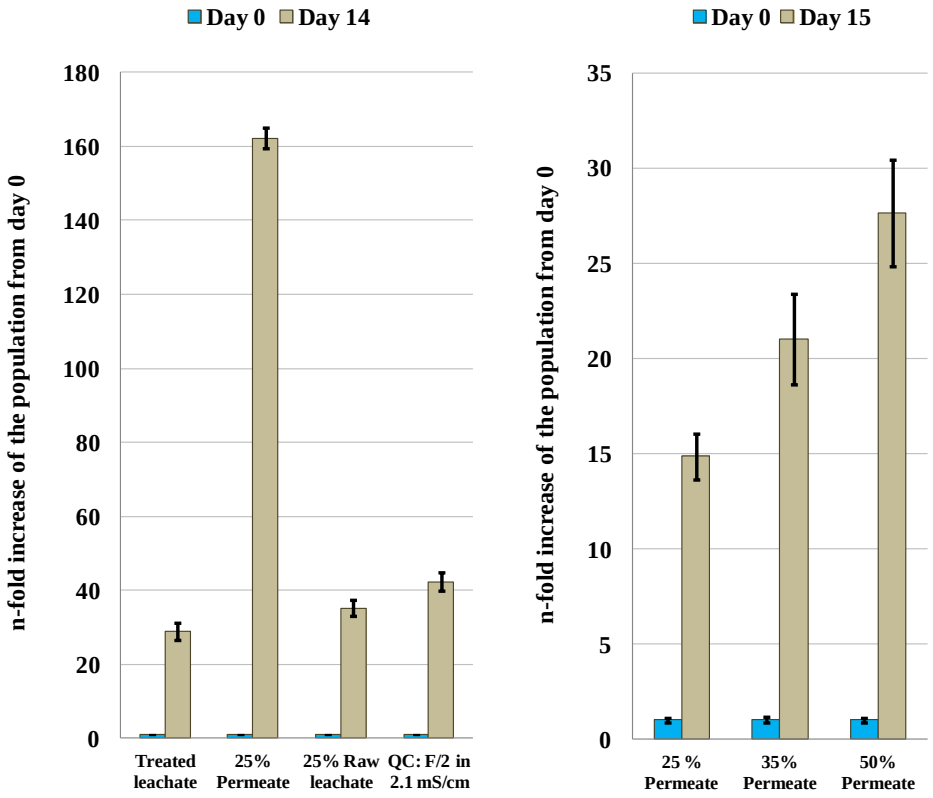


Figure 2.4. Leachate tolerance experiment results for strain SW05aTL.

Table 2.1. Summary of screening results in tolerance experiments

Tolerance	Strain	Taxonomic classification	Origin
High	SW05aTL	*Chlamydomonas* sp.	Treated l. 2013
	SW04aTL	*Chlamydomonas* sp.	Treated l. 2013
	SW13aTL	*Chlamydomonas* sp.	Peat water, Inishbofin, 2013
	OT08aTL	*Scenedemus* sp.	Treated l. 2013
	OT11aTL	*Scenedemus* sp.	Treated l. 2013
Medium	OT10aTL	*Chlorella* sp.	Treated l. 2013
	OT14aMA	Unknown chlorophyte	Sea water, 2012
	OT03aMA	Unknown chlorophyte	Sediment Sligo, 2013
	SW01cMA	*Tetraselmis* sp	Sediment Sligo, 2012
	OT18aLS	Unknown chlophyte	Rock pool Sligo, 2013
	OT09aTL	Unknown chlorophyte	Treated l. 2013
	DI08aTL	*Nitzschia palea*	Treated l. 2013
	SW15aRL	*Chlamydomonas* sp.	Raw l. 2014
	OT19aLS	*Chlamydomonas sp.*	Peat water, Inishbofin 2013
	OT12aTL	*Euglena* sp.	Treated l. 2013
Low	OT17aLS	*Pediastrum* sp.	Rock pool Sligo, 2013
	SW11aLS	*Brachiomonas* sp.	Rock pool Sligo, 2013
	DI07aMA	*Cylindrotheca closterium*	Sediment Sligo, 2013
	OT15aMA	*Rhodella* sp.	Sediment Sligo, 2013
Not suitable	OT04aMA	*Chlamydomonas* sp.	Sediment Sligo, 2013
	OT21aLS	*Scenedesmus* sp.	Rock pool Sligo, 2013
	OT01aMA	Unknown chlorophyte	Sediment Sligo, 2012
	FI03aLS	*Klebsormidium flaccidum*	Peat water, Inishbofin, 2013
	DI10aLS	*Nitzschia* sp.	Peat water, Inishbofin, 2013
	DI05aMA	Unknown diatom	Sea water Sligo 2012
	DI06aMA	*Thalassiosira* sp.	Sediment Sligo, 2013
	DI09aMA	Unknown diatom	Sediment Sligo, 2013
	OT02aMA	*Tabularia* sp.	Sediment Sligo, 2013
	SW06aMA	unknown	Sediment Sligo, 2013
	SW07aMA	unknown	Sediment Sligo, 2013
	SW02aMA	*Chroomonas* sp.	Sediment Sligo, 2012
	SW08aMA	unknown	Sediment Sligo, 2013
	SW10aMA	*Rhodomonas* sp.	Sediment Sligo, 2013
	FI04aLS	*Aphanizomenon* sp.	Peat water, Inishbofin, 2013

2.3.1 Preliminary nutrient depletion experiment

From the growth curves in Figure 2.5 it can be seen that the flasks supplemented with phosphorus both showed increased growth and achieved higher cell densities. In the flasks with no addition of phosphorus the growth stopped after approximately day 36. Nutrient depletion also confirmed the phosphorus limitation as there was clearly higher removal of nitrogen in phosphorus supplemented flasks (Table 2.2).

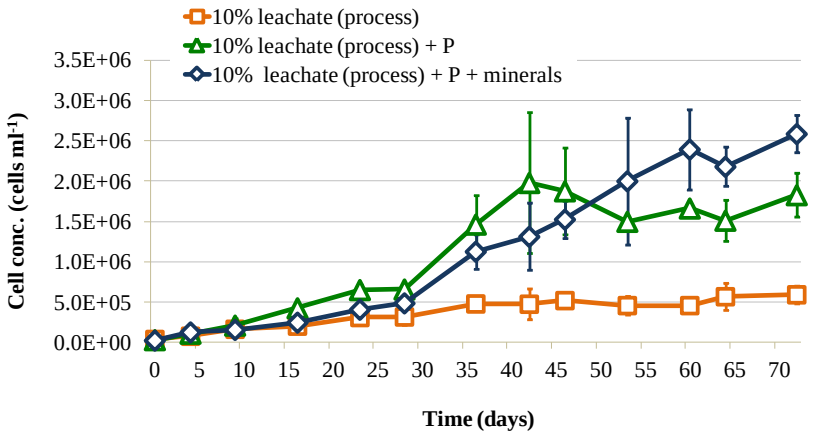


Figure 2.5. Growth curves of strain SW13aLS in 10% permeate with and without nutrient supplementation.

Table 2.2. Change of nutrient concentration due to growth of *Chlamydomonas* sp. in 10% permeate with and without nutrient supplementation. Results on day 60 are averages of duplicates.

	10% permeate			10% permeate (+ PO_4^{3-}-P)			10% permeate (+PO_4^{3-}-P + min)		
	Day 0	Day 60		Day 0	Day 60		Day 0	Day 60	
	Conc.	Conc.	Reduction	Conc.	Conc.	Reduction	Conc.	Conc.	Reduction
	mg l^{-1}	mg l^{-1}	%	mg l^{-1}	mg l^{-1}	(%)	mg l^{-1}	mg l^{-1}	%
PO_4^{3-}-P	1	<0.02	98	41	5	88	41	32	22
TON	95	87	8	95	44	54	95	61	36
NH_4^+-N	23	4.1	82	23	1.5	93	23	*ND	100

*ND – not detected

2.4 Discussion

2.4.1 Screening procedure by tolerance studies

Strains from various environments were included due to their potential to tolerate various substances or conditions. Marine species were chosen to be considered due to their resistance to high concentrations of dissolved salts. Also, a number of freshwater sites were included. For example, bog water (high content of humic substances) was considered due to potentially lesser light demand of the species present in this environment (as leachate can also be very dark). The species from landfill leachate were assumed to be adapted to the high ammonia-nitrogen and other substances present in landfill leachate.

The growth results from leachate/ammonia and conductivity tolerance experiments are indicatory. The effects of various ions concentrations are complex as they mutually influence cell growth through inhibition as well as microalgal nutrient requirements preference. The overall objective was to isolate a small number of microalgal strains best suited to these various conditions. The important factor evaluated was survival but also the ability to grow under new conditions, which was evaluated based on the number of divisions and biovolume increase.

As microalgae strain isolation started concurently with the selection of a wastewater type that could be used in this project, the inital medium used in this project was f/2 medium, which is commercially available in concentrated form. A wide range of cultures had been isolated from various environments: marine, brackish/coastal, peat water and eventually from a landfill leachate sample itself by the time a landfill leachate became available.

The initial screening highlighted that the species from the environment of the landfill tended to perform better in leachate stress studies and not so well in the conductivity tolerance experiments carried out with f/2 medium, in which their growth was relatively poor. In addition, the cultures originating from the landfill sample proved challenging to maintain within the laboratory as the f/2 medium did not appear suitable. It seemed however satisfactory for coastal species, even for the strains isolated from rock pools with low conductivity water. Similar to previous studies

where the chlorophytes dominate wastewater treatment application, in this study they were the most adaptable for growth in polluted waters as well [3, 4, 5, 6, 7].

2.4.2 Importance of selecting a suitable growth medium

Comparison of the average yearly composition of landfill leachate with some commonly used media for microalgal culturing could prove helpful in selecting more suitable media than the initially selected f/2. None completely reflects the leachate profile. However, it can be reasonably assumed that the growth medium into which the microalgae strains are initially isolated provides an early bias in relation to their subsequent selection as it is more likely to promote growth of strains that are suited to that particular composition. Landfill leachate does have a relatively high conductivity caused by high concentrations of various salts, as reflected by high levels of chloride, high concentrations of sodium, calcium and magnesium in comparison with commercially available media (i.e. f/2, BG-11, BBM, TAP, HSM). This could be modulated either by addition of salts or by dillution of landfill leachate.

Nitrogen is present in raw, processed and treated leachate in high concentration. It is present in the form of ammonia rather than nitrate in raw leachate although some nitrate may be present. Processed leachate, which is partially treated leachate, contains both ammoniacal and oxidised nitrogen, while their total cumulative concentration is similar to that of raw leachate. Treated leachate on the other hand contains mostly nitrate at lower concentration and residual ammonia.

In this study, the screening process focused on the three different landfill leachates samples because both raw leachate treatment and treated leachate clarification were considered. Thus far, most raw landfill leachate phycoremediation attempts have required it to be diluted to make any microalgae growth possible [3, 4, 19, 20, 21]. It was suggested that this is mainly due to the toxic effect of ammonia. Thus relating this to the composition of standard media composition, a 10% dilution of average yearly raw leachate composition from the site used in 2013 in this study would have a similar ammonia content (~90 mg l^{-1} NH_4^+-N) to HSM (~131 mg l^{-1} NH_4^+-N), TAP (~98 mg l^{-1} NH_4^+-N) or Sager-Granick (~79 mg l^{-1} NH_4^+-N) media. High N:P molecular ratio in leachate in comparison to the Redfield ratio is often viewed as an

indication of nutrient requirements for algal growth and points to indicate possible phosphorus limitation. Also, if leachate is diluted, possibly some nutrients that were originally present at comparable concentrations to standard media will become reduced (e.g iron, zinc or magnesium).

2.4.3 Nutrient depletion

The nutrient depletion experiment showed that reduction in pollutants is possible. This appeared to be phosphate limited as this nutrient is present at very low concentrations proportionaly to nitrogen. Also the timescale required for growth to take place was very long.

2.4.4 Future work

A number of microalgae strains selected via a screening process will be evaluated for their ability to reduce nutrients, ammonia-nitrogen, total oxidised nitrogen and phosphate. While autoclaved leachate samples were used in isolation of microalgae strains and initial screening, this process is thought to alter the physico-chemical profile of leachate and this will be investigated further. This process is also not sustainable for practical remediation applications. Lastly, the landfill leachate physico-chemical profile indicated possible phosphate limitation for microalgae growth and thus should be investigated further.

2.5 Conclusion

The screening process yielded a number of promising strains for treatment of landfill leachate. These were primarily freshwater species and were mainly isolated from landfill leachate. While these strains performed well in screening tests, it proved difficult to maintain them within the laboratory and interestingly even standard media containing ammonia-nitrogen (TAP, HSM) did not seem to suit some strains. These started to undergo changes (i.e. formation of palmelloids in SW04aTL and SW05aTL). Thus, while certain strains showed major growth in a particular leachate they also seemed more sensitive to composition variations, possibly at a

micronutrient level. On the other hand the species growing across a wider range of substrates showed more moderate growth.

2.6 Acknowledgements

This work has been supported by Institute of Technology Sligo Presidents Bursary Scheme. We also wish to thank employees of the landfill site for their assistance in providing landfill leachate which is greatly appreciated.

2.7 References

[1] Olguín, E.J., Phycoremediation : key issues for cost-effective nutrient removal processes, *Biotechnology Advances*, 22, pp. 81–91, 2003.

[2] Johannessen, L. M., Guidance Note on Leachate Management for Municipal Solid Waste Landfills, The International Bank for Reconstruction and Development/The World Bank, USA, 1999.

[3] Cheng, H.-X., Tian, G.-M., Preliminary Evaluation of a Newly Isolated Microalga *Scenedesmus* sp. CHX1 for Treating Landfill Leachate, 2*013 Third Int. Conf. Intell. Syst. Des. Eng. Appl.*, pp. 1057–1060, 2013.

[4] Lin, L., Chan, G.Y.S., Jiang, B.L., Lan, C.Y., Use of ammoniacal nitrogen tolerant microalgae in landfill leachate treatment, *Waste Management*, 27, pp. 1376–1382, 2007.

[5] Mustafa, E.M., Phang, S.M., Chu, W.L., Use of an algal consortium of five algae in the treatment of landfill leachate using the high-rate algal pond system, *Journal of Applied Phycology*, 24(4), pp. 953–963, 2012.

[6] Abou-Shanab, R.A.I., Ji, M.K., Kim, H.C., Paeng, K.J., Jeon, B.H., Microalgal species growing on piggery wastewater as a valuable candidate for nutrient removal and biodiesel production, *Journal of Environmental Management*, 115, pp. 257–264, 2013.

[7] Zhou, W., Li, Y., Min, M., Hu, B., Chen, P., Ruan, R., Local bioprospecting for high-lipid producing microalgal strains to be grown on concentrated municipal wastewater for biofuel production, *Bioresource Technology*, 102(13), pp. 6909–6919, 2011.

[8] Craggs, R.J., Mcauley, P.J., Smith, V.J., Wastewater Nutrient Removal by Marine Microalgae Grown on a Corrugated Raceway, *Water Research*, 31, pp. 1701–1707, 1997.

[9] Jiang, L., Luo, S., Fan, X., Yang, Z., Guo, R., Biomass and lipid production of marine microalgae using municipal wastewater and high concentration of CO2, *Applied Energy*, 88, pp. 3336–3341, 2011.

[10] Aravantinou, A.F., Theodorakopoulos, M., Manariotis, I.D., Selection of microalgae for wastewater treatment and potential lipids production, *Bioresource Technology*, 147, pp. 130–4, 2013.

[11] Tomas, C. R., *Identifying Marine Phytoplankton*, Academic Press, UK, ISBN-13: 978-0-12-693018-4, 1997.

[12] John, D. M., Whitton, B. A., Brook, A. J., *The Freshwater Algal Flora of the British Isles: An Identification Guide to Freshwater and Terrestrial Algae*, The Natural History Museum and the British Phycological Society, UK, ISBN 0 521 77051 3, 2002.

[13] Touzet, N., Franco, J.M., Raine, R., Characterization of nontoxic and toxin-producing strains of *Alexandrium minutum* (Dinophyceae) in Irish coastal waters, Applied Environmental Microbiology, 73, pp. 3333–3342, 2007.

[14] Castresana J., Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis, *Molecular Biology and Evolution*, 17(4), pp. 540-52, 2000.

[15] Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.F., Guindon, S., Lefort, V., Lescot, M., Claverie, J.M., Gascuel, O., Phylogeny.fr: robust phylogenetic analysis for the non-specialist, *Nucleic Acids Research*, 36(Web Server issue):W465-9. doi: 10.1093/nar/gkn180. Epub 2008 Apr 19, 2008.

[16] Edgar R.C., MUSCLE: multiple sequence alignment with high accuracy and high throughput, *Nucleic Acids Research*, 32(5), pp. 1792-1797, 2004.

[17] Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0., *Systematic Biology*, 59(3), pp. 307-21, 2010.

[18] Anisimova M., Gascuel O., Approximate likelihood ratio test for branchs: A fast, accurate and powerful alternative, *Systematic Biology*, 55(4), pp. 539-52, 2006.

[19] Sforza, E., Al, M.K., Sharif, A., Exploitation of Urban Landfill Leachate as Nutrient Source for Microalgal Biomass Production, *Chemical Engineering Transactions*, 43, 2015.

[20] Thongpinyochai, S., Ritchie, R.J., Using *Chlorella vulgaris* to Decrease the Environmental Effect of Garbage Dump Leachates, *Journal of Bioremediation & Biodegradation*, 5(5), pp. 1-12 2014.

[21] Zhao, X., Zhou, Y., Huang, S., Qiu, D., Schideman, L., Chai, X., Zhao, Y., Characterization of microalgae-bacteria consortium cultured in landfill leachate for carbon fixation and lipid production, *Bioresource Technology*, 156, pp. 322–328, 2014.

Chapter 3

Phycoremediation of landfill leachate with chlorophytes: Phosphate a limiting factor on ammonia nitrogen removal.

This chapter details results from the growth evaluation of a small selection of microalgae in two different leachate samples and their ability to reduce major nutrients (ammonia nitrogen, total oxidised nitrogen and phosphate). It was published as a peer-reviewed article in Water Research 99 (2016) 180-187 and can be accessed on http://dx.doi.org/10.1016/j.watres.2016.04.029.

Authors: Andrea Paskuliakova, Steve Tonry, Nicolas Touzet

Centre for Environmental Research Innovation and Sustainability (CERIS), Institute of Technology Sligo, Ash Lane, Co. Sligo, Ireland.

Water Research 99 (2016) 180–187

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Phycoremediation of landfill leachate with chlorophytes: Phosphate a limiting factor on ammonia nitrogen removal.

Andrea Paskuliakova*, Steven Tonry, Nicolas Touzet

Centre for Environmental Research Innovation and Sustainability (CERIS), Institute of Technology Sligo, Ash Lane, Co., Sligo, Ireland

ARTICLE INFO

Article history:
Received 19 January 2016
Received in revised form
11 April 2016
Accepted 13 April 2016
Available online 16 April 2016

Keywords:
Bioremediation
Landfill leachate
Microalgae
Ammonia nitrogen
Phosphate limitation
Batch culturing

ABSTRACT

The potential of microalgae to bioremediate wastewater has been reported in numerous studies but has not been investigated as extensively for landfill leachate, which may be attributed to its complex nature and toxicity.

In this study we explored if microalgal phycoremediation could constitute an alternative biological treatment option for landfill leachate management in regions with temperate climatic conditions. The aim of this study was to assess the performance of microalgae species at relatively low temperature (15 °C) and light intensity (14:10 h, light: dark, 22 μmol m^{-2} s^{-1}) for reduction in energy inputs. Four chlorophyte strains originating from the North-West of Ireland were selected and used in batch experiments in order to evaluate their ability to reduce total ammonia nitrogen, oxidised nitrogen and orthophosphate in landfill leachate. The *Chlamydomonas* sp. strain SW15aRL isolated from raw leachate achieved the highest level of pollutant reduction whereby a decrease of 51.7% of ammonia nitrogen was observed in 10% raw leachate (~100 mg l^{-1} NH_4^+-N) by day 24 in experiments without culture agitation. However, in the experiment conducted with 10% raw leachate supplemented with phosphate, a decrease of 90.7% of ammonia nitrogen was obtained by day 24 while also achieving higher biomass production. This series of experiments pointed to phosphorus being a limiting factor in the microalgae based phycoremediation of the landfill leachate.

The effective reduction of ammonia nitrogen in landfill leachate can be achieved at lower temperature and light conditions. This was attained by employing native species adapted to such conditions and by improving nutrient balance.

1. Introduction

The liquid phase known as leachate extracted from the bottom of a landfill is a complex, challenging and costly wastewater type to treat. Various biological and physico-chemical treatments exist. Co-treatment of landfill leachate with sewage in waste water treatment plants (WWTP) is one of the most common ways of dealing with this wastewater, although leachate addition to sewage can cause reduction in treatment efficiencies. Alternative ways for treatment are continuously sought (Ahmed and Lan, 2012; Ahn et al., 2002; Deng and Englehardt, 2006; EPA, 2011; Justin and Zupančič, 2009; Kalka, 2012; Kurniawan et al., 2006; Lavrova and Koumanova, 2010; Quant et al., 2009).

Phycoremediation is a process permitting the removal or biotransformation of inorganic and organic pollutants by algae during their growth in wastewaters (Olguín, 2003). The use of this technology is also driven by microalgal biomass generation, which may be suitable for conversion into biofuel and supportive of the development of sustainable environmental solutions (Chisti and Yan, 2011; Prajapati et al., 2013). Countries with temperate climatic conditions are lagging on research providing data for the assessment of the viability of such technology.

Landfill leachate composition is variable and dependant on a number of factors but generally is characterised by high levels of ammonia nitrogen, salts, certain metals as well as a vast array of organic compounds (Heyer and Stegmann, n.d.; Johannessen, 1999). While some of these pollutants may be used by certain species of microalgae as a source of nutrients, the toxicity of landfill leachate to many organisms, including microalgae, is well

Abbreviations and Acronyms: MBR, Membrane Bioreactor; TAN, Total Ammonia Nitrogen; TON, Total Oxidised Nitrogen; PPFD, Photosynthesis Photon Flux Density; WWTP, Waste Water Treatment Plant.

* Corresponding author.
E-mail address: andrea.pask@ymail.com (A. Paskuliakova).

http://dx.doi.org/10.1016/j.watres.2016.04.029
0043-1354/

3.0 Abstract

The potential of microalgae to bioremediate wastewater has been reported in numerous studies but has not been investigated as extensively for landfill leachate, which may be attributed to its complex nature and toxicity.

In this study we explored if microalgal phycoremediation could constitute an alternative biological treatment option for landfill leachate management in regions with temperate climatic conditions. The aim of this study was to assess the performance of microalgae species at relatively low temperature (15°C) and light intensity (14:10 hrs, light:dark, 22 µmol m^{-2} s^{-1}) for reduction in energy inputs. Four chlorophyte strains originating from the North-West of Ireland were selected and used in batch experiments in order to evaluate their ability to reduce total ammonia nitrogen, oxidised nitrogen and orthophosphate in landfill leachate. The *Chlamydomonas* sp. strain SW15aRL isolated from raw leachate achieved the highest level of pollutant reduction whereby a decrease of 51.7% of ammonia nitrogen was observed in 10% raw leachate (~100 mg l^{-1} NH_4^+-N) by day 24 in experiments without culture agitation. However, in the experiment conducted with 10% raw leachate supplemented with phosphate, a decrease of 90.7% of ammonia nitrogen was obtained by day 24 while also achieving higher biomass production. This series of experiments pointed to phosphorus being a limiting factor in the microalgae based phycoremediation of the landfill leachate.

The effective reduction of ammonia nitrogen in landfill leachate can be achieved at lower temperature and light conditions. This was attained by employing native species adapted to such conditions and by improving nutrient balance.

Key words

bioremediation, landfill leachate, microalgae, ammonia nitrogen, phosphate limitation, batch culturing

Abbreviations and Acronyms:

MBR – Membrane Bioreactor, TAN – Total Ammonia Nitrogen, TON – Total Oxidised Nitrogen, PPFD - Photosynthesis Photon Flux Density, WWTP – Waste Water Treatment Plant

Highlights

- Ammonia removal from diluted landfill leachate was achieved with chlorophytes
- The *Chlamydomonas* sp. strain SW15aRL was the most suited to treat 10% raw leachate
- Better efficiency in ammonia removal was achieved with phosphate supplementation
- Phosphate addition to leachate enhanced the biomass production of strain SW15aRL

3.1 Introduction

The liquid phase known as leachate extracted from the bottom of a landfill is a complex, challenging and costly wastewater type to treat. Various biological and physico-chemical treatments exist. Co-treatment of landfill leachate with sewage in waste water treatment plants (WWTP) is one of the most common ways of dealing with this wastewater, although leachate addition to sewage can cause reduction in treatment efficiencies. Alternative ways for treatment are continuously sought (Ahn et al., 2002; Deng and Englehardt, 2006; Kurniawan et al., 2006; Justin and Zupančič, 2009; Quant et al., 2009; Lavrova and Koumanova, 2010; EPA, 2011; Ahmed and Lan, 2012; Kalka, 2012).

Phycoremediation is a process permitting the removal or biotransformation of inorganic and organic pollutants by algae during their growth in wastewaters (Olguín 2003). The use of this technology is also driven by microalgal biomass generation, which may be suitable for conversion into biofuel and supportive of the development of sustainable environmental solutions (Chisti and Yan, 2011; Prajapati et al., 2013).

Countries with temperate climatic conditions are lagging on research providing data for the assessment of the viability of such technology.

Landfill leachate composition is variable and dependant on a number of factors but generally is characterised by high levels of ammonia nitrogen, salts, certain metals as well as a vast array of organic compounds (Heyer and Stegmann, no date; Johannessen 1999). While some of these pollutants may be used by certain species of microalgae as a source of nutrients, the toxicity of landfill leachate to many organisms, including microalgae, is well recognised (Cheung et al., 1993).

Microalgae based phycoremediation could offer an alternative treatment option for removing nutrients such as nitrogenous compounds from landfill leachate. The research in this area has not been extensive. Most of the phycoremediation applications using microalgae have focused on polluted waters such as municipal wastewater, fishpond or agricultural discharges (Wang et al., 2010; Zhou et al., 2011; McGinn et al., 2012; Seng et al., 2012; Ji et al., 2013; Prajapati et al., 2014; Choudhary et al., 2016). Landfill leachate in contrast is more challenging to treat due to the fact that it has higher concentrations of dissolved salts and extremely high levels of ammonia nitrogen ranging from 30-3000 mg l^{-1} NH_4^+-N (Heyer and Stegmann, no date). Less successful remediation results in comparison to other wastewater applications may be attributed to the toxicity of the leachate. Attempts to phycoremediate raw landfill leachate to date have required it to be diluted to reduce its inhibitory effect on microalgal growth. Free ammonia nitrogen is considered the main factor responsible for toxicity in relation to the phycoremediation of landfill leachate. However, many hazardous compounds can be found in landfill leachates often at very low concentrations and their potentiation or synergism may cause substantial toxicity (Oman and Junestedt, 2008; Matejczyk et al., 2011). Suitably tolerant microalgal strains cultured on landfill leachate have been reported to achieve substantial growth at dilutions corresponding to ammonia nitrogen concentrations ranging from approximately 100 to 200 mg l^{-1} NH_4^+-N. Lower leachate dilutions are associated with decreasing growth (Lin et al., 2007; Cheng and Tian, 2013; Thongpinyochai and Ritchie, 2014; Zhao et al., 2014; Sforza et al., 2015). However some success has been achieved when pH is controlled, which aids to counteract the formation of free ammonia (Edmundson and Wilkie, 2013).

Ammonia nitrogen can be readily used by some microalgae. Other pollutants, such as metals, have been known to bioaccumulate by microalgae (Thongpinyochai and Ritchie, 2014). Phosphate on the other hand is in relatively low concentrations in landfill leachate by comparison with nitrogenous compounds. It can be found in the range of 0.1 to 30 (tot. P) mg l^{-1} (Heyer and Stegmann, no date) and is hence expected to be a growth limiting factor that could curb the amount of ammonia nitrogen that microalgae might be capable of assimilating (Chu et al., 1996; Edmundson and Wilkie, 2013; Sforza et al., 2015).

Other issues associated with microalgal phycoremediation include the original colour of the leachate and its nutrient load. The former can affect the maximal amount of biomass that can be supported in relation to light penetration and its decrease throughout the microalgal growth cycle (Dalrymple et al., 2013). Literature on landfill leachate phycoremediation is relatively sparse and has mostly explored batch nutrient depletion, with few studies having been conducted over longer time periods or with a number of different samples (Lin et al., 2007; Mustafa and Phang, 2012; Cheng and Tian, 2013; Thongpinyochai and Ritchie, 2014; Zhao et al., 2014; Sforza et al., 2015; Sniffen et al., 2015).

Although microalgae have previously been shown to reduce nutrients from landfill leachate, the experimental conditions typically used have mostly been associated with temperatures of 25°C and light intensities not prevalent in climates such as in Northwest Europe and Ireland (Lin et al., 2007; Cheng and Tian, 2013; Thongpinyochai and Ritchie, 2014; Zhao et al., 2014). This study hence aimed to isolate and identify native microalgae more suitable for temperate climates in order to remove nutrients from landfill leachate. The amounts of inorganic nitrogenous nutrients TAN (total ammonia nitrogen) and TON (total oxidised nitrogen) removed by selected microalgae strains were quantified and the timeframe required to reach the stationary phase was determined in batch-culture experiments. The effect of phosphate supplementation on microalgal growth and TAN/TON removal was also evaluated. The nitrogen nutrient loading for these experiments was based on data from previously published studies and did not exceed 100 mg(N) l^{-1} (Lin et al., 2007; Cheng and Tian, 2013).

3.2 Materials and Methods

3.2.1 Strain isolation and maintenance

Microalgae were isolated from different environmental habitats (marine sediments, rock pools, high tide seawater, bog/peatland) in the North West of Ireland during the years 2012 and 2013 and from samples of treated and raw leachate collected in March 2013 and April 2014 from a landfill site in Northern Ireland. Non-axenic monocultures were obtained through single cell isolation into f/2 medium (Sigma Aldrich) in the case of marine species. In the case of non-marine species, the cells were isolated using f/2 medium constituents diluted with water of conductivity corresponding to that of the sample or using autoclaved landfill leachate. Cultures were maintained in the incubator with the following settings: a temperature of 15°C, a light cycle of 14:10 hours (light:dark) and a photosynthesis photon flux density (PPFD) of 22 µmol m^{-2} s^{-1}.

3.2.2 Morphogenetic analyses

Species were characterised via light microscopy observations and with the aid of identification manuals (Bellinger et al., 2010; John et al., 2002; Tomas, 1997) as well as partial PCR amplification of the large subunit (28S) ribosomal gene as detailed in Touzet et al. (2007) followed by subsequent sequencing and BLAST analysis.

3.2.3 Strain selection

The 34 strains successfully brought into culture were subjected to a screening process. This involved the evaluation of their survival and growth potential in 24- and 96-well plates in a number of landfill leachates at several dilutions and f/2 medium prepared with a range of salt strengths. The four following strains were eventually selected for further phycoremediation testing: *Chlamydomonas* sp. (strain SW13aLS, Innishbofin 2013), *Scenedesmus* sp. (strain OT08aTL, treated leachate

2013), *Scenedesmus* sp. (strain OT11aTL, treated leachate 2013) and *Chlamydomonas* sp. (strain SW15aRL, raw leachate 2014).

3.2.4 Landfill leachate sample collection

The landfill site in Northern Ireland which supplied the samples has an existing Membrane Bioreactor (MBR) treatment plant. Samples were collected at three different points of the leachate treatment process: raw leachate, process leachate (or permeate) and treated leachate. Process leachate is biologically pre-treated leachate before it is passed through the membrane filtration unit while treated leachate is the final effluent from the MBR plant. Samples were stored at <5°C until used. The leachate samples referred to in this study were collected in March 2013 and April 2014.

3.2.5 Physicochemical analyses

Phosphate (PO_4^{3-}-P), total oxidised nitrogen (TON) and total ammonia nitrogen (TAN) were determined spectrophotometrically based on published methods (American Public Health Association, 2005) adapted to the Aquakem 250 autoanalyser. Samples were filtered through 0.45 µm filter prior to the analyses.
Conductivity was determined electrochemically (HACH conductivity meter sensION5), while pH variation during the experiments was estimated with small aliquots of culture using pH indicator strips (Merck MColorpHast™ pH 5.0-10, pH 7.5-14, Δ0.5 pH, Dosatest® pH 7.0-10.0, Δ0.3 pH).

3.2.6 Selection of landfill leachate pre-treatment and experimental set up

Ammonia in raw leachate may be susceptible to decrease via volatilisation or oxidation, unlike nitrate which is more stable. Commonly used pretreatment techniques such as autoclave sterilisation or techniques often used in microalgae culturing such as aeration may contribute to ammonia losses. This can lead to some level of bias in assuming that the observed ammonia reduction is attributable to microalgae only. Four different experimental arrangements were hence set up to

explore how such treatments may affect TAN concentration in raw leachate. Autoclave sterilisation, mixing by means of aeration and magnetic stirring were compared to static flasks containing diluted raw leachate with microalgae being absent. The experiments were set up in 250 ml Erlenmeyer flasks. Each flask contained 150 ml of 10% raw leachate. The leachate was filtered through a 0.45 µm membrane filter and diluted with autoclaved deionised water. Flasks were covered with cotton plugs and aluminium foil. Each of the four experiments was conducted in triplicate. The first experimental arrangement had the filtered raw leachate autoclaved in addition to filtration and the flasks were left static throughout the duration of the experiment. In the second case, the diluted filtered leachate was left in static flasks. In the third and fourth cases, the diluted filtered leachate was mixed by means of either filtered (0.45 µm) air (30 ml min^{-1}) or a magnetic stirring plate (500 rpm), respectively. The conditions were 15°C and a light cycle 14:10 hours (light:dark). The water samples taken at the start and after 30 days were analysed for TAN and TON.

3.2.7 Comparative nutrient phycoremediation of permeate and raw landfill leachate with the selected microalgae strains

Experiments were conducted as per the second experimental arrangement in section 3.2.6 with diluted leachate in stationary flasks. Permeate and raw leachate (April 2014) were filtered through a 0.45 µm membrane filter. Tests were conducted in 250 ml Erlenmeyer flasks with 150 ml of substrate/microalgae mixture. The final concentration of leachate diluted with autoclaved deionised water was 10% of the final volume. Flasks were inoculated at approximately the same initial biovolume of 0.15 mm^3 ml^{-1} for the four selected strains. All experiments were conducted in triplicate. A control with no microalgae was set up to monitor for any loss of nutrients due to reasons other than microalgal assimilation. A complementary set of flasks supplemented with phosphate was also set up for each culture to evaluate the effect of phosphorus deficiency on the phycoremediation process. Phosphate (1000 mg l^{-1} PO_4^{3-}-P prepared from K_2HPO_4) was added to achieve a molecular ratio of 16:1 N:P in the final volume. Aliquots of 2 ml were sampled every 4 to 7 days and analysed for cell number, TAN, TON and orthophosphate (pH was also noted). The

incubation conditions were as follows: a temperature of 15°C, a light cycle of 14:10 hours (light:dark) and a PPFD of 22 µmol m^{-2} s^{-1}. The strains were maintained in diluted raw leachate and/or permeate prior to the nutrient depletion experiments. The growth progress of individual strains throughout the experiments was monitored by cell counts made with a haemocytometer.

3.2.8 Statistical Analyses

Results were statistically analysed with IBM SPSS Statistics 22 package. All data were checked for normality using the Shapiro-Wilk test. The significance of nutrient decreases was compared via one-way ANOVA (with post hoc analyses; Tukey's HSD; or Games-Howell when homogeneity of variances assumption could not be satisfied). The difference between phosphate non supplemented and supplemented maximal biomass increase, growth rates and ammonia nitrogen removal rates were analysed with independent sample t-tests.

3.3 Results

3.3.1 Strain selection

The four microalgae strains selected for the nutrient depletion experiments were chosen from 34 different isolates obtained from various environments via a screening process mostly based on their potential to grow in landfill leachate (data not shown).

3.3.2 Selection of landfill leachate pre-treatment and experimental set up

Due to the susceptibility of ammonia to volatilisation and oxidation, a short evaluation of leachate pretreatment and experimental set up was conducted prior to the main nutrient depletion experiments. Ammonia is the prime nutrient monitored in raw leachate remediation and occurs at high concentrations at which it may be easily lost to the atmosphere. In all the four treatments tested, the TAN concentration changed significantly (one-way ANOVA, $p<0.05$, $n=24$) between days 0 and 30

(Figure 3.1). Autoclaving caused a TAN decrease of ~25%. There were no significant differences between the TAN decreases in static flasks and the flasks mixed by magnetic stirring (average of 26.5% and 28.4%, respectively) on day 30 and aeration showed the highest impact on TAN decrease (50.5%) by day 30 (one-way ANOVA, $p<0.05$, $n=24$). In none of the experimental arrangements were found statistically significant differences in TON concentration by day 30 (one-way ANOVA, $p>0.05$, $n=24$).

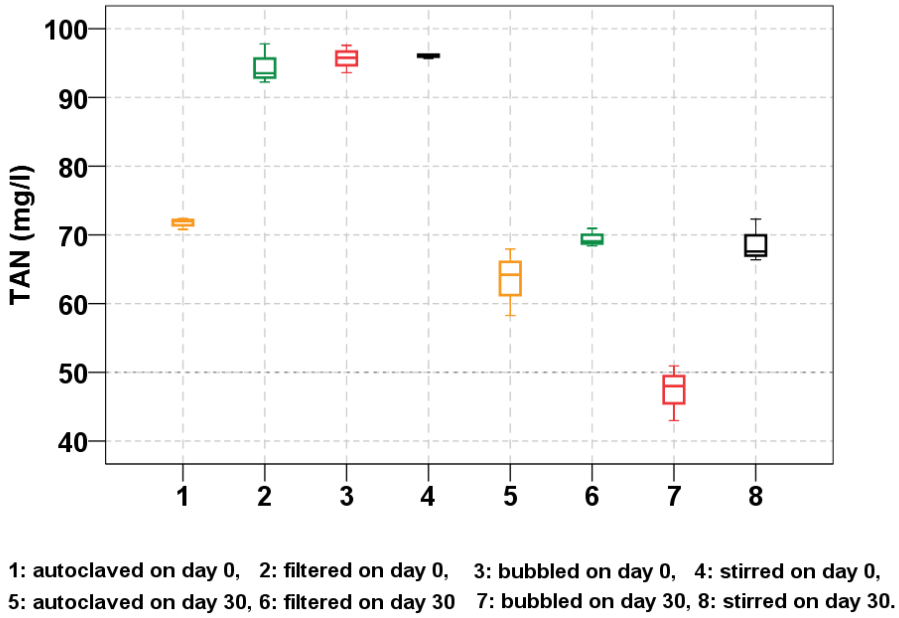


Figure 3.1. Box plot displaying the differences in ammonia nitrogen levels on day 0 and day 30 in diluted raw leachate in the absence of microalgae with four experimental arrangements used in microalgae culturing. Values are an average of three replicates and error bars represent ± one standard deviation.

3.3.3 Comparative growth of the selected strains in landfill leachate samples

Figure 3.2 shows the growth of the four selected microalgae strains in 10% permeate and 10% raw leachate. For comparability, cell concentration was converted into biovolume as there were major cell dimension differences between the strains, ranging from ~25 μm^3 for *Scenedesmus* sp. OT11aTL up to ~1200 μm^3 for

Chlamydomonas sp. SW15aRL. The physicochemical properties of the leachate samples used are summarised in Table 3.1. The main difference between permeate and raw leachate is that permeate is biologically treated raw leachate; ammonia is thus converted to nitrate in permeate.

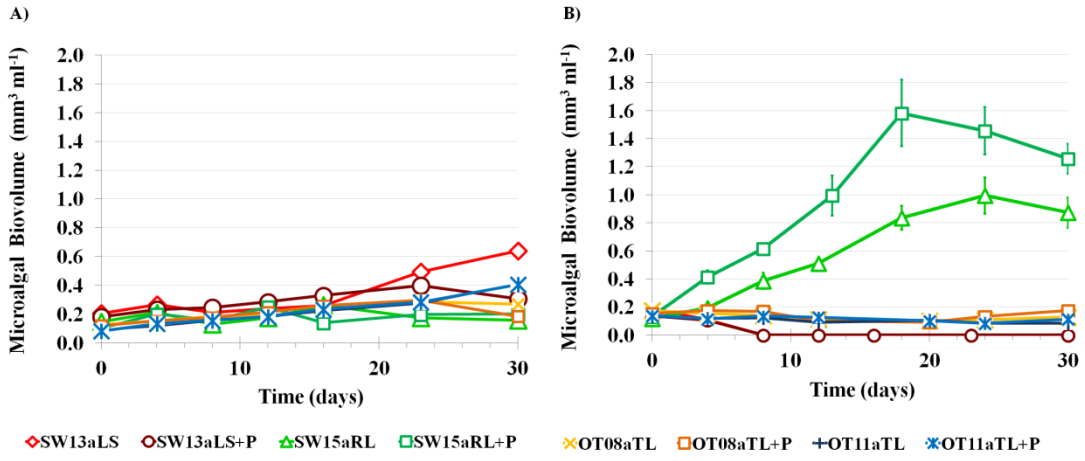


Figure 3.2. Comparison of the growth of the microalgae strains used to bioremediate 10% permeate (A) and 10% raw leachate (B) in flasks supplemented or not with phosphate. Values are an average of three replicates, error bars (± one standard deviation) are not shown for clarity except for strain SW15aRL.

Table 3.1. Physicochemical properties of the leachate samples used in the experiments.

Parameter	Unit	10% Permeate (2014)	10% Raw leachate (2014)
pH		7	8.5
Conductivity	µS cm^{-1}	807	1123
TAN	mg l^{-1}	<0.05	88
TON	mg l^{-1}	85	0.1
PO_4^{3-}-P	mg l^{-1}	0.5	1
Colour	Abs (at 455 nm)	0.065	0.091

In the experiment with 10% permeate, only very slow and minor growth was observed for all the strains. Strain SW15aRL did not grow at all. The highest

biovolume increases at the end of the experiment were observed for strains *Chlamydomonas* sp. SW13aLS and *Scenedesmus* sp. OT11aTL in the range of 3.1 to 4.9 fold biovolume increase.

In the experiment with 10% raw leachate, the culture with *Chlamydomonas* sp. SW13aLS collapsed by day 8. While strains *Scenedesmus* sp. OT08aTL and OT11aTL did not grow, they appeared to be surviving. Major growth was observed with strain *Chlamydomonas* sp. SW15aRL. In the case of the subset supplemented with phosphate a further significant biomass gain was achieved (independent t-test, $p<0.05$) in comparison to the non-supplemented set. The biomass increases for strain SW15aRL were 8.4 and 12.1 fold for phosphate non supplemented and supplemented experiments, respectively. The specific growth rate determined for strain SW15aRL for the PO_4^{3-}-P supplemented series was significantly greater than of the PO_4^{3-}-P non-supplemented series (0.11 ± 0.01 day^{-1} and 0.14 ± 0.02 day^{-1}, respectively, independent t-test, $p<0.05$). Increases in pH throughout the experiments were observed up to values of 10 and did not appear to affect the growth of this particular strain.

3.3.4 Comparative nutrient phycoremediation with the selected microalgae strains in permeate and raw leachate

3.3.4.1 Nutrient removal in 10% permeate

TON decrease was observed in all cases (Figure 3.3). After 30 days the most pronounced reduction (average 41.4%, 41.8% in phosphate supplemented) was observed for the *Chlamydomonas* sp. SW13aLS culture, for which the decrease was significantly higher than those observed in the control treatments (one-way ANOVA, $p<0.05$, $n= 30$).

Phosphate concentration, which was already very low in 10% permeate, was depleted for the SW13aLS, OT08aTL and OT11aTL sets not supplemented with phosphate while there was only a slight decrease in phosphate in the case of strain SW15aRL, which did not display any growth throughout the experiment (Appendix B: Table S1). In the phosphate supplemented experiments, phosphate concentration

was reduced by day 30 by 78%, 90% and 97% in the sets with strains SW13aLS, OT08aTL and OT11aTL, respectively.

While the initial concentration of ammonia in 10% permeate was below 0.05 mg l^{-1} NH_4^+-N, the higher initial concentrations observed when the microalgal inocula were introduced were caused by residual ammonia carried over from the stock cultures, which were initially maintained in leachate as growth medium.

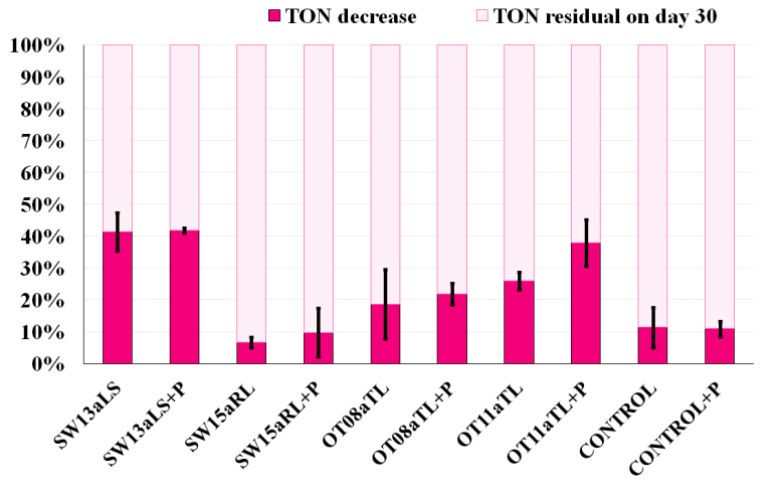


Figure 3.3. Reduction of total oxidised nitrogen in 10% permeate expressed as an average (n=3) percentage decrease in comparison to concentration at day 0 in the individual experimental treatments.

3.3.4.2 Nutrient removal in 10% raw leachate

The main pollutant monitored in 10% raw leachate was ammonia nitrogen and its decreases for the different treatments tested are displayed in Figure 3.4. TAN levels in the control treatments conducted concurrently with the nutrient removal experiments had on average decreased by day 30 by 22.4% and 16.6% in the phosphate non supplemented and phosphate supplemented treatments, respectively. The lowest TAN decrease (0 to 11.4%) was observed in the flasks with the collapsing culture of *Chlamydomonas* sp. SW13aLS. Although no growth was observed for strains OT08aTL and OT11aTL, there was a significant decrease in TAN in comparison to the controls (one-way ANOVA, $p<0.05$, n=27). In addition,

even though the TAN decrease for the two *Scenedesmus* spp. strains was comparable, there was a significantly higher decrease in the flasks supplemented with phosphate (one-way ANOVA, $p<0.05$, $n=27$) that was similar to that achieved by *Chlamydomonas* sp. SW15aRL in the phosphate non supplemented treatment (one-way ANOVA, $p>0.05$, $n=27$). Concomitant to the highest biomass increase, the significantly highest nutrient removal was observed for *Chlamydomonas* sp. SW15aRL (one-way ANOVA, $p<0.05$, $n=27$), the treatment with phosphate supplementation, in particular, showing a ~90% decrease in TAN after 30 days. This decrease was significantly different to any other strain (one-way ANOVA, $p<0.05$, $n=27$). There was no significant difference between the amount of TAN depleted on day 24 and 30 in either phosphate non supplemented and supplemented batches (one-way ANOVA, $p<0.05$, $n=21$) for strain SW15aRL (Figure 3.5). TAN removal rates were calculated for strain SW15aRL between days 0 and 24 in 10% raw leachate; that estimated for the phosphate supplemented treatment was significantly higher than that determined for the non supplemented set (3.67±0.12 and 2.08±0.01 mg l^{-1}.day^{-1}, respectively; independent t-test, $p<0.05$).

Phosphate concentration was not exhausted in the phosphate supplemented subset and reached a level comparable to that originally present in 10% raw leachate (Figure 3.5, Appendix B: Table S2).

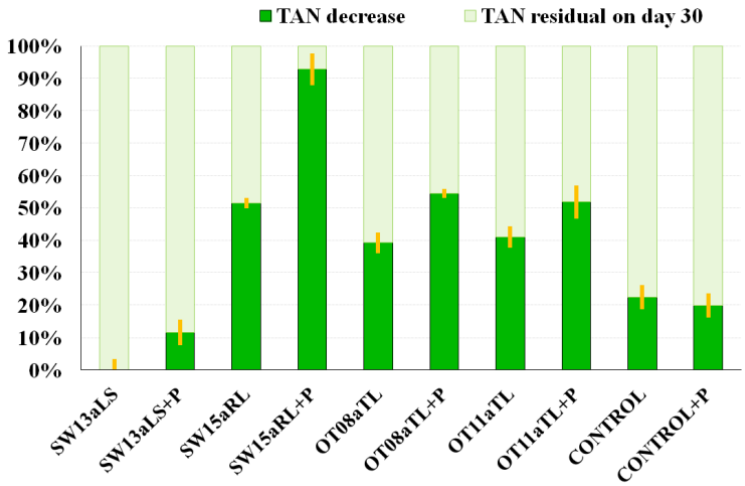


Figure 3.4. Removal of total ammonia nitrogen in 10% raw leachate expressed as an average (n=3) percentage decrease in comparison to concentration at day 0 in the individual experimental treatments.

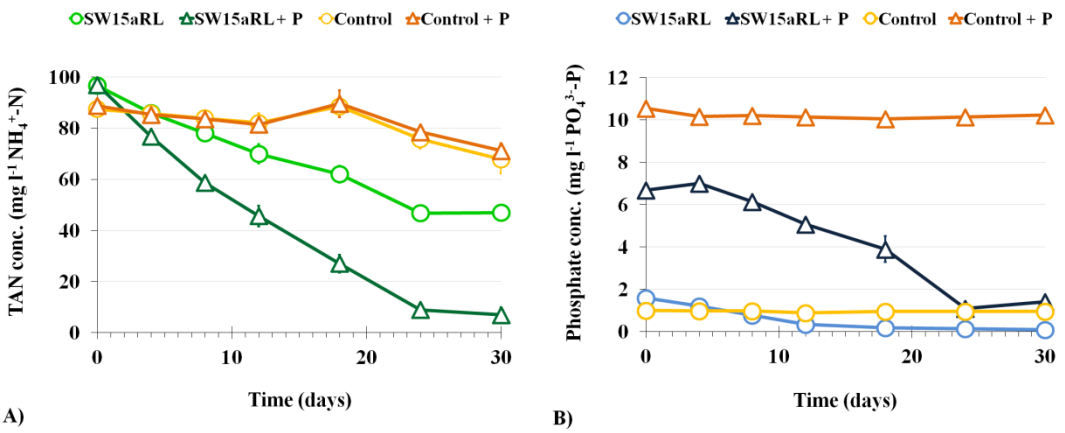


Figure 3.5. Changes in (A) total ammonia nitrogen (B) and phosphate concentration during *Chlamydomonas* sp. strain SW15aRL growth in 10% raw leachate supplemented or not with phosphate. All measurements are replicates of three with ± standard deviation which is in some cases small and does not appear within the chart.

3.4 Discussion

Microalgae with potential to remove pollutants from landfill leachate were used in this study. Four strains inoculated into two different leachate samples showed different growth profiles as a result of their ability to use the nutrients present or the adverse effect the leachate had on their growth. The *Chlamydomonas* sp. strain SW15aRL showed the ability to assimilate most of the total ammonia nitrogen from diluted raw leachate while also producing biomass.

3.4.1 Selection of landfill leachate pre-treatment and experimental set up

Autoclaving or filtration is often carried out for microalgal culturing with respect to catering for the microbiological integrity of experiments. The effects of these sterilisation processes on chemical composition and nutrient availability are not well established with regards to wastewaters, which can be of a very complex nature. Culture agitation is also often employed, either by shaking, air bubbling or stirring. Control experiments prepared without microalgae (Lin et al., 2007) can provide information on the amount of nutrients that are assimilated by biomass for growth or

are otherwise removed via non biological mechanisms. This is an important consideration in assessing the effectiveness of remediation experiments which could also benefit studies focusing on the maximisation of microalgal biomass production on wastewater substrates (Mustafa and Phang, 2012; Edmundson and Wilkie, 2013; Kring et al., 2014). Zhao et al. (2014) also highlighted substantial nutrient losses occurring as a result of experimental arrangement. In their study, instead of a control without microalgae, an elemental analysis of the biomass was conducted and indicated that by day 12 of the aerated phycoremediation of 10% raw leachate, 52% of ammonia nitrogen was assimilated by biomass while the rest was volatilised.

In the present study, the set up chosen for the evaluation of nutrient depletion by microalgae was based on a preliminary assessment of different raw leachate pre-treatments and experimental arrangements. Raw leachate contains high levels of ammonia nitrogen which can be susceptible to volatilisation depending on the experimental conditions (Zhao et al., 2014). Ammonia can act as a nutrient source or have toxic effect on microalgae (Abeliovich and Azov, 1976; Azov and Goldman, 1982; Källqvist and Svenson, 2003). Autoclave sterilisation and air bubbling had major effects on TAN loss within the raw landfill leachate used in this study. Also, a large amount of precipitated matter of unknown composition was present after autoclave sterilisation. This would probably have altered the profile of dissolved micronutrients such as minerals and hence their bioavailability. For this reason this pre-treatment was disregarded. For the subsequent remediation experiments, in order to minimise potential changes in its physicochemical profile, the leachate was only filtered and the culture flasks were left static throughout the experiment and homogenised only for the purpose of sampling.

3.4.2 Comparative growth of the selected strains in landfill leachate samples

The four microalgal strains selected based on the preliminary evaluation of 34 strains (data not shown) belong to the genera *Chlamydomonas* and *Scenedesmus,* similar to other studies where chlorophytes dominate the species grown in landfill leachate (Lin et al., 2007; Mustafa and Phang, 2012; Cheng and Tian, 2013; Edmundson and Wilkie, 2013; Kring et al., 2014; Thongpinyochai and Ritchie, 2014; Zhao et al., 2014; Sforza et al., 2015).

The growth profiles of the strains placed within the two types of landfill leachates were quite different; especially in the case of strain *Chlamydomonas* sp. SW15aRL isolated from raw leachate that could actively grow in raw leachate, which itself seemed highly toxic to the strain *Chlamydomonas* sp. SW13aLS isolated from a bog sample. The moderate growth of the four strains observed in permeate could be attributed to the particular chemical composition of the sample used on this occasion. The selected strains did indeed manifest higher growth potential during the screening process with permeate samples obtained on different dates (data not shown). The constantly varying composition of leachate is one of the major issues in phycoremediation applications and the strains used for such attempts should ideally be able to sustain substantial changes.

The two strains of *Scenedesmus* sp. (OT08aTL and OT11aTL) appeared to be somewhat tolerant to 10% raw leachate although no growth was observed. It is possible that the increase in pH within the flasks had a negative impact on their growth, as previously observed by Edmunson and Wilkie (2013), who achieved higher growth of *Scenedesmus* sp. in landfill leachate when pH was controlled.

The growth rate of strain *Chlamydomonas* sp. SW15aRL was relatively slow in comparison with other studies (Edmundson and Wilkie, 2013; Zhao et al., 2014; Sforza et al., 2015), the low temperature and light settings used in the experiment being the likely contributing factors. Nutrient modulation however proved positive to growth promotion as a significantly higher growth rate was achieved when phosphate was added.

3.4.3 Comparative nutrient phycoremediation with the selected microalgae strains in permeate and raw leachate

Partially treated leachate (permeate) and raw leachate differ in the forms of nitrogen they contain, typically ammonia nitrogen in raw leachate and nitrate mainly in permeate. However the nature of many other compounds differs between the two substrates, which may influence the overall growth of microalgae and their ability to remove specific dissolved pollutants.

3.4.3.1 Nutrient removal in 10% permeate

Although there were statistically significant levels of nitrate removed by the microalgae strains, substantial amounts of TON were still left over after 30 days for all the treatments. There did not appear to be any difference in the amount of nitrate used in the sets either supplemented or non-supplemented with phosphate. In the sets supplemented with phosphate, the cells absorbed more phosphate than originally present in the permeate. Residual amounts were still present after day 30 however. The cells appeared unable to fully avail of these two macronutrients. Permeate is understood to be less toxic than raw leachate as it is already biologically treated and most of the ammonia is oxidised to nitrate. It is likely that the growth of the microalgae in 10% permeate was limited by a micronutrient that was removed during previous treatment stages of the leachate or it is possible that nitrate may be a form of nitrogen that these strains find difficult to utilise.

3.4.3.2 Nutrient removal in 10% raw leachate

The lowest TAN decrease was observed in the flasks with *Chlamydomonas* sp. SW13aLS, which might have been due to the release of ammonia nitrogen from the decomposing biomass of dead cells. Significant TAN decreases were observed with strains *Scenedesmus* spp. (OT08aTL, OT11aTL) and especially *Chlamydomonas* sp. SW15aRL with both phosphate supplemented and non supplemented sets. While the TAN decrease in phosphate supplemented experiments with the *Scenedesmus* spp. strains was comparable to that of the phosphate non supplemented experiment carried out with strain SW15aRL, it is not known how the ammonia was removed in the former. Presence of *Scenedesmus* spp. cells was able to cause TAN reduction, yet no gain of cell number was observed in these cultures. This is similar to the data reported by Lin et al. (2007) in which the growth of *Chlorella pyrenoidosa* in 30% and 50% raw leachate was very moderate but was still accompanied by a major reduction in nutrients. It would be pertinent to consider that this was caused by the use of nutrients for cell maintenance processes even though they were not dividing in these cases.

Chlamydomonas sp. SW15aRL was the only strain that was able to actively grow in 10% raw leachate over the test period. The experiment appeared to be phosphorus limited as higher cell density and growth rate were achieved in phosphate supplemented flasks. In the experiment which was not phosphate supplemented, ammonia nitrogen decreased by ~50% by day 24 and did not change significantly thereafter, suggesting that no more could be removed unless more phosphate was added. With the addition of phosphate, ~91% of ammonia nitrogen was depleted by day 24. However, some residual ammonia and phosphate remained after day 30, which may suggest limitation of the cells by the exhaustion of another micronutrient. In the present study, strain SW15aRL did not appear to be negatively affected by the pH increase observed in the flasks as it was capable of growth at pH up to 10. This could prove a promising trait for future work with this strain given that pH change has been reported to affect the growth of some microalgal species in landfill leachate (Edmundson and Wilkie, 2013).

Whilst it has been demonstrated that microalgae can remove nutrients from landfill leachate at higher temperatures and light intensities, these conditions are not prevalent in the temperate climate of northwest Europe. The light, temperature or mixing conditions used in other studies could be viewed to be more growth promoting than in the set up of the present experiments, although it could be argued that higher temperature would cause ammonium ions to form free ammonia, which is notoriously more toxic. Compared to previous phycoremediation work with landfill leachates, the composition in major macronutrients of the 10% raw leachate used in the present study was similar to that treated with *Scenedesmus* sp. by Cheng and Tian (2013), (90.5 mg l^{-1} NH_4^+-N, TP 0.69 mg l^{-1}) in which 72% of ammonia nitrogen was removed within 20 days (static flasks, 25±2°C, whole day illumination, light intensity 100 µmol m^{-2} s^{-1}). In spite of the lower light and temperature used in the present study, comparable results were obtained with strain SW15aRL in the 10% raw leachate supplemented with phosphate (TAN decrease of ~79% by day 20). Similarly, Lin et al. (2007) conducted experiments on leachate (100% raw leachate composition: pH 7.6, ammonium 1345 mg l^{-1}, nitrate-N 68.4 mg l^{-1}, ortho-P 5.13 mg·l^{-1}) with continuously shaken cultures and temperatures and illuminations of 25-30°C and 2000-3000 lux, respectively. In their experiments carried out with 10% and 30% raw leachate using *Chlorella pyrenoidosa* and *Chlamydomonas snowiae* strains,

decreases of up to 80% TAN were observed in 12 days, a time by which microalgal concentration started to decline. Other studies carried out with landfill leachate (Zhao et al., 2014; Sforza et al., 2015) or other wastewaters with high ammonia nitrogen concentration (Prajapati et al., 2014; Choudhary et al., 2016) have achieved removals above 90% in shorter time. While aeration and higher temperature seem important factors, the likely variations in the individual wastewater chemical compositions also need to be considered.

3.4.4 Phycoremediation considerations

The time required to treat a substantially diluted leachate sample with microalgae is extensive and still probably unsuitable under the current set up for scalability applications. On the other hand, ammonia removal was achieved under relatively low light and temperature in comparison to previous studies and also without mechanical agitation as strain SW15aRL is motile. These factors offer prospects of lower energy requirements. In addition, although the growth rate of strain SW15aRL was relatively low, its substantial cell size can make it easier to separate the biomass from the liquid volume than other smaller microalgal species. Phycoremediation is perceived as an environmentally friendly treatment option but there is an indication that some chemical additions might be needed to adjust its composition either to control pH (Edmundson and Wilkie, 2013) or increase phosphate concentration to make this process effective. To this end, mixing landfill leachate with another waste stream (Zhao et al., 2014) together with the supplementation of CO_2 from flue gas might help with the sustainability of upscaled phycoremediation attempts.

In future work, the performance of strain *Chlamydomonas* sp. SW15aRL will be investigated on various landfill leachate substrates. Nutrient deficiencies are a known factor in biological treatments such as sewage treatment and other sludge based technologies and compensations are used to optimise the processes (Arundel, 1995; Calli et al., 2006; Spellman, 2009; Ahmed and Lan, 2012). Phosphate supplementation might be necessary for the sustainable growth of microalgae in landfill leachates and should be investigated further together with other micronutrients. Optimal concentrations should be established to maximise

microalgal growth and pollutant removal. Landfill leachate constitutes an attractive substrate as a cheap and accessible medium for microalgal biomass production in the context of biofuel production (Edmundson and Wilkie, 2013; Kring, 2014). Many microalgae species can accumulate lipids and tend to do so as a response to stress such as nutrient depletion (Deng et al., 2011; Kropat et al., 2011; Ho et al., 2012). The lipid composition of microalgae grown on landfill leachate, such as *Chlamydomonas* sp. strain SW15aRL, is yet to be evaluated but recent studies have reported values of 14.5-20.8% (Zhao et al., 2014) and 38-48% (Sforza et al., 2015) lipid content in other chlorophytes grown in leachate.

3.5 Conclusions

- Significant amount of ammonia nitrogen was removed from landfill leachate by the microalgal strain *Chlamydomonas* sp. SW15aRL under low regimes of temperature and light.
- The time required to treat a substantially diluted leachate sample is extensive and still probably unsuitable for scalability applications under the set up used.
- Understanding leachate composition in regard to microalgal nutritional demands and the physico-chemical changes occurring during phycoremediation treatment are crucial to the optimisation of the process.

3.6 Acknowledgements

This work has been supported by Institute of Technology Sligo Presidents Bursary Scheme. We also wish to thank employees of the landfill site for their assistance in providing landfill leachate which is greatly appreciated.

3.7 References

Abeliovich, A., Azov, Y., 1976. Toxicity of Ammonia to Algae in Sewage Oxidation Ponds. Applied and Environmental Microbiology 31 (6), 801–806.

Ahmed, F.N., Lan, C.Q., 2012. Treatment of landfill leachate using membrane bioreactors: A review. Desalination 287, 41–54. doi:10.1016/j.desal.2011.12.012.

Ahn, W.-Y., Kang, M.-S., Yim, S.-K., Choi, K.-H., 2002. Advanced landfill leachate treatment using an integrated membrane process. Desalination 149, 109–114. doi:10.1016/S0011-9164(02)00740-3.

APHA (American Public Health Association), 2005. Standard methods for the examination of water and wastewater, 21st ed. Washington, D.C.

Arundel J. ,1995. Sewage and Industrial Effluent Treatment, Blackwell Science, ISBN 0–632–03898-5.

Azov, Y., Goldman, J.C., 1982. Free ammonia inhibition of algal photosynthesis in intensive cultures. Appl. Environ. Microbiol. 43, 735–739.

Bellinger, E. G., Sigee, D. C., Blackwell, W., 2010. Freshwater Algae: Identification and Use as Bioindicators. Wiley Blackwell, UK, ISBN 978-0-470-05814-5.

Calli, B., Mertoglu, B., Roest, K., Inanc, B., 2006. Comparison of long-term performances and final microbial compositions of anaerobic reactors treating landfill leachate. Bioresour. Technol. 97, 641–7. doi:10.1016/j.biortech.2005.03.021.

Cheng, H.-X., Tian, G.-M., 2013. Preliminary Evaluation of a Newly Isolated Microalga *Scenedesmus* sp. CHX1 for Treating Landfill Leachate. 2013 Third Int. Conf. Intell. Syst. Des. Eng. Appl. 1057–1060. doi:10.1109/ISDEA.2012.249.

Cheung, K.C., Chu, L.M., Wong, M.H., 1993. Toxic effect of landfill leachate on microalgae. Water, Air and Soil Pollution 69 (3), 337-349.

Chisti, Y., Yan, J., 2011. Energy from algae: Current status and future trends. Algal biofuels - A status report. Appl. Energy 88, 3277–3279. doi:10.1016/j.apenergy.2011.04.038.

Choudhary, P., Prajapati, S. K., Malik, A., 2016. Screening native microalgal consortia for biomass production and nutrient removal from rural wastewaters for bioenergy applications. Ecol. Eng. 91, 221–230. doi:10.1016/j.ecoleng.2015.11.056.

Chu, L. M., Cheung, K. C., Wong, M. H., 1996. Algal purification of pretreated landfill Leachate. Toxicological and Environmental Chemistry 53, 1-4. http://dx.doi.org/10.1080/02772249609358281.

Dalrymple, O.K., Halfhide, T., Udom, I., Gilles, B., Wolan, J., Zhang, Q., Ergas, S., 2013. Wastewater use in algae production for generation of renewable resources: a review and preliminary results. Aquat. Biosyst. 9, 2. doi:10.1186/2046-9063-9-2.

Deng, X., Fei, X., Li, Y., 2011. The effects of nutritional restriction on neutral lipid accumulation in *Chlamydomonas* and *Chlorella*. African Journal of Microbiology Research 5 (3), 260–270. doi:10.5897/AJMR10.557.

Deng, Y., Englehardt, J.D., 2006. Treatment of landfill leachate by the Fenton process. Water Res. 40, 3683–94. doi:10.1016/j.watres.2006.08.009.

Edmundson, S.J., Wilkie, A.C., 2013. Landfill leachate - a water and nutrient resource for algae-based biofuels. Environ. Technol. 34, 1849–57. doi:10.1080/09593330.2013.826256.

Environmental Protection Agency, 2011. Final Draft BAT Guidance Note on Best Available Techniques for the Waste Sector: Landfill Activities' [accessed: 26/11/2015, online] https://www.epa.ie/pubs/advice/bat/BAT%20Landfill%20-%20Final%20Draft%20-%20December%202011.pdf.

Heyer, K., Stegmann, R., no date. Leachate management : leachate generation, collection, treatment and costs 1–23.

Ho, S., Chen, C., Chang, J., 2012. Bioresource Technology Effect of light intensity and nitrogen starvation on CO_2 fixation and lipid / carbohydrate production of an indigenous microalga *Scenedesmus obliquus* CNW-N. Bioresour. Technol. 113, 244–252. doi:10.1016/j.biortech.2011.11.133.

Ji, M., Abou-shanab, R.A.I., Kim, S., Salama, E., Lee, S., Kabra, A.N., Lee, Y., Hong, S., Jeon, B., 2013. Cultivation of microalgae species in tertiary municipal wastewater supplemented with CO_2 for nutrient removal and biomass production. Ecol. Eng. 58, 142–148. doi:10.1016/j.ecoleng.2013.06.020.

Johannessen, L. M., 1999. Guidance Note on Leachate Management for Municipal Solid Waste Landfills. The International Bank for Reconstruction and Development/ The World Bank, USA.

John, D. M., Whitton, B. A., Brook, A. J., 2002. The Freshwater Algal Flora of the British Isles: An Identification Guide to Freshwater and Terrestrial Algae. The Natural History Museum and the British Phycological Society, UK, ISBN 0 521 77051 3.

Justin, M.Z., Zupančič, M., 2009. Combined purification and reuse of landfill leachate by constructed wetland and irrigation of grass and willows. Desalination 246, 157–168. doi:10.1016/j.desal.2008.03.049.

Kalka, J., 2012. Landfill Leachate Toxicity Removal in Combined Treatment with Municipal Wastewater. The Scientific World Journal, 2012. doi:10.1100/2012/202897.

Källqvist, T., Svenson, A., 2003. Assessment of ammonia toxicity in tests with the microalga, *Nephroselmis pyriformis*, Chlorophyta. Water Res. 37, 477–484. doi:10.1016/S0043-1354(02)00361-5.

Kring, S.A., 2014. Alternative Methods of Microalgae Cultivation and Dewatering to Increase the Sustainability of Biodiesel Production. Ph.D thesis, Clarkson University, ProQuest LLC.

Kropat, J., Hong-Hermesdorf, A., Casero, D., Ent, P., Castruita, M., Pellegrini, M., Merchant, S.S., Malasarn, D., 2011. A revised mineral nutrient supplement increases biomass and growth rate in *Chlamydomonas reinhardtii*. Plant Journal 66 (5), 770–780. doi:10.1111/j.1365-313X.2011.04537.x.

Kurniawan, T.A., Lo, W.-H., Chan, G.Y.S., 2006. Physico-chemical treatments for removal of recalcitrant contaminants from landfill leachate. J. Hazard. Mater. 129, 80–100. doi:10.1016/j.jhazmat.2005.08.010.

Lavrova, S., Koumanova, B., 2010. Influence of recirculation in a lab-scale vertical flow constructed wetland on the treatment efficiency of landfill leachate. Bioresour. Technol. 101, 1756–61. doi:10.1016/j.biortech.2009.10.028.

Lin, L., Chan, G.Y.S., Jiang, B.L., Lan, C.Y., 2007. Use of ammoniacal nitrogen tolerant microalgae in landfill leachate treatment. Waste Management 27, 1376–1382. doi:10.1016/j.wasman.2006.09.001.

Matejczyk, M., Płaza, G.A., Nałecz-Jawecki, G., Ulfig, K., Markowska-Szczupak, A., 2011. Chemosphere Estimation of the environmental risk posed by landfills using chemical , microbiological and ecotoxicological testing of leachates. Chemosphere 82, 1017–1023. doi:10.1016/j.chemosphere.2010.10.066.

McGinn, P.J., Dickinson, K.E., Park, K.C., Whitney, C.G., Macquarrie, S.P., Black, F.J., Frigon, J., Guiot, S.R., Leary, S.J.B.O., 2012. Assessment of the bioenergy and bioremediation potentials of the microalga *Scenedesmus* sp. AMDD cultivated in municipal wastewater effluent in batch and continuous mode. Algal Research 1 (2), 155–165. doi:10.1016/j.algal.2012.05.001.

Mustafa, E., Phang, S., 2012. Use of an algal consortium of five algae in the treatment of landfill leachate using the high-rate algal pond system. Journal of Applied Phycology, 24, 953–963. doi:10.1007/s10811-011-9716-x.

Olguín, E.J., 2003. Phycoremediation: key issues for cost-effective nutrient removal processes. Biotechnol. Adv. 22, 81–91. doi:10.1016/j.biotechadv.2003.08.009.

Oman, C.B., Junestedt, C., 2008. Chemical characterization of landfill leachates – 400 parameters and compounds. Waste Management 28, 1876–1891. doi:10.1016/j.wasman.2007.06.018.

Prajapati, S.K., Kaushik, P., Malik, A., Vijay, V.K., 2013. Phycoremediation and biogas potential of native algal isolates from soil and wastewater. Bioresour. Technol. 135, 232–238. doi:10.1016/j.biortech.2012.08.069.

Prajapati, S.K., Choudhary, P., Malik, A., Vijay, V.K., 2014. Algae mediated treatment and bioenergy generation process for handling liquid and solid waste from dairy cattle farm. Bioresour. Technol. 167, 260–268. doi:10.1016/j.biortech.2014.06.038.

Quant, B., Fudala-Ksiazek, S., Luczkiewicz, A., Kulbat, E., Jankowska, K., Czerwionka, K., Olanczuk-Neyman K., 2009. Influence of landfill leachate on municipal wastewater treatment -model plant (WWTP- MP). Conference: IWA 2nd Specialized Conference Nutrient Management in Wastewater Treatment Processes Kraków.

Sforza, E., Al, M.K., Sharif, A., 2015. Exploitation of Urban Landfill Leachate as Nutrient Source for Microalgal Biomass Production. Chemical Engineering Transaction, 43, ISBN 978-88-95608-34-1, ISSN 2283-9216.

Seng, L.K., Wah, N.B., Chieh, D.C.J., 2012. Biotreatment of Fish Farm Wastewater with Microalgae. Proceedings of International Conference on Engineering and Information Technology “ICEIT2012”, ISBN 978-1-77136-064-7.

Sniffen, K.D., Sales, C.M., Olson, M.S., 2015. Nitrogen removal from raw landfill leachate by an algae-bacterial consortium. Water Science and Technology, doi: 10.2166/wst.2015.499.

Spellman, F. R., 2009. Water and Wastewater Treatment Plant Operations. CRC Press Taylor & Francis Group, Second Edition, ISBN 978–1–4200–7530–4.

Thongpinyochai, S., Ritchie, R.J., 2014. Biodegradation Using *Chlorella vulgaris* to Decrease the Environmental Effect of Garbage Dump Leachates. Journal of Bioremediation and Biodegradation, 5:5. doi:10.4172/2155-6199.1000239.

Tomas, C. R., 1997. Identifying Marine Phytoplankton', Academic Press, UK, ISBN-13: 978-0-12-693018-4.

Touzet, N., Franco, J.M., Raine, R., 2007. Characterization of nontoxic and toxin-producing strains of *Alexandrium minutum* (Dinophyceae) in Irish coastal waters. Appl. Environ. Microbiol. 73, 3333–42. doi:10.1128/AEM.02161-06.

Wang, L., Min, M., Li, Y., Chen, P., Chen, Y., Liu, Y., Wang, Y., Ruan, R., 2010. Cultivation of Green Algae *Chlorella* sp. in Different Wastewaters from Municipal Wastewater Treatment Plant. Appl. Biochem. Biotechnol. 162 (4), 1174–1186. doi:10.1007/s12010-009-8866-7.

Zhao, X., Zhou, Y., Huang, S., Qiu, D., Schideman, L., Chai, X., Zhao, Y., 2014. Characterization of microalgae-bacteria consortium cultured in landfill leachate for carbon fixation and lipid production. Bioresour. Technol. 156, 322–328. doi:10.1016/j.biortech.2013.12.112.

Zhou, W., Li, Y., Min, M., Hu, B., Chen, P., Ruan, R., 2011. Local bioprospecting for high-lipid producing microalgal strains to be grown on concentrated municipal wastewater for biofuel production. Bioresour. Technol. 102, 6909–6919. doi:10.1016/j.biortech.2011.04.038.

Chapter 4

Microalgal bioremediation of nitrogenous compounds in landfill leachate –the importance of micronutrient balance in the treatment of leachates of variable composition

This section directly follows on the findings from the previous experiments. The chapter provides a comparison of growth and nutrient depletion performance of the selected microalgae *Chlamydomonas* sp. strain SW15aRL in several leachate samples. This takes account of leachate variability over time or at different sites and is a necessary initial assessment for the viability of long term studies. Also the growth promoting and inhibiting factors are explored. It is written in a manuscript format and has been submitted to the journal Algal Research.

Authors: Andrea Paskuliakova[1], Ted McGowan[2], Steve Tonry[1], Nicolas Touzet[1]

[1]Centre for Environmental Research Innovation and Sustainability (CERIS), Institute of Technology Sligo, Ash Lane, Sligo.

[2] School of Science, Department of Life Science, Institute of Technology Sligo, Ash Lane, Sligo.

We appreciate the contribution from Ted McGowan who kindly agreed to facilitate the access to use the ICP-MS equipment for analysis of minerals, contributed the analytical standards, discussed and provided help with the development of the method for analysis and provided review of the obtained results and final manuscript.

4.0 Abstract

Landfill leachate is a type of wastewater which is challenging to treat. Phycoremediation has been proposed as an alternative biological treatment for the removal of ammonia nitrogen. Several studies have shown microalgae based bioremediation to be possible with ammonia tolerant microalgal species, provided that an optimal dilution is used and the initial molecular N:P ratio is adjusted.

The composition of landfill leachate varies between sites and throughout the year. The performance of selected microalgal strains and their susceptibility to variation in landfill leachate composition is poorly understood. This study compares the growth of *Chlamydomonas* sp. strain SW15aRL in a variety of leachate samples. The leachate samples are from different sites including leachate sampled on different occasions from the same site. These substrates were diluted to obtain ammonia nitrogen concentration within the range of 30 to 220 mg l^{-1}.

Results showed that strain SW15aRL was capable of growth in a variety of leachates but was dependent on the overall composition profile of the landfill leachate rather than just its dilution. Growth was negatively affected in two of the leachates tested, possibly due to metal toxicity and mineral bioavailability or deficiency. Phosphate addition appeared to be essential for growth in the landfill leachates even though precipitation occurred in some instances. Ammonia nitrogen decrease varied between 70% and 100% in the substrates where microalgae could successfully grow.

This study indicates that due to their overall mineral profile some landfill leachates are more suited for microalgae based remediation than others. Both inhibitory and limiting factors complicate microalgae growth. Dilutions are needed to maintain the solubility of specific constitutents and the toxicity of others in check, yet the dilutions also may reduce the concentrations of key nutrients. Identifying individual contribution from these separate factors is not easy due to the complex nature of landfill leachate. Furthermore, a better understanding of other physicochemical processes that take place concurrently during the growth of microalgae in landfill leachate and which contribute to overall nutrient reduction is required. While small scale, short term studies indicate that landill leachate constitutes a potentially rich source of nutrients for microalgal growth, little is known of the effects that variable

leachate compsisitons can have on the sustainable growth of microalgae should the microalgal cells solely use leachate as their source of nutrients long term.

Keywords

landfill leachate, microalgae remediation, nutrient limitation, leachate toxicity

Abbreviations/ Acronyms

TAN – total ammonia nitrogen, TON – total oxidised nitrogen, APHA – American Public Health Association, TSS – total suspended solid

Highlights

- Strain *Chlamydomonas* sp. SW15aRL can remove TAN in a variety of leachate samples.
- Despite precipitation, better growth was achieved when supplemented with P.
- Indication of secondary nutrient limitations in some leachates.

4.1 Introduction

Phycoremediation generally refers to a type of biological treatment of wastes in which algae remove inorganic and simple organic compounds for their growth while some more complex substances can undergo a certain degree of biotransformation. In addition, the concept of microalgal biomass production as biofuel feedstock has been growing in popularity thereby indicating that the growing of algae on wastewaters offers a dual benefit (Olguín, 2003). While microalgal production is seen as a possible solution for future renewable biofuel needs (Murphy et al., 2013), the studies having assessed the viability of such technology have mostly been conducted in countries with plentiful supply of light and in warm climates. These latter conditions are typically reflected in the laboratory conditions used in previous experimental work. There is therefore limited data on growth rates, nutrient removal

and uptake, and energy consumption for such applications for countries with temperate climates where attaining high light and temperature conditions would come at substantial extra cost.

The possibility of landfill leachate phycoremediation has previously been shown with various chlorophytes (Lin et al., 2007; Cheng and Tian, 2013; Thongpinyochai and Ritchies, 2014; Zhao et al., 2014; Sforza et al., 2015). However, there are few follow up studies comparing the performances of the microalgae strains used with a variety of leachate samples (Mustafa et al., 2012; Thongpinyochai and Ritchie, 2014). Landfill leachate is known for its variable composition depending on the landfill site, age of landfill and weather conditions, while certain trends in physicochemical parameters still apply (Kjeldsen et al., 2002; Oman and Junestedt, 2008). Chemical loading within this type of wastewater is usually high and requires dilution to make the growth of microalgae possible. Nutrient proportions in landfill leachate can also be seen as disadvantageous. The molecular N:P ratio tends to be high with phosphorus being a limiting growth nutrient (Paskuliakova et al., 2016; Pereira et al., 2016). While small scale, short term studies have shown that landfill leachate constitutes a potentially rich source of nutrients for microalgal growth, little is known of the effects that variable leachate compositions can have over time on the sustainable growth of microalgae. Taking into account microalgal nutrient requirements which can be related to composition of this type of wastewater, mineral imbalances have been implied based on calculations rather than experimental observations (Edmundson and Wilkie, 2013). Minerals such as Fe, Co, Mg, Mo, Mn, Zn, Cu and Ni fulfil important physiological functions and their amounts as well as their chemical speciation in solution do matter for the successful growth of microalgae and thus the overall remediation effectiveness (Chen et al., 2011; Juneja et al., 2013). Wastewaters are complex in nature and the proportions of free metal ions rather than their absolute concentrations are important in terms of bioavailability and also toxicity (Chen et al., 2011). Complexation with organic matter or formation of insoluble hydroxides reduces free metal ions in solutions, which can cause mineral deficiencies in plants as well as in microalgae (Nagai et al., 2006; Weger et al., 2006).

The present study involved conducting experiments in batch cultures to verify the capacity of *Chlamydomonas* sp. strain SW15aRL to survive, grow and bioremediate

a range of landfill leachate samples collected from different sites or on varying occasions. In addition the effects of mineral nutrient modulation on the growth and remediation potential of the strain were evaluated.

4.2 Materials and Methods

4.2.1 Microalgae strain

Strain *Chlamydomonas* sp. SW15aRL was isolated from a sample of raw leachate (landfill site in Northern Ireland) in 2014. The ability of this strain to deplete nutrients from landfill leachate was previously studied (Paskuliakova et al., 2016). The strain stocks were maintained in landfill leachates prior to the nutrient depletion experiments

4.2.2 Landfill leachate

Landfill leachate was collected at four different sites during 2015 (Appendix C: Table S1 and S2) either directly from the leachate collection system or from holding tanks. The leachate was collected into plastic bottles and stored at <5°C until used.

4.2.3 Physicochemical analysis

Physico-chemical properties were determined according to published methods (American Public Health Association, 2005). Nutrient profiles (PO_4^{3-}-P, TON, TAN, Cl^-, SO_4^{2-}) were determined spectrophotometrically with an Aquakem 250 autoanalyser on samples filtered through 0.45 µm filter (VWR, Cat. No. 28145-503) prior to analysis. Conductivity and pH were measured electrochemically (HACH conductivity meter sensION5 and 713 pH Meter Metrohm). Colour was estimated by spectrophotometric (VARIAN Cary 50 UV-Visible Spectrophotometer) measurement at λ = 455 nm after filtration through 0.45 µm filter. Alkalinity was determined titrimetrically with 0.1N HCl to pH 4.5 (using Metrohm 713 pH Meter). The metal profiles were determined on raw leachates and also leachates filtered

through 0.7 µm glass filter as all the leachate samples were filtered prior to microalgae remediation experiments in this manner. Leachate samples were digested using a microwave digestion system (Milestone Ethos Plus) with HNO_3 (ROMIL-UpA^{TM}) according to Method 3015A (US EPA, 2007) prior to trace element analysis. Several trace elements (i.e. Fe, Mn, Zn, Co, Cu, Mo, Al, Cr, Ni, Cd, Pb) were determined by ICP-MS (Varian 820MS). Ca, Na, K were measured by flame photometry (Sherwood 360) and Mg was determined by flame AAS (Agilent 200 AA). Suspended solids were quantified gravimetrically by filtering a known volume of sample through 0.7 µm glass filter (VWR, Cat. No. 516-0345) and drying at 105°C until constant weight. Chemical Oxygen Demand (COD) was determined spectrophotometrically after sample digestion using HACH Lange Ltd test kits.

4.2.4 Growth of microalgae in six different leachate samples

Experiments were conducted with raw or diluted leachate using autoclaved deionised water as diluent. The dilution factor depended on nutrient loading with the aim of having a final nitrogen concentration under or near 250 mg l^{-1}. Leachate samples were filtered through a glass fibre filter (VWR 1.6 µm pore size followed by 0.7 µm).

Tests were conducted in 250 ml Erlenmeyer flasks with 150 ml volume of leachate-microalgae mixture in stationary flasks. Flasks were inoculated at approximately the same initial biovolume of 0.15 mm^3 ml^{-1} (~100 000 cells ml^{-1}). All experiments were conducted in triplicate. Controls with no microalgae were set up to monitor losses of nutrients due to reasons other than microalgal assimilation. All solutions were phosphorus supplemented (1000 mg l^{-1} PO_4^{3-}-P prepared from K_2HPO_4) to achieve a molecular ratio 16:1 N:P in the final volume. Aliquots of 2 ml were sampled at intervals and analysed for cell number, TAN, TON and orthophosphate. The incubation conditions were as follows: a temperature of 15°C, a light cycle of 14:10 hours (light:dark) and a photosynthetic photon flux density (PPFD) of 22 µmol m^{-2} s^{-1}.

The growth progress through the experiments was monitored by cell counts using a haemocytometer. Nutrient concentration changes (PO_4^{3-}-P, TON, TAN) were determined spectrophotometrically with Aquakem 250 autoanalyser on samples

filtered through 0.45 µm filter. Variation in pH during the experiments was estimated using pH indicator strips (Merck MColorpHast™ pH 5.0-10, pH 7.5-14, Δ0.5 pH, Dosatest® pH 7.0-10.0, Δ0.3 pH) with small aliquots of culture removed from the flasks.

4.2.5 Growth of microalgae in leachate S1 with three different starting cell concentrations

This was carried out to verify if nutrient removal could improve with increasing starting microalgae in the inocula. The experiment was set up as per section 2.4 with leachate S1 (100%) except that phosphate was adjusted to a molecular ratio ~ 32:1 N:P in the final volume due to the previous observation of extensive precipitation. Three starting cell concentrations were used: 100 000, 200 000 and 500 000 cell ml^{-1}.

4.2.6 Growth of microalgae in three leachate samples supplemented with minerals

This experiment was set up to examine if the addition of specific micronutrients could influence microalgal growth and macronutrient content removal in leachates during the remediation. Three samples were chosen: S3 (10%) where microalgae initially grew but started dying off, S2 (20%) where microalgae growth was slow and S6 (30%) where microalgae grew well. The experiment set up was similar to that in section 2.4 with the addition of extra sets of microalgae treated samples and controls supplemented with iron ($FeCl_3.6H_2O$) and magnesium ($MgSO_4.7H_2O$), which were monitored alongside the mineral non-supplemented flasks. While growth only at one concentration was monitored in S2 (20%) and S3 (10%), several concentrations were monitored in leachate S6 (30%) (Table 4.1).

Table 4.1. Overview of the concentration modulation in iron and magnesium in the individual experiments.

Addition	S2 (20%) P adjusted	S2 (20%) P &min adjusted	S3 (10%) P adjusted	S3 (10%) P &min adjusted	S6 (30%) no addition	S6 (30%) P adjusted	S6 (30%) P & Fe adjusted	S6 (30%) 0.5× min adjusted	S6 (30%) 1× min adjusted	S6 (30%) 2× min adjusted
N:P ratio adjusted	16:1	16:1	16:1	16:1	Not adjusted	16:1	16:1	16:1	16:1	16:1
Fe	0	2 mg l^{-1}	0	2 mg l^{-1}	0	0	2 mg l^{-1}	1 mg l^{-1}	2 mg l^{-1}	4 mg l^{-1}
Mg	0	20 mg l^{-1}	0	20 mg l^{-1}	0	0	0	10 mg l^{-1}	20 mg l^{-1}	40 mg l^{-1}

4.2.7 Biomass and precipitate dry weight determination

Microalgal dry weight and precipitates in the controls were determined by gravimetric quantification by filtering a known volume of sample through 0.7 µm glass filter (VWR, Cat. No. 516-0345) and drying at 105°C until it attained constant weight. As attempts to wash the biomass did not result in the removal of precipitates in previous laboratory experiments (observation), the results of both biomass/precipitate and precipitate determined in the microalgae treated leachate and the controls, respectively, are listed.

4.2.8 Statistical analyses

Data were analysed with the use of IBM SPSS Statistics 22 package. One-way ANOVA (Tukey and Games-Howell where equality of variances could not be assumed) was used to compare means of number of different groups. Paired t-test (2-tailed) was used to compare changes in parameters pre and post treatment. Independent t-test (2-tailed) was applied to compare the effects of the two different treatments.

4.3 Results and Discussion

4.3.1 Composition of the six different leachates used for microalgal growth

The overview of the composition of the raw leachate samples used is summarised in Table 4.2. High strength leachates have been shown to have an inhibitory effect on microalgae growth (Lin et al., 2007; Thongpinyochai and Ritchie, 2014; Zhao et al., 2014; Sforza et al., 2015). Dilutions with TAN content up to ~200 mg l^{-1} seem to contain favourable amounts of nutrients to facilitate the growth of ammonia-tolerant microalgal species. Samples S2, S3, S4 and S6 were diluted for the experiments to 20%, 10%, 10% and 30% respectively, to reduce nitrogen loading up to ~250 mg l^{-1} N. Leachates S1 and S5 did not require any dilution as the main nitrogenous compounds were within the suitable range. Some raw leachate samples had a high concentration of metals such as iron, manganese and copper. However, portions of

some metals were associated with suspended matter given that filtration through 0.7 µm glass membrane caused notable reductions (Table 4.2). In addition to bringing TAN to an appropriate level the leachate dilution would substantially reduce the concentrations of co-occurring metals. Although at high concentrations metals can be toxic, many are also essential as micronutrients for microalgal growth and need to be present in sufficient amounts (Jamers et al., 2013; Juneja et al., 2013; Wan et al., 2014). For example, raw leachate S3 contained 8.6, 0.6 and 0.2 mg l^{-1} of iron, manganese and zinc respectively, and was subjected to a 10% dilution for the remediation trials. Commonly used freshwater media would contain 0.3-2.1 mg l^{-1} of iron, 0.1-2.2 mg l^{-1} manganese and 0.1-8.9 mg l^{-1} zinc as can be seen in the overview of some standard media compositions in Appendix C: Table S3 (John et al., 2002; Kropat et al., 2011), indicating that these minerals would be present in the diluted leachate at levels inferior to those found in standard cultivation media. Similar observation was made by Bohutskyi et al. (2016) in the phycoremediation of wastewater for which the balancing of nutrient content proved beneficial for microalgal growth.

Table 4.2. Physicochemical parameters of the raw landfill leachates (not diluted) used for the growth experiment.

		Leachate sample (not diluted)					
Parameter	**Units**	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**
pH		6.9	6.3	7.8	8.4	7.4	7.7
Conductivity	**mS cm^{-1}**	2.62	10.6	13.8	20.7	2.28	5.60
OD (455 nm)		0.10	0.77	1.42	3.51	0.02	0.27
TAN	**mg l^{-1}**	152	98	1480	2510	122	506
TON	**mg l^{-1}**	0.1	1280	0.1	0.3	0.1	0.2
PO_4^{3-}-P	**mg l^{-1}**	0.5	13.8	16.5	14.8	<0.05	1.7
molecular N:P	**mol/mol**	~700:1	~200:1	~200:1	~400:1	>5000:1	~700:1
COD	**mg l^{-1}**	145	1505	2455	5030	97	526
Suspended solids	**mg l^{-1}**	127	101	37	228	179	223
Alkalinity ($CaCO_3$)	**mg l^{-1}**	105	134	7520	11300	1300	1540
Chloride	**mg l^{-1}**	268	1500	1670	2830	185	522
Sulphate	**mg l^{-1}**	108	<20	51	366	48	141
Ca	**mg l^{-1}**	111	445	295	413	98	194
Mg	**mg l^{-1}**	31	56	72	116	35	38
Na	**mg.l^{-1}**	229	2310	1530	2210	178	496
K	**mg l^{-1}**	142	668	797	1290	98	252
Fe	**mg l^{-1}**	4.0 (*0.4)	5.1 (*3.0)	8.6	2.6	5.3 (*0.2)	3.9 (*1.6)
Mn	**mg l^{-1}**	1.3 (*0.8)	0.5	0.6	0.3	1.0 (*0.6)	>2.0
Zn	**mg l^{-1}**	0.1	0.3	0.2	0.2	1.7	<0.1
Co	**mg l^{-1}**	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Cu	**mg l^{-1}**	0.4	<0.1	<0.1	<0.1	> 4.0	0.2
Mo	**µg l^{-1}**	<100	<100	<100	<100	<100	<100
Al	**µg l^{-1}**	140 (*<100)	710 (*380)	1800	3600	200 (*<100)	280 (*150)
Cr	**µg l^{-1}**	<100	200	450	1100	<100	<100
Ni	**µg l^{-1}**	<100	160	190	500	<100	<100
Cd	**µg l^{-1}**	<10	<10	<10	<10	<10	<10
Pb	**µg l^{-1}**	<10	<10	<10	<10	390 (*<10)	<10

* concentration of metal in cases where filtration through 0.7 µm glass membrane caused more than 25% reduction

4.3.2 Growth of *Chlamydomonas* sp. SW15aRL in six different leachates

The growth profiles of *Chlamydomonas* sp. SW15aRL in each leachate substrate are shown in Figure 4.1. The strain grew in four out of six samples at initial TAN concentrations between 30 to 220 mg l^{-1}. It was not able to grow in leachate sample S5 (100%) in spite of an apparently suitable TAN concentration (122 mg l^{-1}) and started gradually dying off; this experiment was discontinued after day 11. This particular leachate contained a high concentration of copper (>4 mg l^{-1}), which could have caused growth inhibition and toxicity. In the study of Jamers et al. (2013), the optimal free Cu^{2+} concentration was around 0.92 nM for growth of *Chlamydomonas reinhardtii* strain 11-32a while the free Cu^{2+} concentration of 17 nM proved toxic and inhibited growth altogether (the free $Cu^{2+}$was measured in TAP media and added as $CuSO_4$ at concentration 1.6 and 8 mg l^{-1} total Cu^{2+} respectively).

Leachate S3 (10%) supported the growth of strain SW15aRL over the first 6 days with the highest rate of growth of 0.19 day^{-1} (one-way ANOVA (Tukey), n=18, $p<0.05$) (Table 4.3). The number of viable cells in this substrate rapidly declined by the time of the next enumeration, leading to this experiment being also discontinued after day 11. After microscopic examination, the cells were clearly damaged with apparent disruption to their chloroplast structure. This could have been caused by either leachate toxicity or nutrient limitation. Strain SW15aRL was originally isolated from the landfill where S3 was collected and previously showed both substantial growth potential and nutrient removal ability on leachate from this site sampled on an earlier occasion (Paskuliakova et al., 2016).

Strain SW15aRL achieved the highest cell increases (one-way ANOVA (Tukey), n=18, $p<0.05$) in leachates S1 (100%) and S6 (30%), which had initial TAN concentrations similar to that of S3 (10%). Leachates S1, S2 (20%), S4 (10%) and S6 (30%) supported the growth of the strain but with slow growth rates of 0.06 to 0.13 day^{-1}. Leachates S1 and S6 both originated from the same landfill (Appendix C: Table S1 and S2) but were sampled on two different occasions. In S1, the growth of strain SW15aRL was faster at the start of the experiment and similar to that in sample S3, with a growth rate of 0.17 day^{-1} over the first 11 days. In S6 the strain achieved the highest growth rate (0.13 day^{-1}) over the longest period of 23 days (one-way ANOVA (Tukey), n=12, $p<0.05$).

Strain SW15aRL showed slow growth rates in all the substrates used when compared to other similar studies (Table 4.4), yet still similar to that previously achieved with this strain in a different study (Paskuliakova et al., 2016). This is probably attributable to the relatively low light and temperature settings used in the study. Similar growth rates have been obtained for chlorophytes at low temperatures for the treatment of high TAN wastewaters, although such rates can be species and strain dependent (Chen et al., 2012; Pereira et al., 2016). It is also likely that leachate toxicity or micronutrient availability would be contributing factors.

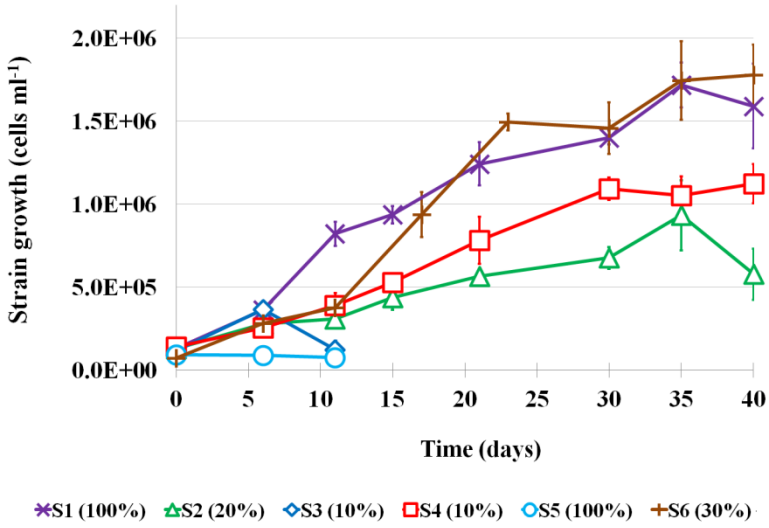


Figure 4.1. Comparison of the *Chlamydomonas* sp. SW15aRL growth in six different leachates. Values are an average of three replicates, error bars ± one standard deviation.

Table 4.3. Comparison of growth rates measured for strain *Chlamydomonas* sp. SW15aRL in the six different leachates.

Leachate	Growth rate (day^{-1})	Period of time over which the rate was calculated
S1 (100%)	0.07 ± 0.00	35 days
S2 (20%)	0.06 ± 0.00	35 days
S3 (10%)	0.19 ± 0.01	6 days
S4 (10%)	0.07 ± 0.00	30 days
S5 (100%)	None	Decreasing cell conc.
S6 (30%)	0.13 ± 0.01	23 days

Table 4.4. Comparison of microalgal growth rates in different studies carried out with landfill leachate.

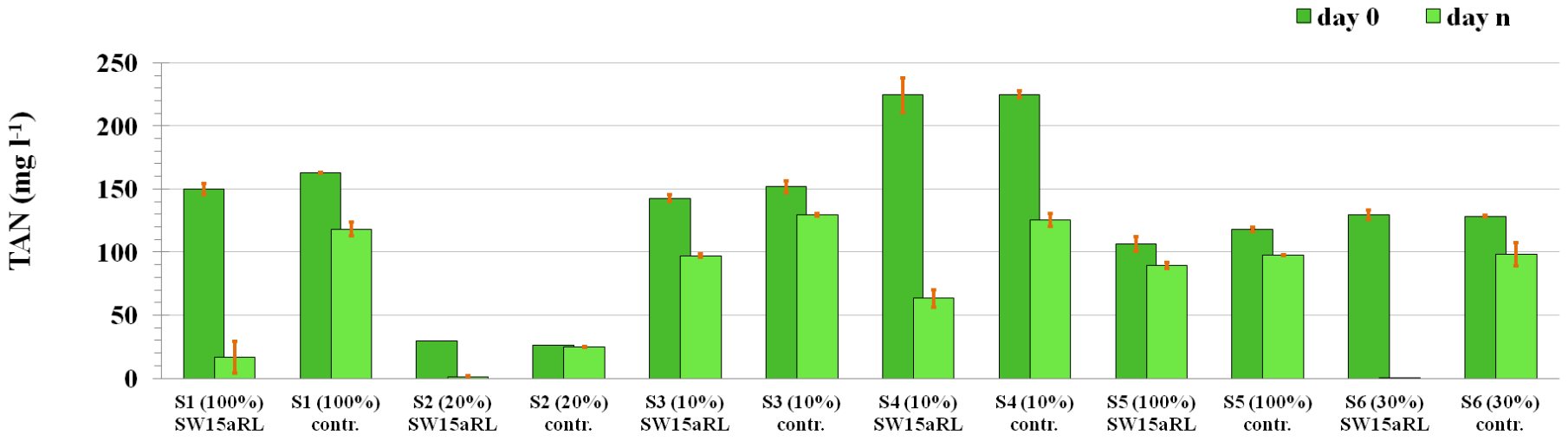
Growth rate (day^{-1})	Microalgal strain	Substrate	Conditions	Reference
0.67 0.83	*Chlorella* sp. *Scenedesmus* sp.	raw leachate with adjusted pH	150 µmol photons m^{-2} s^{-1} 24:0 hours of light:dark 25°C; aeration 0.065 L min^{-1}	Edmundson and Wilkie (2013)
0.28	*Chlorella* sp.	10% raw leachate diluted with municipal wastewater	8000 lux 20:4 hours of light:dark 25°C; aeration 100 mL min^{-1}	Zhao et al. (2014)
0.14	*Chlamydomonas* sp. strain: SW15aRL	10% raw leachate	22 µmol photons m^{-2} s^{-1} 14:10 hours of light:dark 15°C; no agitation	Paskuliakova et al. (2016)
0.4 (cell no.) 0.7 (dry weight basis)	*Acutodesmus obliquus*	10% raw leachate	100 µmol photons m^{-2} s^{-1} 24:0 hours of light:dark magnetic stirring bubbled with 5% v/v CO_2/air 1 L h^{-1}	Sforza et al. (2015)
0.03 – 0.23	Various chlorophytes and *Euglena* sp.	25 and 50% treated leachate	42 µmol photons m^{-2} s^{-1} 12:12 hours of light:dark 25°C; continuous shaking 150 rpm pH adjusted initially to 7.0	Mustafa et al. (2012)
0.39	*Chlorella pyrenoidosa* (P)	10% raw leachate	2000-3000 lux 16:8 hours of light:dark 25-30°C; continuous shaking 100 rpm	Lin et al. (2007)
0.03-0.13	*Chlorella. vulgaris* CCAP 211/11B	3 different treated leachates	32-42 µmol photons m^{-2} s^{-1} 24:0 hours of light:dark 16-21°C; aeration 90 L h^{-1}	Pereira et al. (2016)

4.3.3 Nutrient decrease in six different leachates

The overall changes for the nutrients monitored (TAN, TON and orthophosphate) and the initial and final pH for the leachates treated with microalgae and their corresponding controls are summarised in Appendix C: Table S4.

4.3.3.1 Nitrogenous compounds changes in the six leachates during phycoremediation

The overview of ammonia nitrogen decreases is shown in Figure 4.2. The highest absolute ammonia nitrogen reduction of 161 mg l^{-1} in 40 days was observed in sample S4 (10%), representing ~70% of the overall content. However, losses in the corresponding controls for this leachate were also quite high, suggesting a contribution to the reduction by other biological or non biological processes. Ammonia nitrogen in the controls was more susceptible to losses (23-44%) at higher initial TAN concentration, as can be observed in samples S1 (100%), S4 (10%) and S6 (30%, initial TAN 128-225 mg l^{-1}), unlike sample S2 (20%, initial TAN 26 mg l^{-1}) where only a ~4% reduction was observed in the control during the experimental period. Reductions of 89% and almost 100% were observed in samples S1 (100%) and S6 (30%) respectively, both from the same landfill site, when treated with strain SW15aRL.



	S1 (100%) SW15aRL	S1 (100%) contr.	S2 (20%) SW15aRL	S2 (20%) contr.	S3 (10%) SW15aRL	S3 (10%) contr.	S4 (10%) SW15aRL	S4 (10%) contr.	S5 (100%) SW15aRL	S5 (100%) contr.	S6 (30%) SW15aRL	S6 (30%) contr.
TAN decrease (mg l^{-1})	133.2	44.3	28.3	1.1	45.6	22.2	161.2	99.5	16.8	20.2	129.0	30.1
TAN decrease (%)	88.9	27.2	95.9	4.2	31.9	14.6	71.7	44.2	15.8	17.1	99.6	23.4

Figure 4.2. Comparison of TAN reduction in six leachates with *Chlamydomonas* sp. strain SW15aRL and the corresponding controls. Values are an average of three replicates ± one standard deviation. n = 40 days (S1, S2, S4, S6) while n = 11 days (S3, S5).

The only substrate with substantial level of TON initially was sample S2. It is possible that this nutrient is accessible only to a limited extent to strain SW15aRL as there was a significant yet relatively small (~21 mg l^{-1}) reduction of TON concentration over the test period (paired t-test, 2-tailed $p<0.05$) in comparison to the control, in which TON remained unchanged (paired t-test, 2-tailed $p>0.05$).

Some microalgae are known to have a preference for TAN, which is a less energy demanding nitrogen source (Dortch et al., 1990). Strain SW15aRL was shown in previous work to be unable to grow solely on substrate containing TON only as an inorganic nitrogen source (Paskuliakova et al., 2016).

An increase in oxidised nitrogen (9.6 ± 11.6 mg l^{-1}) was also observed in the case of leachate S6 (30%) in the control, which was of no statistical significance (paired t-test, 2-tailed $p>0.05$) possibly due to the variability of results in TON concentration across the three replicates. TON did not increase, however, in the leachates treated with microalgae (Appendix C: Table S4).

As the samples were not sterilised, ammonia oxidisation might have been the result of microbial activity over the course of the experiment, which appeared to be suppressed in the presence of microalgal cells. While the bacterial interactions with microalgae in phycoremediation experiments have been emphasized in a number of studies (Lau et al., 1995; Zhao et al., 2014; Krustok et al., 2015), the composition of such a community within landfill leachate has not been described yet.

4.3.3.2 Phosphate changes in the six leachates during phycoremediation

For consistency in experimental design phosphate concentrations were adjusted to a molar ratio N:P 16:1 at the start of the experiment in all the leachates. Phosphate was not completely depleted in any of the treatments at the end of the experimental period (Appendix C: Table S4). Within the first few days of the experiments, a visible precipitate was formed within flasks with some leachate samples. In these cases, the added phosphate probably underwent co-precipitation with other inorganic constituents as its concentration in the solution was reduced dramatically (paired t-test, 2-tailed $p<0.05$) within the first 6 days, especially for leachate S1 (100%) and S5 (100%), which can be seen from the control treatments (Figure 4.3). Less drastic

but still significant change within the first 6 days was also recorded in leachate S6 (30%) (paired t-test, 2-tailed $p<0.05$). Not as extensive but also significant (paired t-test, 2-tailed $p<0.05$) gradual change in phosphate concentration was observed in the controls of leachates S3 (10%) and S4 (10%). No significant difference was observed in leachate S2 (20%) controls, where the amount of dissolved phosphate in the leachate remained stable (paired t-test, 2-tailed $p>0.05$).

The issue of precipitation was to be expected as landfill leachates are heavily polluted with various substances and precipitation with chemical agents is another form of treatment which can be employed (Huang et al. 2014). The amount of phosphate which would precipitate depends on the amounts of several other ions present in solution. According to Abou-Shanab et al. (2013), precipitates formed during the microalgal remediation of piggery wastewater were made mainly of carbon, oxygen and calcium, with small amounts of phosphorus, nitrogen and magnesium based on a weight by weight basis. In aquatic systems phosphate speciation is dependent on pH, temperature, concentration and the relative proportions of cations and competitive complexing anion ligands. Calcium and magnesium co-precipitate with phosphate. Increasing pH, which would be typically associated with microalgal activity would enhance precipitation of mineral hydroxides. Phosphate bioavailability is known to be reduced via precipitation and surface adsorption onto other inorganic particles (Cembella et al., 1982). Thus factors which reduce the amount of minerals that are readily available to microalgae in the solution could consequently reduce the speed at which they can be absorbed and thereby affect the rate of the growth. While the lower temperature used in this study may reduce the metabolic rate of microalgae, it would also influence the chemical solubility of the nutrients. Pereira et al. (2016) also observed higher ammonia nitrogen and phosphate removal during microalgae growth in landfill leachate when phosphate was added, although, some reduction in phosphate concentration was also attributed to precipitation.

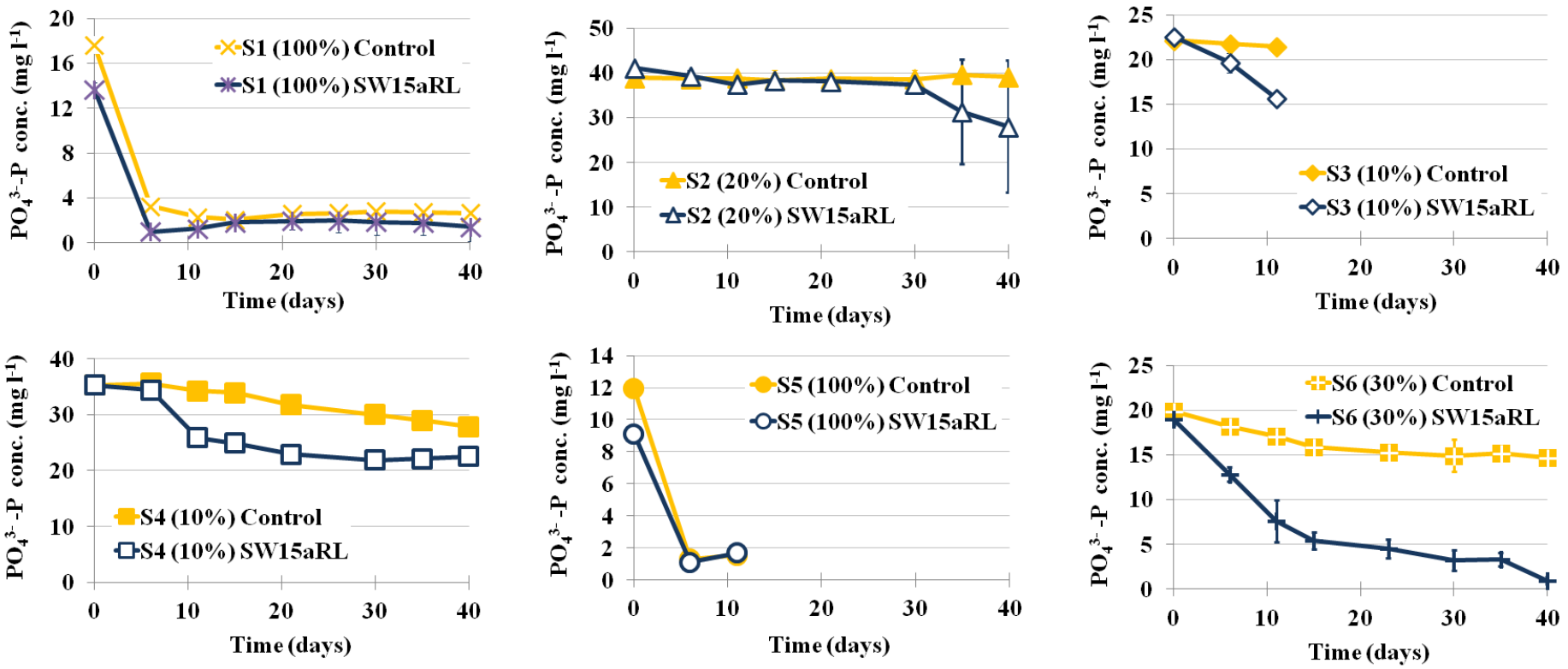


Figure 4.3. Phosphate changes throughout duration of experiments in leachate S1 and S6. Significant reductions of the dissolved phosphate were observed in the leachate samples S1, S5 and S6 in controls within the first 6 days. The phosphate concentration in the other three leachate samples was less immediate. Values are an average of three replicates; error bars ± one standard deviation.

Phosphate was not completely depleted in any of the experiments. It appeared this nutrient was accessible to strain SW15aRL in spite of precipitation occurring, but possibly at a slower rate. Additional experiments showed that if the precipitate was removed from the flasks, the culture stopped growing (observation). In the cases of leachates S1, S2 and S4 there were still residual macronutrients which were not used up at the end of the experiment even though the strain was in its stationary phase of growth. This supports the assumption that another nutrient might have been limiting. Similar assumption was made after remediation of concentrated municipal wastewater using microalgae which resulted in nutrient reduction but with the culture ceasing to grow before the macronutrients from the solution (total nitrogen and phosphate compounds) were depleted (Zhou et al., 2011).

4.3.4 Experiment with different starting cell concentrations

Leachate S1 was used to verify whether or not increasing the size of the microalgal inoculum could improve the rate of TAN removal based on the assumption that more cells would require more nutrients. It was previously shown that increased biomass productivity and faster nutrient removal can be achieved with higher starting inocula for some microalgal species, providing that sufficient amounts of nutrients are available (Lau et al., 1995; Becerra-Dórame et al., 2010; Bohutskyi et al., 2016). Leachate S1 was chosen because no dilution was required for treatment due to its initial TAN. Also, one of the highest overall TAN reductions was achieved for S1 in the earlier experiment (Figure 4.2), however the time required was quite long.

The highest cell concentrations achieved throughout the experiment were between 1.3-1.6 $\times 10^6$ cell ml^{-1} and were not significantly different amongst the three sets of tests or the cell concentration reached with S1 described in section 4.3.1 (one-way ANOVA (Tukey), $n=12$, $p>0.05$). The specific growth rates significantly decreased with increasing starting cell concentration (one-way ANOVA (Tukey), $n=9$, $p<0.05$). The significantly highest (one-way ANOVA (Tukey), $n=12$, $p<0.05$) decrease in TAN over 40 days was observed with the highest cell concentration and this

decrease was higher by ~10 mg l^{-1} in comparison to the next highest TAN decrease achieved. There was no significant difference between the other two.

Regardless of the starting cell concentration, TAN decrease was very similar in the three sets (Figure 4.4; Appendix C: Table S5). This could have been caused by precipitation and the possible slow release of minerals, including phosphorus, necessary for the cells to grow. Trials modulating the starting cell concentrations across several orders of magnitude might be warranted in the future.

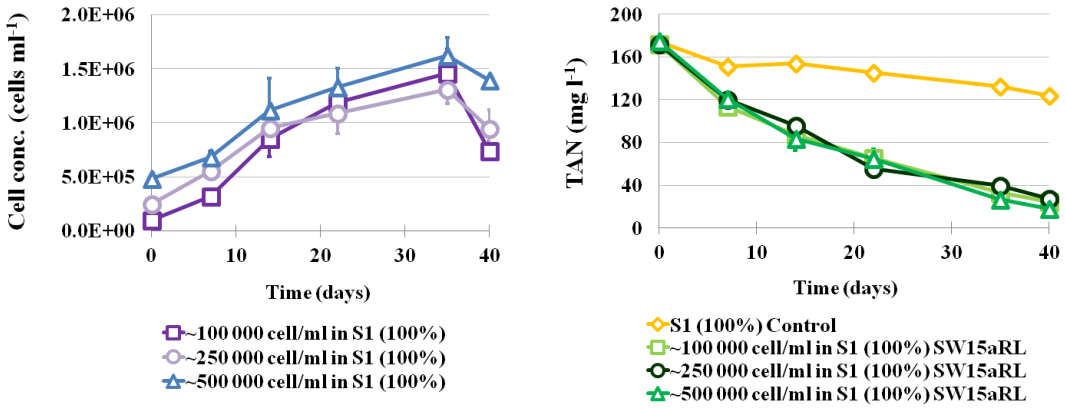


Figure 4.4. Growth progress of *Chlamydomonas* sp. SW15aRL in leachate S1 (100%) when inoculated at three different starting cell concentrations and corresponding TAN reduction. Phosphate concentration was adjusted to molecular ratio N:P ~ 32:1. Values are an average of three replicates ± one standard deviation.

4.3.5 Growth and nutrient depletion in the three leachates supplemented by minerals

Leachate S3 did not support the growth of strain SW15aRL and was either nutrient limited and/or too toxic for this strain. A short experiment conducted in well plates and cells removed from flasks containing leachate S3 (10%) showed that the addition of some minerals (e.g. Fe, Mg and Ca) helped the damaged cells to recover while cells in wells non-supplemented with such minerals died off, suggesting certain micronutrient deprivation (observation). As a follow up, three leachate samples were supplemented by minerals to observe how this would influence cell growth and

nutrient reduction. These experiments were carried out with leachates S2 (20%), S3 (10%) and S6 (30%). The results for the growth of strain SW15aRL and nutrient changes are displayed in Figure 4.5 and Appendix C: Table S6 to S8.

4.3.5.1 Mineral additions to leachate S3 (10%)

Strain SW15aRL initially grew in leachate S3 (10%) and then started dying off, similar to results in section 4.3.1. No significant (independent t-test, 2-tailed $p>0.05$) difference between growth rates was observed over the first 23 days between the mineral supplemented and non supplemented treatments. After 23 days the microalgae in the flasks supplemented with minerals continued to grow while those in the non-supplemented started dying off (Figure 4.5A). There was no significant (one-way ANOVA (Tukey), $n=24$, $p>0.05$) reduction of ammonia nitrogen observed after day 30 in the mineral non supplemented experiment while it continued to decrease further in the experiment supplemented with minerals, resulting in a significant (independent t-test, $p<0.05$) difference in the overall amount of ammonia reduced (~36 $mg.l^{-1}$) between the two experiments by day 40. The difference in the biomass obtained on day 40 was significant (independent t-test, $p<0.05$) between mineral supplemented and non supplemented sets (Table 4.4). The addition of minerals had no significant impact (independent t-test, $p>0.05$) on ammonia losses within the control treatment in comparison to the control without minerals added. There was no significant (independent t-test, $p>0.05$) difference in phosphate decrease in the mineral supplemented and non supplemented treatments on day 40. However a phosphate decrease in the mineral non-supplemented treatment took place only after cells starting dying off.

4.3.5.2 Mineral additions to leachate S2 (20%)

While there was no significant difference between the maximum cell concentrations achieved (independent t-test, $p>0.05$) in the microalgae-treated flasks with leachate S2 (20%), the addition of minerals did significantly increase the growth rate (Figure 4.5B) (independent t-test, $p<0.05$) and the amount of solids (Table 4.4) (one-way ANOVA (Tukey), $n=12$, $p<0.05$) in comparison to the mineral non supplemented

set. Addition of minerals in leachate S2 (20%) had no significant influence on precipitate formation in the controls (one-way ANOVA (Tukey), n=12, $p>0.05$) either. Nutrients, both phosphate and ammonia, were used faster when minerals were added (Figure 4.5B). After 40 days, there were significant (paired t-test, $p< 0.05$) decreases in TON concentration in the experiments with microalgae both with and without minerals addition (~15 and 34 $mg.l^{-1}$ respectively) while there was no significant (paired t-test, $p>0.05$) change in the controls (Appendix C: Table S7).

4.3.5.3 Mineral additions to leachate S6 (30%)

While strain SW15aRL grew well in the leachates from site A (S1 and S6 (30%)), it was of interest to see how distinct mineral increase regimes would affect cell growth and nutrient reduction. The results showed the same overall growth trend in all the sets except for the treatment in which no phosphate was added and where the growth was minimal (Figure 4.5C). Precipitate formation was significantly (one-way ANOVA (Games-Howell), n=33, $p<0.05$) higher in all the control treatments with addition of either phosphate or more minerals when compared to the leachate without any additions (Table 4.4). There was no difference (one-way ANOVA (Games-Howell), n=36, $p>0.05$) in TAN reduction between all the treatments supplemented with nutrients over 40 days. These were however significantly higher (one-way ANOVA (Games-Howell), n=36, $p<0.05$) than the microalgae treated leachate not supplemented at all and the controls. Addition of minerals had no impact on ammonia losses in the control treatments in comparison to the phosphate only supplemented set. Increasing mineral additions did however increase phosphate reduction in the controls at higher concentrations in this leachate sample (Figure 4.5C. and Appendix C: Table S8).

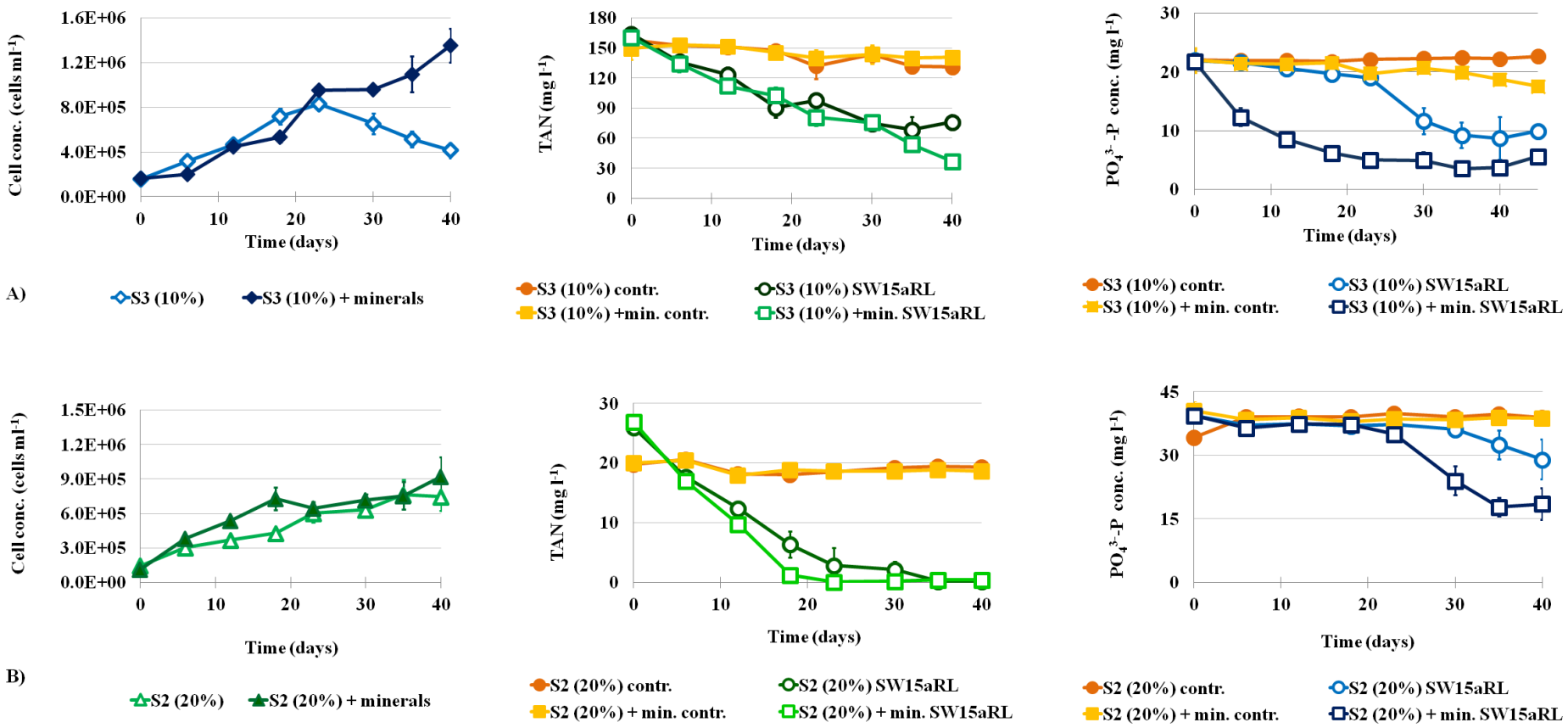
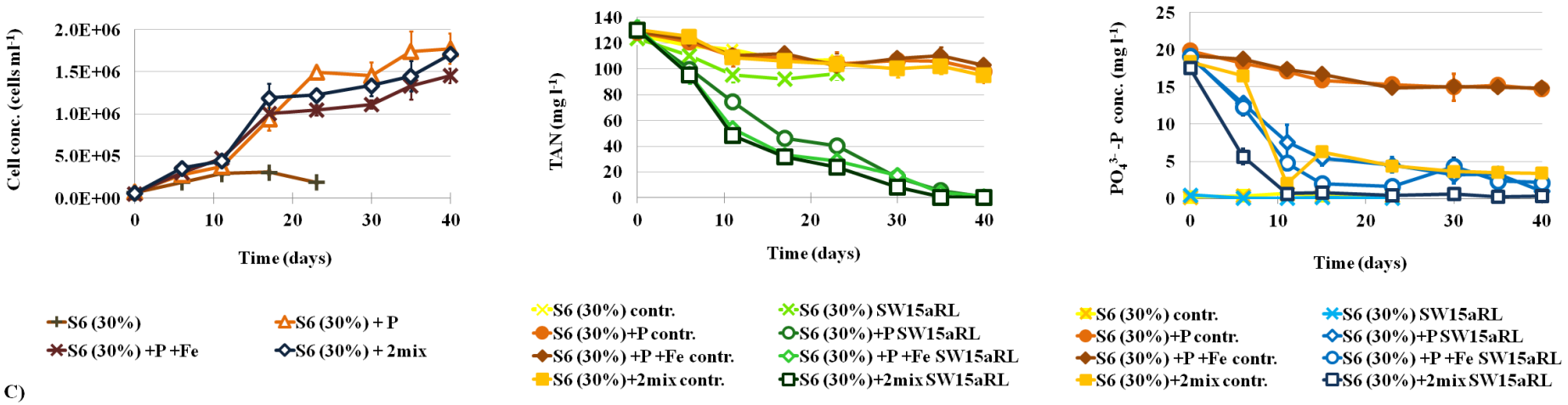


Figure 4.5 A) and B). Growth of *Chlamydomonas* sp. SW15aRL in leachate A) S3 (10%) B) S2 (20%) and C) S6 (30%) with and without mineral addition and corresponding nutrient decreases. Phosphate concentration was adjusted in all cases to molecular ratio N:P ~ 16:1 except for S6 as indicated. Values are an average of three replicates ± one standard deviation.



C)

Figure 4.5 C). Growth of *Chlamydomonas* sp. SW15aRL in leachate A) S3 (10%) B) S2 (20%) and C) S6 (30%) with and without mineral addition and corresponding nutrient decreases. Phosphate concentration was adjusted in all cases to molecular ratio N:P ~ 16:1 except for S6 as indicated. Values are an average of three replicates ± one standard deviation.

Table 4.4. Overview of biomass and precipitate in the treated leachates S3 (10%), S2 (20%) and S6 (30%) and their controls with and without mineral addition.

Treatment	TSS±std (g l^{-1}) SW15aRL	TSS±std (g l^{-1}) Control
10% S3	0.54 ± 0.07	0.00 ± 0.00
10% S3 + minerals	0.86 ± 0.07	0.04 ± 0.01
20% S2	0.81 ± 0.14	0.00 ± 0.00
20% S2 + minerals	1.17 ± 0.09	0.02 ± 0.01
30% S6	-	0.01 ± 0.00
30% S6 + P	1.20 ± 0.06	0.04 ± 0.01
30% S6 + P + Fe	1.18 ± 0.12	0.05 ± 0.00
30% S6 + P + 0.5 × min.	1.17 ± 0.15	0.04 ± 0.00
30% S6 + P + 1 × min.	1.09 ± 0.05	0.05 ± 0.00
30% S6 + P + 2 × min.	1.33 ± 0.04	0.05 ± 0.00

4.3.5.4 Comparison of the effects of mineral additions to the leachates

While small scale, short term studies have shown that landfill leachate constitutes a potentially rich source of nutrients for microalgal growth, little is known of the long term effect of maintaining a microalgal culture solely in landfill leachate or of the effects that variable leachate compositions can have on sustainably allowing the growth of microalgae. The suggested influence of mineral imbalances on microalgal growth was previously based on calculations rather than observation in experiments (Edmundson and Wilkie, 2013). Possibly this might be because the monitoring period is concluded when TAN is depleted. It is also acknowledged that there would be substantial contribution to TAN losses via volatilisation facilitated by aeration, as typically employed to support microalgae growth during experiments, rather than just microalgae assimilation (Zhao et al., 2014). Therefore the complete nutrient requirements for microalgae to fully absorb macronutrients in landfill leachate are still largely unknown.

Here, the addition of minerals increased the growth rate of strain SW15aRL in leachate S2 (20%) and biomass production in leachates S2 (20%) and S3 (10%), and did not have any obvious effects on growth in leachate S6 (30%). The experiment confirmed that when supplemented by minerals, the growth of strain SW15aRL was extended in leachate S3 (10%) whereas cells non-supplemented with minerals started dying off after a certain period of time. Although strain SW15aRL managed to grow for a longer time in the repeated experiment with leachate S3 (10%), it was still impacted negatively when the minerals were not added. Disruption to chloroplast structure was also observed. Iron and magnesium are important components of the photosynthesis mechanism in microalgae. Iron in particular is also required for the functioning of the nitrate and nitrite reductases, which facilitate the assimilation of nitrate as a nitrogen source (Liu and Qiu et al., 2012). The amount of solids (biomass inclusive of precipitates) produced in leachate S3 (10%) supplemented with minerals was lower than in leachate S2 (20%) supplemented with minerals, despite higher cell counts in the former. This could also reflect the fact that the culture in S3 (10%) even when supplemented with the two minerals (which allowed it to grow further than without supplementation) might have still suffered physiologically due to a shortage of some other minerals in the leachate S3 (10%) itself and resulting in smaller cells and lower solids yield. To allow cell growth to achieve its maximum potential, the correct proportions of bioavailable nutrients are crucial. Mandalam and Palsson (1998) showed that balancing the nutrient proportions in growth media (N8 medium) increased the production of high density culture requiring four elements to be added together while individually they would have no effect. Iron concentrations in S1 (100%) and S3 (10%) were ~400 and ~860 µg l^{-1} respectively. Strain SW15aRL could grow in S1 (100%) even though total iron concentration was lower in S1 (100%) than in S3 (10%) while TAN concentrations in the two substrates were comparable.

Metals such as iron can be complexed with organic matter or present as insoluble oxyhydroxides, thereby affecting their bioavailability. This has been known to cause iron deficiencies in plants as well as in microalgae in freshwater systems (Nagai et al., 2006; Weger et al., 2006). The ability to adsorb complexed iron ion from chelators differs between microalgae species (Weger et al., 2006). Humic substances represent a substantial portion of organic matter within the landfill leachate and have

strong complexing ability (Jones and Bryan, 1998). Parameters indirectly reflecting the amount of humic substances in leachate samples S1 and S3 are colour and COD. These were higher for S3 (10%) than S1 (100%).

Microalgae growth in landfill leachate is associated with pH increase due to reduction in carbon dioxide. In addition metal speciation is also primarily governed by pH. Metal solubility typically decreases at higher pH as a result of higher proportions being adsorbed on particulate matter and hence reducing their bioavailability. These interactions are complex and depend on the overall composition profile of the leachate sample to be treated (Gundersen and Steinnes, 2003).

In addition to mineral bioavailability in solution, the microalgae utilisation of the nitrogen reduced in the solution needs to be further assessed through material balance in order to quantify the losses of ammonia nitrogen or other pathways of its transformation. The amount of solids (biomass inclusive of precipitates) obtained from the experiments with S2 (20% + minerals) was comparable to that produced in the more polluted leachate S6 (30%) with higher TAN content. Ammonia volatilisation is known to be directly proportional to the concentration of ammonia nitrogen in the solution, pH and also depends on the presence of other chemical species in the solution. Turbulence in solution or air movement above the liquid surface can further enhance this process, and so can increases in temperature (Vlek and Stumpe, 1978). Microalgal activity and growth typically cause pH increases in batch cultures, therefore ammonia losses in treated flasks are likely to exceed those in the controls. For both leachates S2 (20%) and S6 (30%) ~1.2 g l^{-1} of dry matter material was recovered while the total nitrogen reductions were ~55 mg l^{-1} (TAN+TON) and ~130 mg l^{-1} (as TAN only) respectively.

Mineral additions improved macronutrient reduction (N and P) in the present study, suggesting that the availability of certain minerals in landfill leachates can be the cause of secondary nutrient limitation during phycoremediation. The addition of minerals did not contribute to any losses of TAN although phosphate reduction in the controls was increased when minerals (Fe and Mg) were added. Both inhibitory and limiting factors complicate microalgae growth in landfill leachate. Dilutions are needed to maintain the solubility of specific constituents and the toxicity of others in check, yet the dilutions also reduce the concentrations of key nutrients and minerals.

Identifying and quantifying individual contribution from these separate factors is not easy due to the complex nature of landfill leachate. While small scale, short term studies have shown that landfill leachate constitutes a potentially rich source of nutrients for microalgal growth, little is known of the effects that variable leachate compositions can have on the sustainable growth of microalgae should the microalgal cells solely use leachate as their source of nutrients.

4.4 Conclusion

Growth of *Chlamydomonas* sp. strain SW15aRL was compared in six different landfill leachate samples and the resulting ammonia reduction was evaluated.

- Strain SW15aRL can grow in a variety of leachate samples of varying origins with variable success.
- Data indicated that landfill leachate composition variations impact upon microalgal growth and therefore bioremediation treatability.
- During treatment, several chemical, physical and biological processes concurrently interplay and result in precipitation, TAN volatilisation, TAN oxidation and TAN sorption for biomass growth.
- Phosphate addition promotes microalgal growth and TAN removal, and appears to be utilisable despite the precipitation observed is some cases.
- When strong leachates are diluted to acceptable TAN levels prior to remediation with microalgae, the reduced concentration of other nutrients or their bioavailability probably contribute to slow microalgal growth and may also cause secondary nutrient limitations.

In this study, microalgal growth rate and biomass production were enhanced by the addition of the minerals in some leachate samples. Additional work is required on the long term sustainability of microalgae growth in landfill leachate and on better understanding the implications of composition variations to this process.

4.5 Acknowledgements

This work has been supported by Institute of Technology Presidents Bursary Scheme. We would particularly like to thank the employees of the landfill sites whose assistance in providing landfill leachate is greatly appreciated.

4.6 References

Abou-Shanab. R.A.I., Ji, M.K., Kim, H.C., Paeng, K.J., Jeon, B.H., 2013. Microalgal species growing on piggery wastewater as a valuable candidate for nutrient removal and biodiesel production. Journal of Environmental Management 115, 257-264. http://dx.doi.org/10.1016/j.jenvman.2012.11.022.

APHA (American Public Health Association), 2005. Standard methods for the examination of water and wastewater, 21st ed. Washington, D.C.

Becerra-Dórame, M.J., López-Ellas, J.A., Enríquez-Ocaña, F., Huerta-Aldaz, N., Voltolina, D., Osuna-López, I., Izaguirre-Fierro, G., 2010. The effect of initial cell and nutrient concentrations on the growth and biomass production of outdoor cultures of *Dunalliella* sp.. Ann. Bot. Fennici 47, 109-112. ISSN 1797-2442.

Bohutskyi, P., Kligerman, D.C., Byers, N., Nasr, L.K., Cua, C., Chow, S., Sua, C., Tangc, Y., Betenbaugh, M.J., Bouwer, E.J., 2016. Effects of inoculum size, light intensity, and dose of anaerobic digestion centrate on growth and productivity of *Chlorella* and *Scenedesmus* microalgae and their poly-culture in primary and secondary wastewater. Algal Research 19, 278–290. http://dx.doi.org/10.1016/j.algal.2016.09.010.

Cembella, A.D., Antia, N.J., Harrison, P.J., 1982. The Utilization of Inorganic and Organic Phosphorus Compounds as Nutrients by Eukaryotic Microalgae: A Multidisciplinary Perspective: Par I. Critical Reviews in Microbiology 10:4, 317-391. doi: 10.3109/10408418209113567.

Chen, M., Tang, H., Maa, H., Holland, T.C., Ng, K.Y.S., Salley, S.O., 2011. Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. Bioresource Technology 102, 1649–1655. doi:10.1016/j.biortech.2010.09.062.

Chen, R., Li, R., Deitz, L., Liu, Y., Stevenson, J., Liao, W., 2012. Freshwater algal cultivation with animal waste for nutrient removal and biomass production. Biomass and Bioenergy 39. 128-138. doi:10.1016/j.biombioe.2011.12.045.

Cheng, H.-X., Tian, G.-M., 2013. Preliminary Evaluation of a Newly Isolated Microalga *Scenedesmus* sp. CHX1 for Treating Landfill Leachate. 2013 Third Int. Conf. Intell. Syst. Des. Eng. Appl. 1057–1060. doi:10.1109/ISDEA.2012.249.

Dortch, Q., 1990. The interaction between ammonium and nitrate uptake in phytoplankton. Mar. Ecol. Prog. Ser. 61, 183-201.

Edmundson, S.J., Wilkie, A.C., 2013. Landfill leachate-a water and nutrient resource for algae-based biofuels. Environ. Technol. 34, 1849–57. doi:10.1080/09593330.2013.826256.

Gundersen, P., Steinnes, E., 2003. Influence of pH and TOC concentration on Cu, Zn, Cd, and Al speciation in rivers. Water Research 37, 307–318. http://doi.org/10.1016/S0043-1354(02)00284-1.

Huang, H., Xiao, D., Zhang, Q., Ding, L., 2014. Removal of ammonia from landfill leachate by struvite precipitation with the use of low-cost phosphate and mangesium sources. Journal of Environmental Management. 145, 191-198. doi:10.1016/j.jenvman.2014.06.021.

Jamers, A., Blust, R., De Coen, W., Griffin, J.L. Jones, O. A. H., 2013. Copper toxicity in the microalga *Chlamydomonas reinhardtii*: an integrated approach. Biometals 26,731–740. DOI 10.1007/s10534-013-9648-9.

John, D.M., Whitton, B.A., Brook, A.J., 2002. The Freshwater Algal Flora of the British Isles: an Identification Guide to Freshwater and Terrestrial Algae. The Natural History Museum and the British Phycological Society, UK.ISBN 0 521 77051 3.

Jones, M.N., Bryan, N.D., 1998. Colloidal properties of humic substances. Advances in Colloid and Interface Science 78, 1-48. http://doi.org/10.1016/S0001-8686(98)00058-X.

Juneja, A., Ceballos, R.M., Murthy, G.S., 2013. Effects of Environmental Factors and Nutrient Availability on the Biochemical Composition of Algae for Biofuels Production: A Review. Energies 2013, 6, 4607-4638. ISSN 1996-1073. doi:10.3390/en6094607.

Kjeldsen, P., Barlaz, M.A., Rooker, A.P., Baun, A., Ledin, A., Christensen, T.H., 2002. Present and Long-Term Composition of MSW Landfill Leachate: A Review. Critical Reviews in Environmental Science and Technology, 32 (4), 297–336.

Kropat, J., Hong-Hermesdorf, A., Casero, D., Ent, P., Castruita, M., Pellegrini, M., Merchant, S.S., Malasarn, D., 2011. A revised mineral nutrient supplement increases biomass and growth rate in *Chlamydomonas reinhardtii*. Plant Journal 66 (5), 770–780. doi:10.1111/j.1365-313X.2011.04537.x.

Krustok, I., Truu, J., Odlare, M., Truu, M., Ligi, K., Tiirik, E., Nehrenheim, E., 2015. Effect of lake water on algal biomass and microbial community structure in municipal wastewater-based lab-scale photobioreactors. Applied Microbiology and Biotechnology, 99 (15), 6537–6549. doi: 10.1007/s00253-015-6580-7.

Lau, P.S., Tam, N.F.Y., Wong, Y.S., 1995. Effect of Algal Density on Nutrient Removal from Primary Settled Wastewater. Environmental Pollution 89, 59-66. https://doi.org/10.1016/0269-7491(94)00044-E.

Lin, L., Chan, G.Y.S., Jiang, B.L., Lan, C.Y., 2007. Use of ammoniacal nitrogen tolerant microalgae in landfill leachate treatment. Waste Management 27, 1376–1382. doi:10.1016/j.wasman.2006.09.001.

Liu, S.W., Qiu, B.S., 2012. Different responses of photosynthesis and flow cytometric signals to iron limitation and nitrogen source in coastal and oceanic *Synechococcus* strains (Cyanophyceae). Mar. Biol. 159. 519–532. doi:10.1007/s00227-011-1832-2.

Mandalam, R.K., Palsson, B. Ø., 1998. Elemental Balancing of Biomass and Medium Composition Enhances Growth Capacity in High-Density *Chlorella vulgaris* Cultures. Biotechnology and Bioengineering 59 (5), 605-611. http://dx.doi.org/10.1002/(SICI)1097-0290(19980905)59:5<605::AID-BIT11>3.0.CO;2-8.

Murphy, F., Devlin, G., Deverell, R., McDonnell, K., 2013. Biofuel Production in Ireland-An Approach to 2020 Targets with a Focus on Algal Biomass. Energies 6, 6391–6412. doi:10.3390/en6126391

Mustafa, E., Phang, S., 2012. Use of an algal consortium of five algae in the treatment of landfill leachate using the high-rate algal pond system. Journal of Applied Phycology, 24, 953–963. doi:10.1007/s10811-011-9716-x.

Nagai, T., Imai, A., Matsushige, K., Fukushima, T. 2006. Effect of iron comlexation with dissolved organic matter on the growth of cyanobacteria in a eutrophic lake. Aquatic Microbial Ecology 44, 231-239.

Olguín, E.J., 2003. Phycoremediation : key issues for cost-effective nutrient removal processes. Biotechnol. Adv. 22, 81–91. doi:10.1016/j.biotechadv.2003.08.009.

Oman, C.B., Junestedt, C., 2008. Chemical characterization of landfill leachates – 400 parameters and compounds. Waste Management 28, 1876–1891. doi:10.1016/j.wasman.2007.06.018.

Paskuliakova, A., Tonry, S., Touzet, N., 2016. Phycoremediation of landfill leachate with chlorophytes: Phosphate a limiting factor on ammonia nitrogen removal. Water Res. 99, 180–187. doi:10.1016/j.watres.2016.04.029.

Pereira, S.F.L., Gonçalves, A.L., Moreira, F.C., Silva, T.F.C.V., Vilar, V.J.P., Pires, J.C.M., 2016. Nitrogen Removal from Landfill Leachate by Microalgae. Int. J. Mol. Sci. 2016, 17 (11), 1926; doi:10.3390/ijms17111926.

Sforza, E., Al, M.K., Sharif, A., 2015. Exploitation of Urban Landfill Leachate as Nutrient Source for Microalgal Biomass Production. Chemical Engineering Transaction, 43, ISBN 978-88-95608-34-1, ISSN 2283-9216.

Thongpinyochai, S., Ritchie, R.J., 2014. Biodegradation Using *Chlorella vulgaris* to Decrease the Environmental Effect of Garbage Dump Leachates. Journal of Bioremediation and Biodegradation, 5:5. doi:10.4172/2155-6199.1000239.

US EPA 2007. Test Method 3015A, Microwave Assisted Acid Digestion of Aqueous Samples and Extracts, SW-846, US EPA.

Vlek, P.L.G., Stumpe, J.M., 1978. Effects of Solution Chemistry and Environmental Conditions on Ammonia Volatilization Losses From Aqueous Systems. Soil Science Society of America Journal 42 (3), 416-421. doi:10.2136/sssaj1978.03615995004200030008x.

Wan, M., Jin, X., Xia, J., Rosenberg, J.N., Yu, G., Nie, Z., Oyler, G.A., Betenbaugh, M.J., 2014. The effect of iron on growth, lipid accumulation, and gene expression profile of the freshwater microalga *Chlorella sorokiniana*. Appl. Microbiol. Biotechnol. 98, 9473–9481. doi:10.1007/s00253-014-6088-6.

Weger, H.G., Matz, C. J., Magnus, R. S., Walker, C. N., Fink, M.B., Treble, R. G., 2006. Differences between two green algae in biological availability of iron bound to strong chelators. Canadian Journal of Botany. 84 (3), 400-411. doi: 10.1139/BO6-013.

Zhao, X., Zhou, Y., Huang, S., Qiu, D., Schideman, L., Chai, X., Zhao, Y., 2014. Characterization of microalgae-bacteria consortium cultured in landfill leachate for carbon fixation and lipid production. Bioresour. Technol. 156, 322–328. doi:10.1016/j.biortech.2013.12.112.

Zhou, W., Li, Y., Min, M., Hu, B., Chen, P., Ruan, R., 2011. Local bioprospecting for high-lipid producing microalgal strains to be grown on concentrated municipal wastewater for biofuel production. Bioresour.Technol. 102, 6909–6919. doi:10.1016/j.biortech.2011.04.038.

Chapter 5

Phycoremediation of landfill leachate with the Chlorophyte *Chlamydomonas* sp. SW15aRL and evaluation of toxicity pre and post treatment

This chapter provides findings on the changes in toxicological profile of a leachate sample pre and post phycoremediation treatment with the selected microalgae *Chlamydomonas* sp. strain SW15aRL. Additional tests were conducted to ascertain potential losses of nutrients via other processes. The experiments were carried out using leachates S7 and S8, which were sampled from the site where leachate samples S1 and S6 were collected and previously proved suitable for growth of the microalgal strain SW15aRL. It is written in a manuscript format and is currently under review in the journal Ecotoxicology and Environmental Safety.

Authors: Andrea Paskuliakova[1], Ted McGowan[2], Steve Tonry[1], Nicolas Touzet[1]

[1] Centre for Environmental Research Innovation and Sustainability (CERIS), Institute of Technology Sligo, Ash Lane, Sligo.

[2] School of Science, Department of Life Science, Institute of Technology Sligo, Ash Lane, Sligo.

We appreciate the contribution from Ted McGowan who kindly agreed to facilitate the access to use the ICP-MS equipment for analysis of minerals, contributed the analytical standards, discussed and provided help with the development of the method for analysis and provided review of the obtained results and final manuscript.

5.0 Abstract

Landfill leachate treatment is an ongoing challenge in the wastewater management of existing sanitary landfill sites due to heavy pollution and the complex nature of leachates. There is a continuous interest in treatment biotechnologies with expected added benefits for resource recovery; microalgal bioremediation is seen as promising in this regard.

Toxicity reduction of landfill leachate subsequent to phycoremediation was investigated in this study. The treatment eventuated from the growth of the ammonia tolerant microalgal strain *Chlamydomonas* sp. SW15aRL with N:P ratio adjustment in diluted leachate for facilitation of the process. Toxicity tests ranging over a number of trophic levels were applied, including bacterial-yeast (MARA), protistean (microalgae growth inhibition test), crustacean (daphnia, rotifer) and higher plant (monocot, dicot) assays.

Ammonia nitrogen in the diluted landfill leachate containing up to 158 mg l^{-1} NH_4^+-N (60% dilution of the original) was reduced by 83% during the microalgal treatment. Testing prior to remediation indicated the highest toxicity in the crustacean assays *Daphnia magna* and *Brachionus calyciflorus* with EC50s at 24 h of ~35% and 40% leachate dilution, respectively. A major reduction in toxicity was achieved with both bioassays post microalgal treatment with effects below the EC20s. The microalgae inhibition test on the other hand indicated increased stimulation of growth after treatment as a result of toxicity reduction but also a presence of residual nutrients. Several concurrent processes of both biotic and abiotic natures contributed to pollutant reduction during treatment. Modifying phosphate dosage especially seems to require further attention. As a by-product of the remediation process up to 1.2 g l^{-1} of microalgal biomass was obtained with ~18% DW lipid content.

Keywords

microalgae, landfill leachate, phycoremediation, toxicity testing, biomass generation

Abbreviations and Acronyms:

MARA – Microbial Assay for Risk Assessment; TSS – total suspended solids; OECD – Organisation for Economic Co-operation and Development; COD – chemical oxygen demand; TAN – total ammonia nitrogen; DW – dry weight; EC20 - 20 percent maximal effective concentration; EC50 – half maximal effective concentration.

Highlights

- TAN 158 mg l^{-1} in landfill leachate was reduced by 83% by phycoremediation.
- Optimal phosphate dosage requires further study.
- Major toxicity reduction was achieved in crustacean assays.
- Generated microalgal biomass was 1.2 g l^{-1} with ~18% DW lipid content.

5.1 Introduction

Landfill leachate is high strength wastewater saturated with various compounds leaching out of decomposing municipal waste. Its composition is very complex because of the wide range of toxicants it contains (Cecilia and Junestedt, 2008). Ammonia nitrogen is considered one of the main toxicants therein and can be present at very high concentration, as can many other inorganic compounds. Other organic and metal-organic compounds are present at very low concentrations and are often difficult to detect by standard analytical procedures; with the possibility that many have not yet even been identified (Cecilia and Junestedt, 2008). Many of the 'priority' or 'priority hazardous' substances listed in Directive 2008/105/EC (Daughter Directive to the Water Framework Directive) typically do not exceed the limit values within landfill leachates, which can still exhibit high toxicity as shown by ecotoxicological testing. It is thought that the combined toxic effects of many compounds at sub-detection levels are the causes (Brito-Pelegrini et al., 2007; Płaza et al., 2011).

Biological testing has been used as an indicatory means of evaluating the ecotoxicological impact due to the complex composition of some wastewater samples such as landfill leachate. Multispecies assays that cover a number of trophic levels are usually recommended. In this way, different groups of pollutants can be detected by species sensitive to them. Several standardised and/or commercially available bioassays currently exist (Persoone and Gillett, 1990; Bernard et al., 1996; Brito-Pelegrini et al., 2007). According to Bernard et al. (1996) the toxicity of the majority of leachate samples can be assessed with a battery of tests including bacterial assays, protozoan assays and microalgae assays jointly with higher plants, rotifers or crustaceans. The methods for these tests are well established. The OECD publishes procedures (i.e. Guidelines for the Testing of Chemicals) that are generally accepted internationally as standard methods for assessing the potential risk of chemicals on the environment and these procedures can also be used for the testing of multi-constituent matrices such as landfill leachate.

Microalgae have been explored for wastewater treatment purposes for over two decades and are actively considered for biofuel production. The most successful species usually come from the chlorophytes group, including *Scenedesmus* sp., *Chlorella* sp. or *Chlamydomonas* sp., but cyanobacteria or other phylogenetic groups appear occasionally within the literature (Mandal and Mallick, 2011; Zhou et al., 2011; Kothari et al., 2013; Choudhary et al., 2016). Microalgae can be used to effectively remove ammonia nitrogen and other inorganic constituents to build their biomass (Lin et al., 2007; Mandal and Mallick, 2011; Prajapati et al., 2014; Zhao et al., 2014; Sforza et al., 2015; Sutherland et al., 2016). Some studies have reported that certain microalgal species are capable of removing, biodegrading or biotransforming organic compounds (Hirooka et al., 2003; Pinto et al., 2003; Lima et al., 2004; Yan & Pan, 2004; Li et al., 2009) and also extracting metals from solutions (Thongpinyochai and Ritchie, 2014; Li et al., 2015). However, it has also been shown that remediation of landfill leachate with microalgae can require a certain process control such as pH adjustment (Edmundson and Wilkie, 2013) or the need for nutrient compensations (Paskuliakova et al., 2016a; Pereira et al., 2016) to facilitate growth of the microalgal cells. It is therefore sometimes a requirement to add certain chemicals to overcome these limitations. Whilst this can initially increase the pollution load, it also aids the overall remediation process by overcoming the

limitations identified. Toxicological assays can be subsequently employed to demonstrate that the treatment process not only removes specific pollutants of interest but also reduces the overall ecotoxicity of treated wastewaters. This has previously been demonstrated for landfill leachate in small scale microalgae-based remediation experiments (Lin et al., 2007; Kumari et al., 2016).

Associating microalgal remediation to biomass valorisation for biofuel production has been suggested to increase the economic viability of 3rd generation biofuels (Pittman et al., 2011). Several options for energy generation from microalgal biomass have been described depending on its composition (Brennan and Owende, 2010; Juneja et al., 2013). The use of microalgal biomass grown on possibly toxic wastewaters such as landfill leachate for other than bioenergy purposes is however limited due to the possible accumulation of toxicants within the biomass.

Phosphate supplementation appears to be essential for the successful use of microalgae in the treatment of landfill leachate (Paskuliakova et al., 2016a). The present study compares phosphoric acid to dipotassium hydrogen phosphate for their suitability as a phosphorus source for microalgal growth in landfill leachate. In addition to nutrient reduction, the remediation capability of *Chlamydomonas* strain SW15aRL was evaluated by assessing the toxicity of the leachate, which was determined both pre- and post-treatment across several trophic levels. The lipid content achieved in the microalgal biomass was also determined post treatment in order to verify its potential for possible conversion into bioenergy commodities.

5.2 Materials and methods

5.2.1 Landfill leachate

The landfill leachate samples S7 and S8 were collected in October 2015 and January 2016, respectively, from a site in the Republic of Ireland and stored at <5°C until use.

5.2.2 Physicochemical analyses

Physicochemical properties were determined according to published methods (APHA, 2005). Nutrient profiles (PO_4^{3-}-P, TON, TAN, Cl^-, SO_4^{2-}) were determined spectrophotometrically with the Aquakem 250 autoanalyser on samples passed through 0.45 µm filters (VWR, Cat. No. 28145-503) prior to analysis. Conductivity (using HACH conductivity meter sensION5) and pH (using Metrohm 713 pH Meter) were measured electrochemically. Lovibond® test discs were used to categorise the colour of the samples after filtration through 0.45 µm filters. Alkalinity was determined titrimetrically with 0.1N HCl to pH 4.5 (using Metrohm 713 pH Meter). The metal profiles were determined on both, the raw leachates and leachates filtered through 0.7 µm (VWR, Cat. No. 516-0345) glass filters given that all the leachate samples were filtered through 0.7 µm glass filters prior to microalgae remediation experiments. Leachate samples were processed by microwave digestion (Milestone Ethos Plus) with HNO_3 (ROMIL-UpA^{TM}) according to Method 3015A (US EPA, 2007) prior to trace element analysis. Several trace elements (i.e. Fe, Mn, Zn, Co, Cu, Mo, Al, Cr, Ni, Cd, Pb) were determined by inductively coupled plasma mass spectrometry (ICP-MS, Varian 820). Major elements (i.e. Ca, Na and K) were determined by flame photometry (Sherwood 360) while Mg was determined by flame Atomic Absorption Spectroscopy (Agilent 200 AA). Suspended solids were quantified gravimetrically by filtering a known volume of sample through a 0.7 µm glass filter and drying it at 105°C until constant weight. Chemical Oxygen Demand (COD) was determined spectrophotometrically after sample digestion with HACH Lange Ltd test kits.

The variation in pH during the experiments was estimated with small aliquots of culture using pH indicator strips (Merck MColorpHast™ pH 5.0-10, pH 7.5-14, Δ0.5 pH, Dosatest® pH 7.0-10.0, Δ0.3 pH).

5.2.3 Microalgal strain

The *Chlamydomonas* sp. strain SW15aRL (previously isolated from a sample of raw leachate in 2014 from a landfill site in Northern Ireland) was maintained in raw

leachate or diluted raw leachate samples with a phosphate concentration adjusted to a molar N:P ratio ~16:1 prior to the experiments.

5.2.4 Growth in leachate S7 with two different phosphate sources

Leachate S7 was filtered through a 1.2 µm glass filter (VWR, Cat. No. 516-0869) followed by filtration through a 0.7 µm glass filter (VWR, Cat. No. 516-0345). Dilution to 30% with autoclaved deionised water was carried out to decrease the inhibitory effect of TAN on microalgal growth. The experiment was set up in triplicate in 250 ml conical flasks stoppered with cotton plugs and covered by tin foil. The total volume of leachate-microalgal mixture was set to 150 ml and the flasks were incubated stationary, homogenised only for sampling at intervals to monitor nutrient depletion and microalgae growth. The starting cell concentration was ~100 000 cells ml^{-1}. Phosphate was added in the form of dipotassium hydrogen phosphate (K_2HPO_4) and phosphoric acid (H_3PO_4) in the same concentration and molar ratio ~ N:P=16:1 in the final volume, giving ~100 mg l^{-1} NH_4^+-N and 18 mg l^{-1} PO_4^{3-}-P content. For comparison, experimental controls were set up to account for changes in nutrient content otherwise occurring in the leachate substrate. These were prepared in the same manner as described above but the microalgae cells were not added.

5.2.5 Phycoremediation experimental set up in leachate S8

The raw leachate S8 was filtered through a 1.2 µm glass filter (VWR, Cat. No. 516-0869) followed by filtration through a 0.7 µm glass filter (VWR, Cat. No. 516-0345). The phycoremediation was conducted in six 250 ml conical flasks stoppered with cotton plugs and covered by tin foil. The total volume of leachate-microalgal mixture was 150 ml and the flasks were incubated stationary, homogenised only for sampling at intervals to monitor the nutrient depletion and microalgae growth. The starting cell concentration was ~280 000 cells ml^{-1}. A complementary set of six flasks was set up as a control where no microalgae were inoculated. The raw leachate (January 2016) was diluted to 60% with autoclaved deionised water to

adjust the ammonia nitrogen to ~150 mg l^{-1} NH_4^+-N. Phosphate (as H_3PO_4) was added to achieve the concentration of 22 mg l^{-1} for a final N:P molecular ratio 16:1.

5.2.6 Biomass and precipitate dry weight determination

The microalgal dry weight together with the amount of precipitate formed was determined by total suspended solids (TSS) gravimetric quantification by filtering a known volume of sample through 0.7 µm glass filters (VWR, Cat. No. 516-0345) and drying them at 105°C until constant weight. As attempts to wash the biomass did not result in complete removal of precipitates in previous laboratory experiments (data not shown), the results of biomass/precipitate and precipitate determined for the microalgae treated leachate and the controls, respectively, are both indicated to highlight the amount of precipitate associated with the biomass.

5.2.7 Lipid determination

Lipid content within the biomass was quantified using the sulpho-phospho-vanillin colorimetric method according to Mishra et al. (2014). Analysis was carried out on a portion of microalgal culture volume with known TSS content to convert the lipid mass concentration into percentage of DW.

5.2.8 Elemental analysis of the suspended matter

The solids from the phycoremediation experiments in 5.2.4 and 5.2.5 with *Chlamydomonas* sp. SW15aRL were concentrated by centrifugation at 5000×*g* and freeze dried. The matter from all the replicates was then pooled. Elemental analysis testing of C:H:N percentages in the dry weight was subcontracted to UCL, School of Pharmacy, London. Analysis was conducted in duplicate.

5.2.9 Toxicological testing

Toxicological testing was carried out on non treated 60% raw leachate and microalgae-treated leachate as well as the controls that were set up alongside the

experiment described in section 5.2.5. All the samples for the toxicological studies were filtered through 0.7 µm glass filters (VWR, Cat. No. 516-0345). Volumes from the replicates were combined and aliquots were used according to the requirements of the toxicological test.

The toxicological tests performed spanned several trophic levels (bacteria/yeast, protists/microalgae, invertebrates and higher plants).

5.2.9.1 Microbiological testing

The commercial test kit (MARA), comprising 10 bacterial species and one yeast species was used for toxicity screening of undiluted samples and was conducted following the manufacturer's instructions. Testing was conducted in duplicate. The samples tested were the original 60% raw leachate, the leachate treated with strain SW15aRL after 35 days, and the 60% raw leachate supplemented with phosphate but without microalgae (control treatment) after 35 days.

5.2.9.2 Microalgae growth inhibition test

Microalgal growth inhibition toxicity test was based on the OECD Guideline 201 (OECD, 2011). The test strain used was *Chlorella* sp. OT10aTL, which was not selected for remediation studies due to its weaker performance in previous leachate tolerance experiments in comparison to other strains (Paskuliakova et al., 2016b). This species, although not listed in the OECD guideline, was deemed fit for the purpose of toxicity studies as it satisfied the growth criteria of the guideline. These were determined in OECD medium and are as follows: 1) growth rate 1.1±0.2 day^{-1} with coefficient of variation 21% between the section by section growth rates measured on a daily basis; and 2) the coefficient of variation of the average specific growth rate of 1.3% under the test conditions (21°C and continuous light with intensity of 8500 lux over 72 hrs). The suitability of the strain was established prior to its use for this test in 24 well plates and with four replicates. The linear relationship between the measured parameter (cell number) and dry weight over the used range was also confirmed ($R^2 = 0.994$) as required by the OECD guideline.

The specific growth rate of the microalgae strain in the OECD medium was compared to that in 60% raw leachate and the leachate treated by strain SW15aRL as well as the control which was not treated using microalgae cells. The experiment was set up in 24 well plates with three replicates for each substrate at the same temperature and light conditions as noted above, over a 72 h test period and with a starting cell concentration of ~28 000 cell ml^{-1}.

Biomass increases were monitored through cell counts by focal view in 96 well plates at ×200 magnification by inverted microscopy, which allowed for the removal of minimal amount of culture from the experiment. Prior to the tests, the focal view counts method was also compared to counts achieved using a haemocytometer with the same strain and the correlation between the two methods (R^2=0.88, n(pairs) = 38) was established as satisfactory.

5.2.9.3 Terrestrial Plant Test

The toxicity to higher plants was assessed according to the protocol developed for the PHYTOTOXKIT (MicroBio Tests Inc) with some adjustments as described in the following. This test evaluates seedlings emergence based on comparing roots and shoot length of three higher plants. For the purpose of this work one dicotyl plant *Lepidium sativum* (garden cress) and two monocotyl plants *Allium cepa* (onion) and *Secale cereale* (Rye) were used as suggested in the Annex 2 OECD Guideline for the Testing of Chemicals; 208: Seedling emergence and Seedling Growth Test (OECD, 2006). These seeds are capable of short germination times under the experimental conditions outlined for this test. The leachate substrates that were tested, and tap water used as control, were mixed with soil (air dried at 105°C, sieved through 2 mm, Water Holding Capacity was determined as described in the PHYTOTOXKIT protocol) and placed into containers in which the seeds were allowed to germinate. Ten seeds of each species for each tested substrate were used, each in triplicates. For the test to be valid at least eight of the control seeds had to emerge. The percentage of growth reduction of the shoots and roots was calculated in comparison to the controls. The assay was conducted in a controlled temperature environmental chamber at 25°C in the dark for three days.

5.2.9.4 Crustaceans acute immobilisation test

The DAPHTOXKIT F^{TM} test kit (MicroBio Tests Inc.) was used and the protocol followed for this test complied with ISO norm 6341 and OECD Guideline for Testing of Chemicals; 202 (OECD, 2004). It assessed the immobilisation of young daphnids (<24 hours, *Daphnia magna*) after 24 hours in landfill leachate and its four dilutions (ranging from 100% to 6.5%) plus the control. The results were analysed to estimate the EC50 at 24h. This assay is typically conducted with four replicates for every tested substrate and the effect in the control must not be higher than 10% for a test to be deemed valid.

5.2.9.5 Rotifer, 24 h mortality test

The test kit Rotoxkit F (MicroBio Tests Inc.) is an assay adhering to the ASTM Standard Guide E1440-91. It was used to evaluate the mortality of the rotifer *Brachionus calyciflorus* after 24 hours. The assay was conducted in the three leachate substrates tested as per section 2.9 with four dilutions (ranging from 100% to 6.5%) and a control. This was followed by determining the lethal concentration 50 (LC50) at 24h. The assay was conducted with 6 replicates with the effect in the control needing to be less than 10% for the test to be valid.

5.2.10 Statistical analysis

Data were analysed using IBM SPSS Statistics 22 package. One-way ANOVA (Tukey and Games-Howell where equality of variances could not be assumed) was used to compare the means of the different groups. Paired t-test (2-tailed) was used to compare changes in parameters pre- and post- treatment. Independent t-test (2-tailed) was applied to compare the effects of two different treatments.

The results for EC50 were determined for the toxicity tests that were conducted across a range of concentrations (Rotifer, 24h mortality test, Crustaceans acute immobilisation test). The dose response curves were visualised with the one-way spline add-on function in MS Excel.

5.3 Results

5.3.1 Physicochemical parameters of leachate samples

The physicochemical properties of the leachates used for the experiments are summarised in Table 5.1. The two samples were relatively weak in terms of typical leachate composition, with TAN below 400 mg l^{-1}, conductivity less than 6 mS cm^{-1} and COD less than 500 mg l^{-1} yet still substantially polluted from a treatment point of view. Dilution of 30% and 60% for S7 and S8 respectively were used for the microalgal treatments.

Table 5.1. Physicochemical parameters of the raw leachate samples used in the study

Parameter	Units	Leachate S7 (not diluted)	Leachate S8 (not diluted)
pH		7.0	7.6
Conductivity.	**mS cm^{-1}**	5.63	4.08
Colour	**PCU**	800-900	500-600
TAN	**mg l^{-1}**	367	261
TON	**mg l^{-1}**	89	0.1
PO_4^{3-}-P	**mg l^{-1}**	0.9	1.2
molecular N:P	**mol:mol**	1120:1	469:1
COD	**mg l^{-1}**	469	290
Suspended solids	**mg l^{-1}**	64	72
Alkalinity ($CaCO_3$)	**mg l^{-1}**	618	1180
Chlorides	**mg l^{-1}**	546	340
Sulphates	**mg l^{-1}**	177	137
Ca	**mg l^{-1}**	240	135
Mg	**mg l^{-1}**	41	28
Na	**mg l^{-1}**	508	336
K	**mg l^{-1}**	254	182
Fe	**mg l^{-1}**	6.8 (*3.0)	2.1 (*0.6)
Mn	**mg l^{-1}**	>2.0	0.6 (*0.3)
Zn	**mg l^{-1}**	0.2	<0.1
Co	**mg l^{-1}**	<0.1	<0.1
Cu	**mg l^{-1}**	<0.1	<0.1
Mo	**µg l^{-1}**	nd	nd
Al	**µg l^{-1}**	420 (*<100)	200 (*<100)
Cr	**µg l^{-1}**	<100	<100
Ni	**µg l^{-1}**	<100	<100
Cd	**µg l^{-1}**	nd	<10
Pb	**µg l^{-1}**	nd	nd

nd = not detected
* concentration of metals in cases where filtration through 0.7 µm glass membrane caused more than 25% reduction

5.3.2 Comparison of microalgal growth and nutrient reduction using two different inorganic phosphorus compounds

The growth of strain SW15aRL in leachate S7 at 30% dilution and supplemented with either potassium hydrogen phosphate (K_2HPO_4) or phosphoric acid (H_3PO_4) is displayed in Figure 5.1. There was a moderately significant difference (independent t-test, $p=0.051$) between the biomass obtained depending on the phosphorus compound used and no significant difference in the amount of precipitate formed in controls (Appendix D: Table S1) (independent t-test, $p>0.05$). There was however a significant (independent t-test, $p<0.05$) difference in the maximum number of cells achieved throughout the experiment between the two treatments (Figure 5.1) but no significant difference in the growth rates (independent t-test, $p>0.05$). The progress of nutrient reduction is displayed in Figure 5.1 and the overall results are summarised in Appendix D: Table S2. There was no significant difference (independent t-test, $p>0.05$) between the amount of TAN reduced between the two experiments using microalgae nor was there any difference (independent t-test, $p>0.05$) between TAN losses in the corresponding controls between day 0 and 40. This represented ~98% TAN removal within 30 to 35 days in the microalgae-treated leachate. A portion of TAN in the controls became oxidised following day 30, resulting in an average increase in TON by 26 and 16 mg l^{-1} in the K_2HPO_4 and H_3PO_4 supplemented controls, respectively. There was no significant (paired t-test, $p>0.05$) change between the initial and final concentration of oxidised nitrogen in the treatments with microalgae. Finally, there was no significant (independent t-test, $p>0.05$) difference in cell lipid content between the two treatments on day 40, which was ~10% DW.

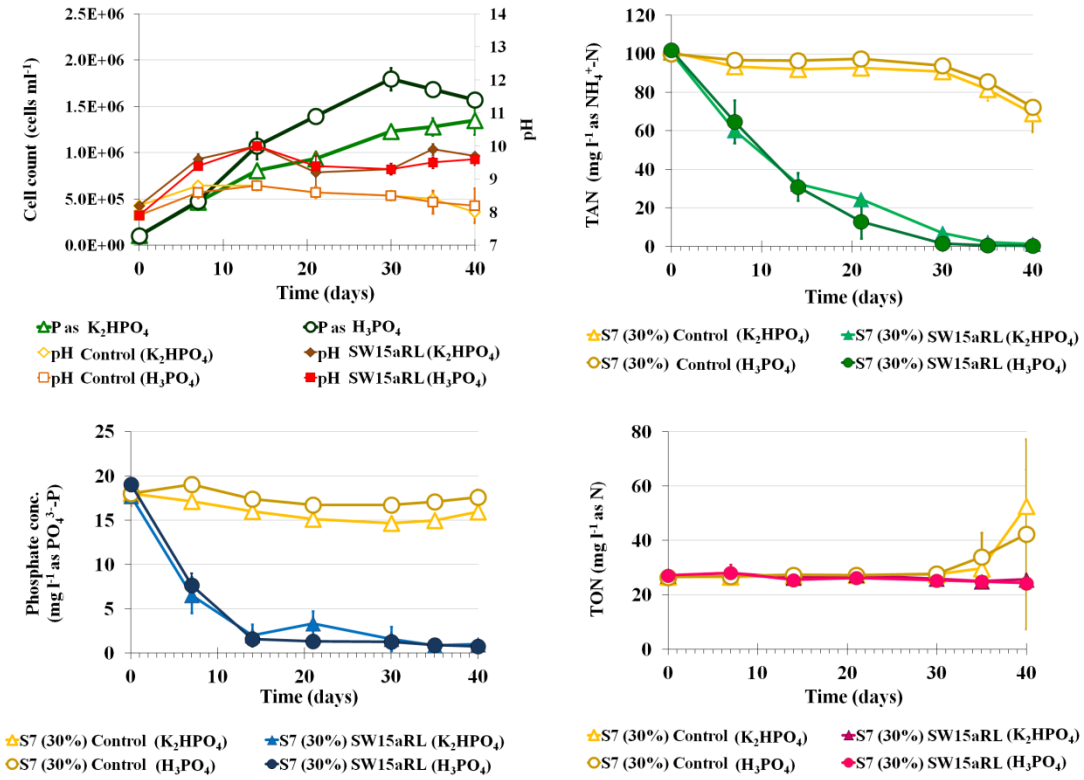


Figure 5.1. Comparison of the *Chlamydomonas* sp. SW15aRL growth in a leachate sample S7 as 30% dilution with two different phosphate compounds and corresponding nutrient decreases. Values are averages of three replicates ± std.

5.3.3 TAN and phosphate removal in S8 (60%) by *Chlamydomonas* sp. SW15aRL

The growth of strain SW15aRL during the phycoremediation experiment in 60% leachate S8 and the corresponding reduction of major nutrients are displayed in Figure 5.2. The overall data for changes in nitrogenous compounds and phosphate are summarised in Appendix D: Table S3.

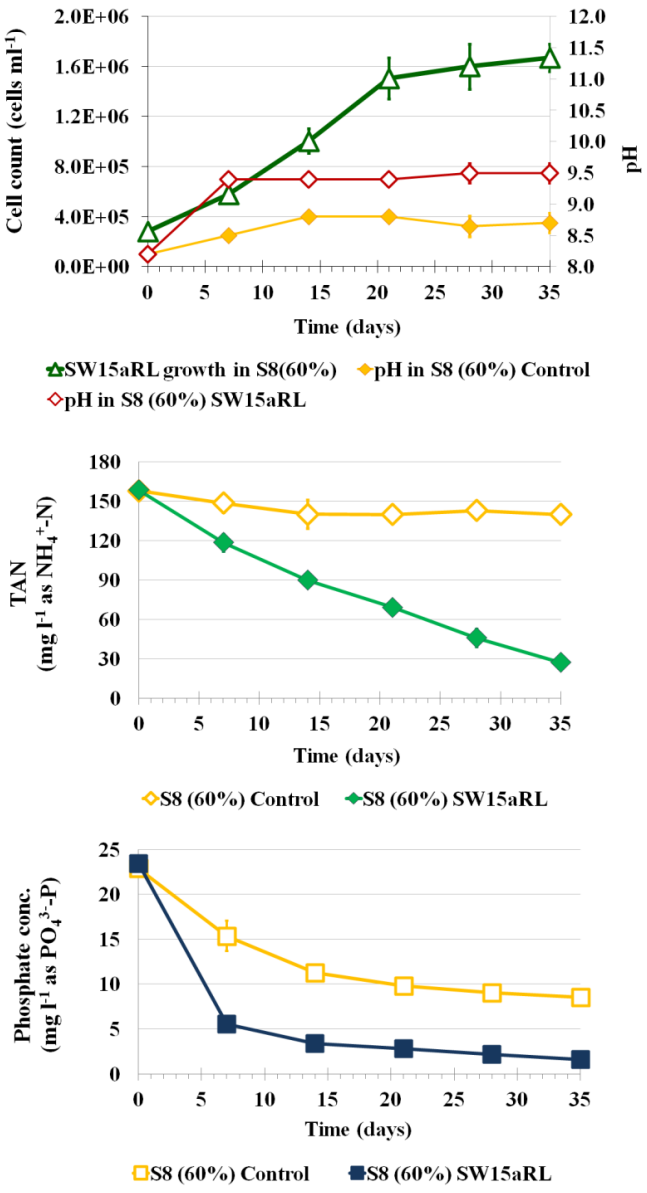


Figure 5.2. Growth curve of *Chlamydomonas* sp. SW15aRL in leachate S8 (60%) and corresponding changes in pH and nutrient concentration during the experiment. Values are average of three replicates ± one std.

Over 83 % (131 mg l^{-1}) of ammonia nitrogen was reduced during the remediation experiment in 35 days while 11% (18 mg l^{-1}) was lost in the controls. Phosphate decreased within the first ~14 days of the experiment substantially, not so rapidly thereafter, coinciding with the formation of precipitates in the solution. There was no significant difference in TON concentration at the start and end of the experiment in

the microalgae treated leachate (paired t-test, $p>0.05$) while there was a minor increase yet statistically significant (paired t-test, $p<0.05$) of 2.5 mg l^{-1} in TON concentration in the control.

Strain *Chlamydomonas* sp. SW15aRL reached the stationary phase near day 21 with an average growth rate 0.08±0.01 day^{-1}. The amount of biomass and precipitated matter obtained was 1.21±0.07 g l^{-1} while precipitated matter alone represented 0.12±0.00 g l^{-1} in the control without microalgae. The amount of lipid estimated at the end of the experiment represented 18±1 % dry weight which was significantly (one-way ANOVA, $n=9$, $p<0.05$) higher than the lipid content achieved when treating leachate S7 as outlined in Section 5.3.2.

5.3.4 Efficiency of nitrogen uptake and losses during the remediation with microalgae

In addition to the overall nitrogen concentration changes (TAN and TON) determined in the leachate, the amount of nitrogen that was actually integrated into biomass was established by the elemental analysis of the dried suspended solids for experiments 5.3.2 and 5.3.3 with microalgae (Appendix D: Table S4). Consequently the amount of nitrogen that was possibly volatised could be estimated. Nitrogen redistribution is indicated in Figure 5.3 and summarised in Appendix D: Table S5.

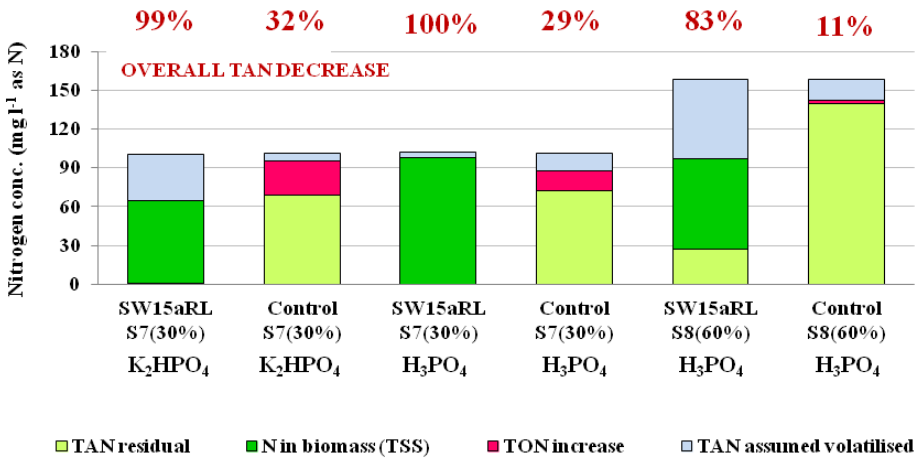


Figure 5.3. TAN reduction during remediation with microalgae and distribution of nitrogen at the end of experiments expressed as N in mg l^{-1}. TAN changes in controls are also included.

5.3.5 Toxicological evaluation of microalgal remediated leachate S8 (60%)

5.3.5.1 Microbiological testing

The results from microbiological toxicity screening of the three leachate substrates are displayed in Figure 5.4. The 60% raw leachate S8 and the microalgae-treated leachate showed similar profiles and were of low toxicity. The most sensitive organisms were number 9 and 11 in the case of the treated leachate while the growth of the other microorganisms was substantially stimulated by the nutrients present in the leachate. The experimental control with added phosphate was however toxic to a number of microorganisms (especially numbers 1, 4, 9 and 10).

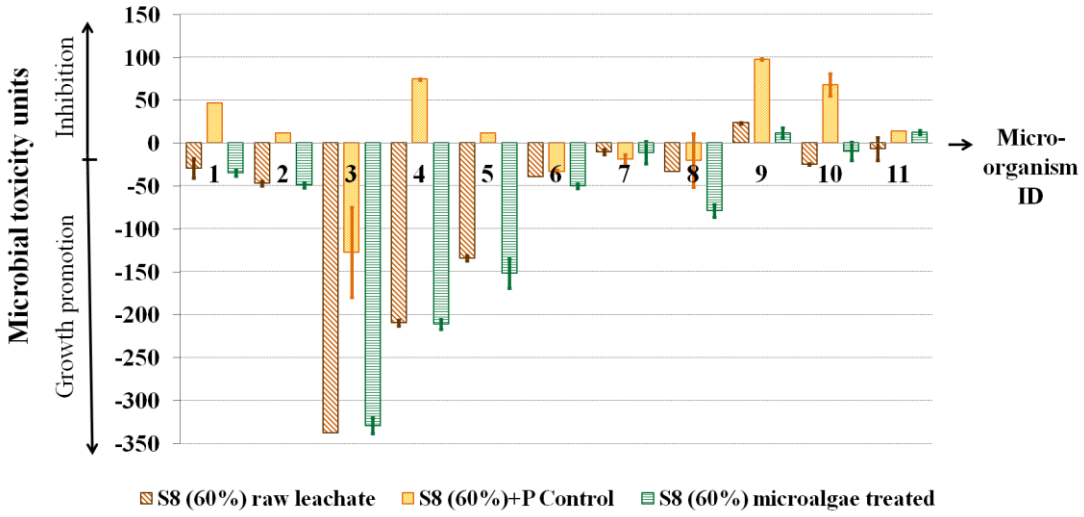


Figure 5.4. MARA toxicity screening results of undiluted leachate smaples. The results are average of two ± std.

5.3.5.2 Microalgae inhibition test

The growth data measured in the microalgae inhibition test are summarised in Appendix D: Table S6. There was no observed growth inhibition in comparison to the control (OECD media). On the contrary, growth promotion was observed in

increasing order from “60% S8” to “60% S8+P Control” to the highest in “60% S8+P treated with microalgae” with significant differences between all (one-way ANOVA, n=12, p<0.05). The progress of growth stimulation in the tested leachate samples is depicted in Figure 5.5.

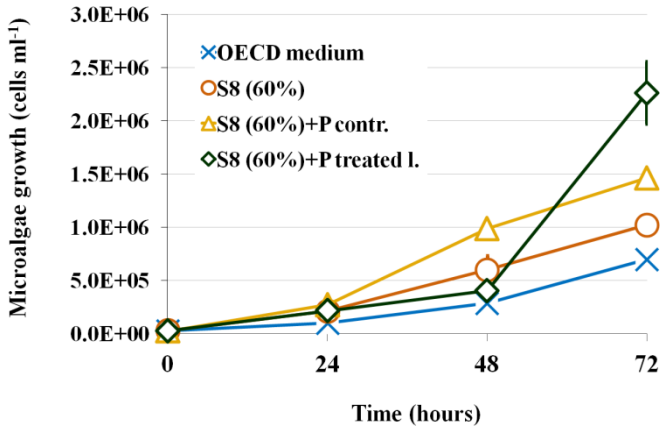


Figure 5.5. Growth curves of *Chlorella* sp. OT10aTL in the microalgae growth inhition toxicity test.

5.3.5.3 Terrestrial Plant Test

The results for the germination and the root and shoot lengths of one dicot (cress) and two monocot (onion and rye) plants are displayed in Figure 5.6.

There was no significant (one-way ANOVA, n=12, p>0.05) effect of any of the examined substrates, whether treated or untreated, in comparison to water as control on the germination of Rye and Cress.

The germination of onion seeds in the treated leachate was significantly (one-way ANOVA, n=12, p<0.05) lower by 37% in comparison to the control but there was no significant (one-way ANOVA, n=12, p>0.05) difference in onion root length in germinated seeds between the different substrates.

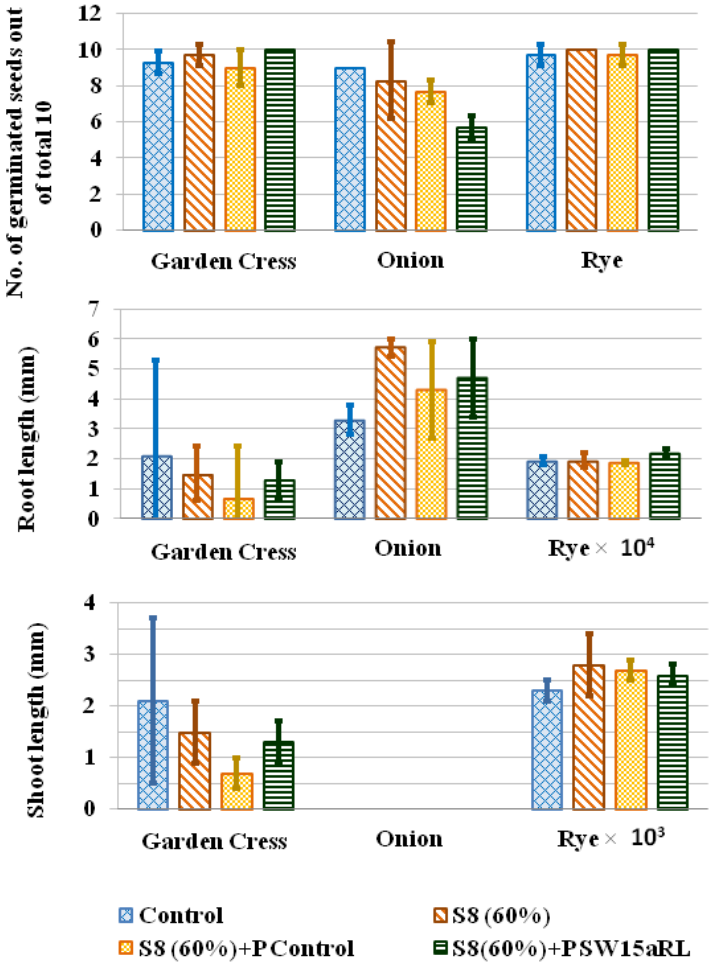


Figure 5.6. Results for toxicological test with higher plants. The values are averages of three experimental replicates ± std.

5.3.5.4 Crustaceans acute immobilisation test and Rotifer, 24 h mortality test

The dose-response curves for the individual invertebrate assays are displayed in Figure 5.7. Pronounced reduction in toxicity was observed for both invertebrate models. It was lowered to well below the EC50 for the microalgae-treated leachate in comparison to the non treated 60% raw leachate or the experimental control, which had an EC50 within the range of 35 to 45% for the crustacean and rotifer assays.

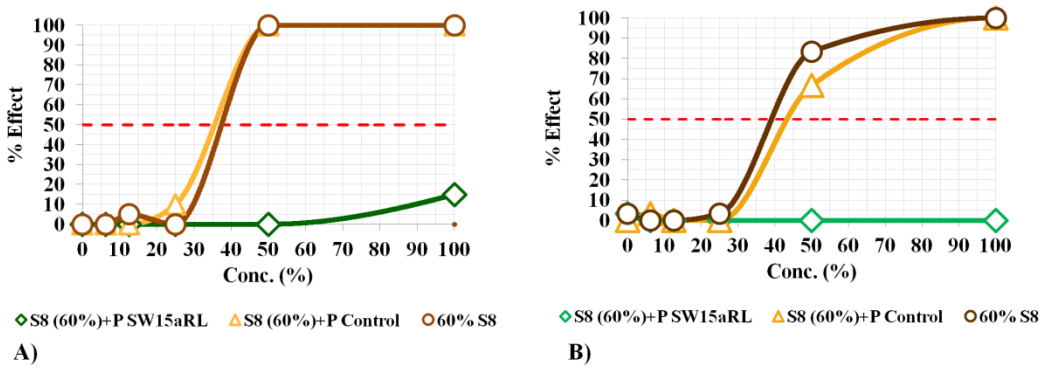


Figure 5.7. Invertebrate dose response curves A) Crustaceans acute immobilisation test at 24 h and B) Rotifer, 24 h mortality test.

5.4. Discussion

The treatment of landfill leachate with microalgae has been demonstrated in laboratory experiments but knowledge in this area is still relatively limited and improvements are sought. Although microalgae can efficiently reduce inorganic substances, especially TAN and minerals, the complex nature of landfill leachate makes the monitoring of individual pollutants and/or their degradation products difficult and expensive. Toxicological testing at a number of trophic levels was used to assess the efficacy of the microalgal treatment of landfill leachate.

5.4.1 Effect of phosphorus source on microalgal remediation

Addition of hydrochloric acid to decrease pH has previously been shown to be positive for the growth of microalgae in landfill leachate (Edmundson and Wilkie, 2013). As the addition of phosphate also appears to be important to balance high N:P ratio in landfill leachate (Paskuliakova et al. 2016a; Pereira et al. 2016), phosphoric acid (H_3PO_4) was substituted for dipotassium hydrogen phosphate (K_2HPO_4) to verify if it would offer any advantage. The experiment showed no major difference in growth data or overall nutrient reduction between the two phosphate compounds used. Phosphoric acid was then further used for the remediation of leachate S8.

5.4.2 Landfill leachate nutrient reduction during phycoremediation

The applicability of landfill leachate treatment with microalgae has its constraints, one being the dilutions required, sometimes up to 10% for strong landfill leachates (Lin et al., 2007; Cheng and Tian, 2013; Thongpinyochai and Ritchie, 2014; Zhao et al., 2014; Sforza et al., 2015; Paskuliakova et al., 2016a). Previous work carried out with *Chlamydomonas* sp. strain SW15aRL has shown that some landfill sites can produce leachates that are of relatively lower strength and do not require such high dilutions. Based on the variability over the year, the dilutions of 30% to undiluted raw leachate could be used with this strain (as per results in Chapter 4).

TAN reduction in the diluted landfill leachates S7 and S8 ranged between ~80% and almost 100%, which is consistent with the performance of *Chlamydomonas* sp. SW15aRL in previous study (Paskuliakova et al., 2016a). In comparison to other studies the time required here to observe substantial reduction in TAN and microalgal growth is quite lengthy (Lin et al., 2007; Cheng and Tian, 2013; Thongpinyochai and Ritchie, 2014; Zhao et al., 2014; Sforza et al., 2015) yet consistent with previous results under the conditions used. Other microalgae based remediation studies with other wastewaters have reported much shorter nutrient reduction times. These are usually reported from countries with warm climates where experimental temperatures range between 25°C and 30°C (Chokshi et al., 2014; Prajapati et al. 2014; Choudhary et al.; 2016). This might not be achievable naturally in countries with temperate climates without employing engineered systems with extra energy inputs.

Although the initial TAN concentration in S7 (30%) was 50% lower than in S8 (60%), the biomass obtained on the two leachates was similar. Elemental analysis was used to provide insights into how much nitrogen was incorporated into the biomass and other aggregates collected by centrifugation. Although microorganisms and microalgae can produce extracellular nitrogen containing organic substances, ammonia volatilisation is a more likely route of loss. Elemental analysis of the biomass grown in leachate with a TAN concentration of ~100 mg l^{-1} showed that the loss of ammonia by volatilisation in the treatment with microalgae was apparently higher when dipotassium hydrogen phosphate was used and similar to that in the corresponding controls without microalgae. However, it should be noted that some

of the reduced TAN in the controls was actually oxidised into TON. At a TAN concentration in the leachate of ~160 mg l^{-1} ammonia loss in the microalgae treated test (39%) exceeded that in the control (11%). Volatilisation in the microalgae treated leachate S8 was likely to be enhanced by increased pH due to microalgal activity and this effect could be more pronounced at higher leachate/TAN concentration when compared with S7 leachate. Ammonia stripping during microalgal treatment has also been observed by Zhao et al., (2014) where just 52% of the TAN reduced in 10% landfill leachate was apparently biologically absorbed.

In contrast to leachate S7 treated with microalgae there was formation of oxidised nitrogen in the experimental controls. The TON increase coincided with a decrease of TAN after day 30 in the controls. This was likely due to developing nitrifying microbial community (small sized, <0.7 μm) which could possibly have been suppressed in the environment containing the microalgae.

At lower concentration of leachate S7 (30%) the added phosphate in the controls remained stable. The phosphate concentration reduction in the controls in the experiment with S8 (60%) coincided with the formation of precipitates. Precipitate formation has been reported in microalgal treatment with another wastewater at alkaline pH. It consisted mostly of calcium carbonate with small amounts of phosphorus, nitrogen and magnesium (Abou-Shanab et al., 2013). Results for metal reduction during the remediation experiment with S8 (60%) showed that metals were not removed entirely due to microalgal assimilation but also due to precipitation and/or adsorption, as their concentration was also lowered in the control treatment with no microalgae (data not displayed). This suggests that precipitation could contribute to reducing nutrient bioavailability for microalgae cells and hence result in premature growth inhibition. Landfill leachate dilution for phycoremediation might be beneficial not just for lowering the toxic effect of TAN but also to help maintain other nutrients in the solution for balanced growth.

5.4.3 Biomass quantification

Increasing needs in the production of renewable energy (Murphy et al., 2013) have also placed focus on algae as a source of 3rd generation biofuels. Growing microalgae on wastewaters has fostered renewed interests in this area. In the present

study, it was established that up to 1.2 g l^{-1} of microalgal biomass could be produced on diluted landfill leachate in a batch culture mode. The lipid content of such biomass varied between 10–18% dry weight for *Chlamydomonas* sp. strain SW15aRL, which is lower than the values reported in other similar remediation attempts by Zhao et al. (2014) with microalgae *Chlorella pyrenoidosa* (14.5–20.8%) or Sforza et al. (2015) with *Acutodesmus obliquus* (38-48%). Although some microalgae can accumulate large amounts of lipids, these are often under controlled conditions which might not be feasible in remediation applications where wastewater composition can be variable. Indeed, while some microalgae species are known to accumulate as much lipid as ~60% DW (Mata et al., 2010), microalgae produced on wastewater accumulate lipids mostly in the region of 25% DW (Pittman et al., 2011).

5.4.4 Toxicological evaluation

The possibility of generating toxic by-products and degradation compounds in the treatment processes exists, as outlined in several studies, including in bioremediation (Stalter et al., 2010; Watson et al., 2012; Chibwe et al., 2015).

The landfill leachate S8 used in the present study was diluted to 60% (corresponding to TAN concentration of ~160 mg l^{-1}) prior to remediation due to the inhibitory effects on microalgal growth typically observed at higher leachate concentration (Lin et al., 2007; Cheng and Tian, 2013; Thongpinyochai and Ritchie, 2014; Zhao et al., 2014; Sforza et al., 2015). At this dilution, S8 leachate was found to be of low to no toxicity in a number of bioassays and similar to that of the corresponding microalgae treated leachate. However, the treated leachate appeared to have a slightly toxic effect on the germination of onion seeds. The 60% raw leachate S8 was particularly toxic to invertebrates, which could be mainly attributed to TAN. This is based on the observed reduction in toxicity well below the EC50 for the microalgae treated leachate, while the experimental control supplemented with phosphate still exhibited high toxicity comparable to or slightly higher than that of the 60% raw leachate.

The 60% S8 leachate did not cause significant inhibition of the microalgae *Chlorella* sp. OT10aTL compared to the toxicity assay control. However, it was obvious that its growth was inhibited in comparison to the microalgae treated leachate. This appeared to be both through nutrient limitation and inhibition based on the fact that

the 60% S8 leachate supplemented with phosphate supported higher growth than the 60% S8 substrate but was lower than the microalgae treated leachate. The growth rates of microalgae in landfill leachates rarely reach 0.80 day^{-1} (e.g. 0.83 day^{-1} for *Scenedesmus* cf. *rubescens* - Edmundson and Wilkie (2013)). The *Chlorella* sp. strain OT10aTL was chosen for toxicity studies due to its lesser tolerance to landfill leachates compared to *Chlamydomonas* sp. SW15aRL in previous screening tests at low light and temperature (Paskuliakova et al. 2016b). In spite of this, it achieved high growth rates (1.2–1.5 day^{-1}) when higher temperatures and light intensity were employed. Other contributory factors to such growth rates are likely to be the small initial inocula and small volume of the 24 well plates for non-agitated cultures and also the fact that OECD nutrients were supplemented into each of the substrates tested.

Overall, the microalgae treatment contributed to the reduction of pollutant levels and ecotoxicity. The improvement in the quality of 20% leachate by phycoremediation over a 10 day period with a microalgae-bacterial co-culture has also been reported in Kumari et al., (2016), which used the Methyl Tetrazolium Assay for cytotoxicity and Alkaline Comet genotoxicity Assay on the hepatoma HepG2 human liver cancer cell line. Another study by Lin et al. (2007) involving the 4-week treatment of diluted leachate samples (10-50%) with microalgae (*Chlorella pyrenoidosa*) and the subsequent use of the seed germination test with *Brassica chinensis* also demonstrated a reduction in toxicity.

Phycoremediation is unlikely to be used as a standalone treatment and, depending on additional landfill leachate processing, complementary toxicity tests could be used to assess any other residual effects which might need to be mitigated. Several options exist. Comet assay is useful in genotoxicity testing and theoretically can use any eukaryotic cells that could be considered as target to the exposure of toxicant under investigation. Also fish cell lines can be employed in ecotoxicity tests where depending of experimental set up, different mechanism of action can be elucidated. Apart from a number of advantages at experimental level, these tests offer an alternative to unnecessary animal testing at higher trophic levels (Tice et al., 2000; Bols et al., 2005).

The toxicity of raw landfill leachates varies greatly depending on site and its environmental circumstances. Mostly they are very toxic (Brito-Pelegrini, 2007;

Płaza et al., 2011). Although the dilution in this study contributed to reduction in toxicity, the overall ecotoxicity seemed relatively low and mostly attributable to ammonia.

It is important to determine the potential toxicity of waste streams in the context of water quality and the biological diversity of the aquatic habitats into which the treated wastes are eventually discharged. Water quality in ecology is often determined by community composition and abundance of particular indicator species (Persoone and Gillett, 1990; Jackson et al., 2016). While some species can be suppressed upon the introduction of substances of anthropogenic origin, others can be stimulated. This was reflected here with the freshwater crustaceans being very sensitive to untreated leachate while the growth of the microalgae *Chlorella* sp. OT10aTL was enhanced. These contradictory effects can cause imbalances within ecosystems upon the release of inadequately treated wastewaters.

5.5 Conclusions

Using microalgae for the treatment of landfill leachate is possible but has its constraints. A limited amount of information is available to help understanding the requirements of the species used in this biological treatment method, such as nutrient balance. Microalgal bioremediation reduced ammonia nitrogen in this study by incorporation into biomass whilst a portion was also likely volatilised. Accumulation of oxidised nitrogen in the treated leachate was suppressed in the presence of microalgae. Toxicity reduction was also achieved by treating diluted landfill leachate with microalgae. Co-occurring precipitation also probably contributed to pollutant and toxicity reduction. Future work should focus on attempting to remove residual nutrients and optimising the dosage of phosphate.

5.6 Acknowledgements

This work has been supported by Institute of Technology Presidents Bursary Scheme. Also the assistance from the employees of the landfill site for providing landfill leachate is greatly appreciated.

5.7 References

Abou-Shanab. R.A.I., Ji, M.K., Kim, H.C., Paeng, K.J., Jeon, B.H., 2013. Microalgal species growing on piggery wastewater as a valuable candidate for nutrient removal and biodiesel production. Journal of Environmental Management 115, 257-264. http://dx.doi.org/10.1016/j.jenvman.2012.11.022.

APHA (American Public Health Association), 2005. Standard methods for the examination of water and wastewater, 21st ed. Washington, D.C.

Bernard, C., Guido, P., Colin, J., Anne, L.D.D., 1996. Estimation of the hazard of landfills through toxicity testing of leachates - I. Determination of leachate toxicity with a battery of acute tests. Chemosphere 33, 2303–2320. doi:10.1016/0045-6535(96)00322-0.

Bols, N.C., Dayeh, V.R., Lee, L.E.J., Schirmer, K., 2005. Chapter 2: Use of fish cell lines in the toxicology and ecotoxicology of fish. Piscine cell lines in environmental toxicology. Biochemistry and Molecular Biology of Fishes 6, 43-84. doi.org/10.1016/S1873-0140(05)80005-0.

Brennan, L., Owende, P., 2010. Biofuels from microalgae - A review of technologies for production, processing, and extractions of biofuels and co-products. Renewable and Sustainable Energy Reviews 14, 557-577. doi:10.1016/j.rser.2009.10.009.

Brito-Pelegrini, N.N., Pelegrini, R.T., Paterniani, J.E.S., 2007. Ecotoxicological evaluation of leachate from the Limeira sanitary landfill with a view to identifying acute toxicity. Ambi-Agua, Taubaté 2 (3), 34-43. doi:10.4136/ambi-agua.31.

Cecilia, B.O., Junestedt, C., 2008. Chemical characterization of landfill leachates – 400 parameters and compounds. Waste Management 28, 1876–1891. doi:10.1016/j.wasman.2007.06.018.

Cheng, H.-X., Tian, G.-M., 2013. Preliminary Evaluation of a Newly Isolated Microalga *Scenedesmus* sp. CHX1 for Treating Landfill Leachate. 2013 Third Int. Conf. Intell. Syst. Des. Eng. Appl. 1057–1060. doi:10.1109/ISDEA.2012.249.

Chibwe, L., Geier, M.C., Nakamura, J., Tanguay, R.L., Aitken, M. D., Simonich, S.L.M., 2015. Aerobic Bioremediation of PAH Contaminated Soil Results in Increased Genotoxicity and Developmental Toxicity. Environ Sci Technol. 49 (23), 13889–13898. doi:10.1021/acs.est.5b00499.

Chokshi, K., Pancha, I., Ghosh, A., Mishra, S., 2016. Microalgal biomass generation by phycoremediation of dairy industry wastewater: An integrated approach towards sustainable biofuel production. Bioresource Technology 221, 455–460. http://dx.doi.org/10.1016/j.biortech.2016.09.070.

Choudhary P., Prajapati, S.K., Malik, A., 2016. Screening native microalgal consortia for biomass production andnutrient removal from rural wastewaters for bioenergy applications. Ecological Engineering 91, 221–230. http://dx.doi.org/10.1016/j.ecoleng.2015.11.056.

Edmundson, S.J., Wilkie, A.C., 2013. Landfill leachate-a water and nutrient resource for algae-based biofuels. Environ. Technol. 34, 1849–57. doi:10.1080/09593330.2013.826256.

Hirooka, T., Akiyama, Y., Tsuji, N., Nakamura, T., Nagase, H., Hirata, K., Miyamoto, K., 2003. Removal of Hazardous Phenols by Microalgae under Photoautotrophic Conditions. Journal of Bioscience and Bioengineering 95, 200–203. doi:10.1016/S1389-1723(03)80130-5.

Jackson, M.C., Weyl, O.L.F., Altermatt, F., Durancek, I., Friberg, N., Dumbrell, A.J., Piggott, J.J., Tiegs, S.D., Tocknerkk, K., Krug, C.B., Leadley, P.W., Woodward, G., 2016. Chapter twelve: Recommendations for the Next Generation of Global Freshwater Biological Monitoring Tools. Advances in Ecological Research 55, 615-636. http://dx.doi.org/10.1016/bs.aecr.2016.08.008.

Juneja A., Ceballos, R.M., Murthy, G.S., 2013. Effects of Environmental Factors and Nutrient Availability on the Biochemical Composition of Algae for Biofuels Production: A Review. Energies 6, 4607-4638. doi:10.3390/en6094607.

Kumari, M., Ghosh, P., Thakur, I.S., 2016. Landfill leachate treatment using bacto-algal co-culture: An integrated approach using chemical analyses and toxicological assessment. Ecotoxicol. Environ. Saf. 128, 44–51. doi:10.1016/j.ecoenv.2016.02.009.

Li, R., Chen, G., Fung, N., Tam, Y., Luan, T., Shin, P.K.S., Cheung, S.G., Liu, Y., 2009. Toxicity of bisphenol A and its bioaccumulation and removal by a marine microalga *Stephanodiscus hantzschii*. Ecotoxicology and Environmental Safety 72, 321–328. doi:10.1016/j.ecoenv.2008.05.012.

Li, T., Lin, G., Podola, B., Melkonian, M., 2015. Continuous removal of zinc from wastewater and mine dump leachate by a microalgal biofilm PSBR. Journal of Hazardous Materials 297, 112–118. http://dx.doi.org/10.1016/j.jhazmat.2015.04.080.

Lima, S.A.C., Raposo, M.F.J., Castro, P.M.L., Morais, R.M., 2004. Biodegradation of p-chlorophenol by a microalgae consortium 38, 97–102. doi:10.1016/j.watres.2003.09.005.

Lin, L., Chan, G.Y.S., Jianga, B.L., Lan, C.Y., 2007. Use of ammoniacal nitrogen tolerant microalgae in landfill leachate treatment. Waste Management 27, 1376–1382. doi:10.1016/j.wasman.2006.09.001.

Mandal, S., Mallick, N., 2011. Waste Utilization and Biodiesel Production by the Green Microalga *Scenedesmus obliquus*. Applied and Environmental Microbiology 77, 374-377. doi:10.1128/AEM.01205-10.

Mata, T.M., Martins, A.A., Caetano, N.S., 2010. Microalgae for biodiesel production and other applications: A review. Renewable and Sustainable Energy Reviews 14, 217–232. http://dx.doi.org/10.1016/j.rser.2009.07.020.

Mishra, S.K., Suh, W.I., Farooq, W., Moon, M., Shrivastav, A., Park, M.S., Yang, J., 2014. Rapid quantification of microalgal lipids in aqueous medium by a simple colorimetric method. Bioresour. Technol. 155, 330–333. doi:10.1016/j.biortech.2013.12.077.

Murphy, F., Devlin, G., Deverell, R., McDonnell, K., 2013. Biofuel Production in Ireland - an Approach to 2020 Targets with a Focus on Algal Biomass. Energies 6, 6391-6412. doi:10.3390/en6126391.

OECD, 2004. Test No. 202: *Daphnia* sp. Acute Immobilisation Test, OECD Publishing, Paris. doi: http://dx.doi.org/10.1787/9789264069947-en.

OECD, 2006. Test No. 208: Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test, OECD Publishing, Paris. doi: http://dx.doi.org/10.1787/9789264070066-en.

OECD, 2011. Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test, OECD Publishing, Paris. doi: http://dx.doi.org/10.1787/9789264069923-en.

Paskuliakova A., Tonry, S., Touzet, N., 2016a. Phycoremediation of landfill leachate with chlorophytes: Phosphate a limiting factor on ammonia nitrogen removal. Water Research 99, 180–187. http://dx.doi.org/10.1016/j.watres.2016.04.029.

Paskuliakova A., Tonry, S., Touzet, N., 2016b. Microalgae isolation and selection for the treatment of landfill leachate. WIT Transactions on Ecology and The Environment, 209, 65-78. © 2016 WIT Press. ISSN 1743-3541. doi:10.2495/WP160071.

Pereira, S.F.L., Gonçalves, A. L., Moreira, F.C., Silva, T.F.C.V., Vilar, V.J.P., Pires, J.C.M., 2016. Nitrogen Removal from Landfill Leachate by Microalgae. Int. J. Mol. Sci. 17, 1926. doi:10.3390/ijms17111926.

Pinto, G., Pollio, A., Previtera, L., Stanzione, M., Temussi, F., 2003. Removal of low molecular weight phenols from olive oil mill wastewater using microalgae. Biotechnology Letters 25, 1657–1659. doi: 10.1023/A:1025667429222.

Pittman, J.K., Dean, A.P, Osundeko, O., 2011. The potential of sustainable algal biofuel production using wastewater resources. Bioresource Technology 102, 17–25. doi:10.1016/j.biortech.2010.06.035.

Płaza, A., Nałe, G., Ulfig, K., 2011. Estimation of the environmental risk posed by landfills using chemical, microbiological and ecotoxicological testing of leachates. Chemosphere 82, 1017–1023. doi:10.1016/j.chemosphere.2010.10.066.

Prajapati, S.K., Choudhary, P., Malik, A., Vijay, V.K., 2014. Algae mediated treatment and bioenergy generation process for handling liquid and solid waste from dairy cattle farm. Bioresource Technology 167, 260-268. http://dx.doi.org/10.1016/j.biortech.2014.06.038.

Sforza E., Khairallah Al Emara, M.H., Sharif, A., Bertucco A., 2015. Exploitation of urban landfill leachate as nutrient source for microalgal biomass production. Chemical Engineering Transactions, 43, 373-378. doi: 10.3303/CET1543063.

Stalter, D., Magdeburg, A., Weilb, M., Knackerb, T., Oehlmann, J., 2010. Toxication or detoxication? In vivo toxicity assessment of ozonation as advanced wastewater treatment with the rainbow trout. Water Research 44, 439-448. doi:10.1016/j.watres.2009.07.025.

Sutherland, D.L., Turnbull, M.H., Craggs, R.J., 2014. Increased pond depth improves algal productivity and nutrient removal in wastewater treatment high rate algal ponds. Water Res. 53, 271–281. doi:10.1016/j.watres.2014.01.025.

Tice, R. R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.-C., Sasaki, Y. F., 2000. Single Cell Gel/Comet Assay: Guidelines for In Vitro and In Vivo Genetic Toxicology Testing.

Environmental and Molecular Mutagenesis 35, 206-221. doi: 10.1002/(SICI)1098-2280(2000)35:3<206::AID-EM8>3.0.CO;2-J.

Thongpinyochai, S., Ritchie, R.J., 2014. Biodegradation Using *Chlorella vulgaris* to Decrease the Environmental Effect of Garbage Dump Leachates. J. Bioremed. Biodeg. 5 (5), 239-251. doi:10.4172/2155-6199.1000239.

Persoone, G., Gillett, J., 1990. Chapter 17: Toxicological versus Ecotoxicological Testing. Short-term Toxicity Tests for Non-genotoxic Effects. Edited by P. Bourdeau et al., Published by John Wiley & Sons Ltd.

US EPA 2007. Test Method 3015A, Microwave Assisted Acid Digestion of Aqueous Samples and Extracts, SW-846, US EPA.

Watson, K., Shaw, G., Leusch, F.D.L., Knight, N.L., 2012. Chlorine disinfection by-products in wastewater effluent: Bioassay-based assessment of toxicological impact. Water Research 46, 6069-6083. http://dx.doi.org/10.1016/j.watres.2012.08.026.

Yan, H., Pan, G., 2004. Increase in biodegradation of dimethyl phthalate by *Closterium lunula* using inorganic carbon. Chemosphere 55, 1281–1285. doi:10.1016/j.chemosphere.2003.12.019.

Zhao, X., Zhou, Y., Huang, S., Qiu, D., Schideman, L., Chai, X., Zhao, Y., 2014. Characterization of microalgae-bacteria consortium cultured in landfill leachate for carbon fixation and lipid production. Bioresour. Technol. 156, 322–328. doi:10.1016/j.biortech.2013.12.112.

Zhou, W., Li, Y., Min, M., Hu, B., Chen, P., Ruan, R., 2011. Local bioprospecting for high-lipid producing microalgal strains to be grown on concentrated municipal wastewater for biofuel production. Bioresource Technology 102, 6909–6919. doi:10.1016/j.biortech.2011.04.038.

Chapter 6
Overview Discussion

6.1 General Overview

Microalgae have attracted attention for the development of potential wastewater treatment technologies (phycoremediation) with the added benefit of using the obtained biomass as a valuable resource for energy production (Olguín 2003; Murphy et al., 2013). In phycoremediation, the reduction or transformation of inorganic or organic substances results from the activity and growth of microalgae and associated compatible bacteria (Zhao et al., 2014; Krustok, 2016). A variety of industrial and agricultural wastewaters has been studied with some promising results. Landfill leachate has however been less explored. Leachate accumulates at the bottom of landfills, which requires regular removal and appropriate management for decades even after the landfill site closure. It is an extremely complex type of wastewater saturated with decomposition products from a variety of materials deposited in the landfill, which makes it very difficult to treat. Although some of the already developed treatment technologies can be fairly effective, the combination of biological and physico-chemical processes is often used. Phycoremediation is considered as a complementary treatment option in this case for removing nutrients such as nitrogenous compounds. Prerequisite to exploring this type of treatment as a possible technological option is the isolation of suitable microalgae strains with tolerance to high ammonia nitrogen. Although several small scale studies have indicated that microalgae can use leachate as a source of nutrients, the conditions of these experiments did not reflect the realistic climatic conditions found in countries with temperate climates (Lin et al., 2007; Cheng and Tian, 2013; Edmundson and Wilkie, 2013; Zhao et al., 2014). Adopting more realistic conditions would allow a more appropriate assessment of the timescale required for treatment, or alternatively to assess the extra energy demand needed to achieve the desired treatment. Treatment timescale also relates to the volume that can be treated and consequently the footprint and design of possible up-scale facilities. Further knowledge gaps relate to treatment efficiencies and the repeatability or consistency of microalgal performance in relation to landfill leachate composition changes throughout the year

or from different sites. All these factors need to be taken into consideration for viability assessment or further research of this technology.

6.2 Overview of the main results and their significance

6.2.1 Isolation and selection of microalgae for phycoremediation experiments (Chapter 2)

Microalgae species were isolated from various environments and subjected to screening based on their tolerance to various leachate compositions and salt strength in order to find strains most suitable for the treatment of landfill leachate (Paskuliakova et al., 2016a). It was found that the applied screening procedure introduced a number of biases and proved more suitable for eliminating unsuitable species rather than necessarily selecting the best performing strains. This was also proved by the fact that the strain eventually selected for the phycoremediation experimentation was not in the highest ranking category during the screening tests. Although the conductivity tolerance tests indicated species adaptable to certain variable compositions which was a desired characteristic, the cultivation media used were also low in macronutrients, with nitrate being the sole source of nitrogen. On the other hand, the leachate tolerance screening clearly showed species absolutely not suitable for leachate treatment, which were either intolerant to high ammonia toxicity or the other present pollutants in conjunction with the overall nutrient profile (e.g. marine Cryptophytes and Diatoms). While some strains showed major growth in leachates of particular composition (e.g. some *Chlamydomonas* spp.), these strains had difficulties in adapting to changing composition, resulting in these two screening approaches yielding contradictory results. The species that performed well in both were generally universally well growing Chlorophytes (e.g. *Scenedesmus* and *Chlorella* spp.) that commonly appear across many wastewater applications and display good tolerance to wide composition profiles. These however showed relatively slower growth; the decrease in growth performance was more pronounced as pollution levels increased or if the leachate composition profile was changed. Although the results of the tolerance screening were taken into account as a guide for selecting species for further work it was not considered a particularly good tool for

finding the most suitable strains. The microalgae *Chlamydomonas* sp. SW15aRL for example did not perform particularly well in the screening but seemed to have a good potential for growth in raw leachates and, in spite of a lower overall scoring, was selected alongside another three strains for further studies. Another observation was that the strains performing the best in the leachate tolerance tests were difficult to maintain in a laboratory set up and commercial media did not seem to meet their needs (e.g. some *Chlamydomonas* spp.). Some of these strains seemed to have suffered after long-term laboratory maintenance and started forming pallmeloids, which might have been also the consequence of malnutrition. It is possible that the most suitable strains of wild species could be excluded during such selection or screening process, as during the isolation process the commercial media introduce bias which does not reflect the intended purpose of leachate treatment. In this study, the strains isolated from the leachate itself and maintained in it were eventually the most successful. It is recommended that this approach should be adopted in future studies of a similar nature.

6.2.2 Selection of a strain with high nutrient reduction potential: *Chlamydomonas* sp. SW15aRL (Chapter 3)

A narrowed selection of strains subjected to prior screening was used for the assessment of their nutrient reduction potential (Paskuliakova et al., 2016b). The comparison of growth of four selected microalgal strains in two different leachates was in agreement with the previous study (Paskuliakova et al., 2016a) where the best performing strains (*Scenedesmus* and *Chlorella* spp.) adapted well to growing in both. However their growth was slow. A substantial growth depression was also observed in larger culturing volumes in comparison to the screening tests carried out in well plates. This is likely associated with inadequate gas exchange and light penetration in larger non agitated volumes. *Chlamydomonas* sp. strain SW15aRL showed substantial growth in raw leachate while it did not grow well in substrates containing nitrate as the sole source of nitrogen. This was also in agreement with the screening tests where this strain returned low scores in the conductivity tolerance tests where f/2 medium, which contains nitrate only, was used. The amounts of nutrients reduced in leachates followed the trend of microalgal biovolume increases

and was the highest for strain SW15aRL. Phosphate limitation was also experimentally confirmed and its addition resulted in increased growth and ammonia nitrogen reduction. Although phosphate requirements for microalgae are typically species and strain specific, phosphate limitation had been previously suggested based on elemental analysis of microalgal biomass compared to elemental profiling of leachate substrates (Chu et al., 1996; Edmundson and Wilkie, 2013; Sforza et al., 2015). A reason why phosphate limitation might not have been identified in previous studies with landfill leachate could be linked with the fact that the aeration which is commonly employed in the experiments can contribute to substantial losses of ammonia nitrogen from landfill leachate during microalgal cultivation (Zhao et al., 2014). The volatilised ammonia nitrogen would then skew the nitrogen to phosphate ratio assumed to have been assimilated by microalgae to be higher if this was based entirely on nutrients being removed without allocating a process for its reduction. Another cause can be associated to the fact that the strains used in some other studies would have been maintained in standard media such as Bold Basal medium, which is rich in phosphate, prior to the remediation experiments (Lin et al., 2007; Edmundson and Wilkie, 2013). This way, it is likely that the microalgae would have stored intracellular reserves that could have sustained their growth for longer than if otherwise grown exclusively in landfill leachate. There is hence a need for additional studies with selected strains which should be grown in a variety of leachate samples over longer experimental periods. Culture maintenance in commercial media in such experiments should be omitted to confirm the suitability of such strains for the purpose of landfill leachate treatment. Further clarification of the processes taking place during phycoremediation and contributing to nutrient reduction need to be also ascertained.

6.2.3 Comparative performance of one selected strain across a number of leachates (Chapter 4)

The possibility of using microalgae for the treatment of landfill leachate has been shown in a number of isolated small scale experiments. There were no follow up studies of these to demonstrate the performance of the selected species on leachate from various sites or on samples over longer periods of time. It is indeed known that

landfill leachate composition at one site may vary substantially throughout the year. This could pose difficulty for microalgae in a pilot scale scenario.

Previous studies have shown that microalgae grow best in dilutions of landfill leachate with corresponding ammonia nitrogen concentrations of up to approximately 200 mg l^{-1}, reporting growth inhibition in less diluted leachates. This has been mostly attributed to the toxicity of ammonia. The work carried out with strain SW15aRL returned similar results whereby the strain could grow at these concentrations of ammonia nitrogen while it was also observed that its growth was impeded at lower concentrations of ammonia. The variations in growth can be in part attributed to mineral deficiencies or bioavailability issues, possibly contributing to secondary deficiencies in microalgae grown in diluted leachates. While this can be assumed to have resulted from dilution of less abundant constituents, these were as well possibly caused by the binding ability of the humic substances present in the landfill leachate samples. Depending on their nature and concentration, both stimulatory and inhibitory effects have been reported throughout the literature (Prakash et al., 1973; Doblin et al., 1999; Imai et al., 1999; Gagnon et al., 2005; Zhang et al., 2016). Also, the decreased nutrient availability could have been exacerbated by the formation of precipitates resulting from high carbonate and calcium content, phosphate addition or possibly increased pH from microalgal metabolic activity. Leachate dilution seemed to have served two purposes in the work carried out with strain SW15aRL: 1) to reduce the inhibitory effect of ammonia and other toxicants and 2) to reduce salt loading to maintain the solubility of some inorganic components while also decreasing the concentrations of other less abundant minerals. This implies that the composition of leachate for treatment with microalgae would need to be continuously monitored so that appropriate adjustments for dilutions and phosphate additions are made to find an appropriate balance.

Unlike the previous studies conducted to date, strain SW15aRL seems to be the only strain which has been continuously maintained in landfill leachate, demonstrating its ability to survive long term effects in this substrate. Comparative studies of microalgal growth with this strain using different leachates showed a need for phosphate addition. However, this was further complicated by variations in the overall leachate composition profiles where high concentrations of inorganic

constituents could result in precipitation. An important point here is that some leachates from some sites appear to be more suitable than others.

6.2.4 Effect of phycoremediation on the ecotoxicity of landfill leachate (Chapter 5)

In addition to decreases of nutrients it was confirmed that the toxicity of leachate was substantially reduced by treatment with the microalgae strain SW15aRL. The main toxicity appeared to be caused by ammonia. The most sensitive groups of organisms which showed measureable responses were the invertebrates *Daphnia magna* and *Brachionus calyciflorus* followed by some sensitivity observed in the microalgae *Chlorella* sp. OT10aTL. While the acute toxicity effects in the invertebrates were alleviated post-phycoremediation, microalgal growth was actually stimulated when compared to raw leachate, in spite of the fact that the available macronutrients were reduced (inorganic nitrogen) in the treated leachate in comparison to the raw leachate for the microalgae cells to use. Nutrient limitation was also reflected in toxicity testing in that the growth of *Chlorella* sp. OT10aTL was more stimulated in the control with added phosphate than in the raw leachate of the same concentration. The toxicity/pollutants decrease during phycoremediation with strain SW15aRL was achieved not only by microalgal assimilation but also via other abiotic processes such as precipitation and ammonia volatilisation which could have been amplified by microalgal activity and associated pH increase. It was established in this study that such ammonia losses, as high as 40%, can be experienced in leachates with TAN ~200 mg l^{-1}. If the production of microalgal biomass were a co-aim of the phycoremediation process (for potential conversion into bioenergy for example), this aspect would need to be taken into account as some nitrogen is lost to the algae in that way.

6.3 Implications and potential follow-up work

In spite of numerous small scale studies having indicated that microalgae can use wastewaters as a source for their nutrients, scaled up studies are not common. This technology seems to be struggling to be developed on an industrial scale (Brennan

and Owende, 2010; Krustok, 2016). The main reasons for this as highlighted throughout the literature are related to engineering solutions for cultivation, biomass separation and biofuel deriving technologies, which are possible but apparently not economically viable as of yet (Brennan and Owende, 2010; Chen et al. 2011; Pires et al., 2013; Richardson et al., 2014; Chiu et al., 2015).

Phycoremediation is considered a complementary treatment option for removing nutrients such as nitrogenous compounds from landfill leachate. One of the main drawbacks is the requirement for dilutions. This might be problematic at sites which often produce 50–70 m^3 of raw leachate per day when considering the design and footprint of the potential microalgae treatment plant and associated energy requirements for heat, light and medium circulation.

Most studies have focused to date on monoalgal cultures or simple consortia. The metabolic capability of these to treat complex wastewater streams might be insufficient. New studies of complex bacteria-algal consortia could possibly provide more sustainable solutions. It is assumed that these would have a better adaptability to changing composition of wastewaters unlike monocultures which can be quite vulnerable from the point of view of nutrient demands or microbial contamination (Krustok, 2016).

6.4 Perspectives on future research areas in phycoremediation

A. In order to reduce several biases which were experienced at the beginning of the present study and in any future work where the ability of microalgae to treat landfill leachate is assessed, it would be beneficial to develop a new cultivation medium that more realistically reflects the composition of this type of wastewater. This would allow for more targeted cell isolations from leachate substrates and the better maintenance of the corresponding microalgal strains successfully brought into culture. A cultivation medium of such defined composition could be used to study the individual influences of several of its components on the metabolism of the selected microalgae strains, thus possibly alleviating the difficulties encountered in experimental work resulting from varying landfill leachate composition. In addition, using stock culture inocula brought up on media of a composition similar to landfill leachates would allow for more realistic and consistent experiments as the cultures

would be in the same physiological state at the start of the experiments and would have shorter adaptation periods. This can prove a challenging aspect when using landfill leachate for stock culture maintenance due to its constant composition variation either as a result of prolonged storage or from being sampled at a given site on separate occasions, requiring it to be often analysed to determine its composition. Important aspects which should be taken into consideration for the development of a new defined medium are the ammonia nitrogen concentration within the range of values reported throughout literature, the relatively high molecular N:P ratio and the profile of other major inorganic substances (i.e. carbonate, chloride, sodium and calcium). The addition of humic substances should also be considered as these are highly abundant in landfill leachate and they also play an important role in influencing microalgal growth. The iterative approach described in Kropat et al. 2011 for tailoring the micronutrient profile of existing media could be used as a starting point in this context.

B. Understanding the nutrient requirements of microalgae entails also understanding their bioavailability. The overall concentration of minerals does not appropriately reflect the amounts actually available to cells and establishing this in landfill leachate may not be easy due to the complexity of its composition. It is suspected that this is a contributory factor responsible for the slower growth rates or the limited growth observed in landfill leachate with *Chlamydomonas* sp. strain SW15aRL. Metals seemed to be associated with different fractions of suspended matter and possibly humic substances which have substantial complexing ability. The measurement of free metals and available minerals in landfill leachate for microalgae could clarify these issues. A number of techniques exist to do so, which include Ion selective electrode (ISE), Donnan Membrane Technique (DMT) and diffuse gradients in thin films (DGT) in conjunction with ICP-MS (Rensing and Maier, 2003; Kalis et al., 2006; Wu et al., 2011).

C. The landfill leachates used in this study were neither sterilised nor treated with antibiotics and thus the treatment performance achieved after adding microalgae also resulted from the coexisting bacterial community. Synergistic relationships between microalgae and bacteria include nutrient exchange, signal transduction or gene

transfer, which can prove beneficial for improved biomass production and pollutant reduction (Krustok, 2016). Most phycoremediation studies to date have been conducted with unialgal cultures or by using the most dominant species in the phycoremediation system. These are simplified scenarios which are easier to study but might not reflect well the complexities of the treatment process in an upscaled system in practice. Wastewater streams typically contain their own complex microbiomes, which can be affected by the introduction of for example particular microalgae, which alter the biological and physicochemical properties of this environment and vice versa the success of establishing the introduced microalgae strain can depend on prior presence of other microorganisms. Understanding the coexistence of bacterial and microalgal communities is probably critical to the success and efficiency of the phycoremediation process. The microbial profiles of such communities in landfill leachates during treatment have not been described yet. The area of culture-independent approaches in genetic profiling is developing rapidly. Metagenomic community profiling in environmental samples is becoming more accessible so describing the prokaryotic and protistean communities in landfill leachate is now becoming possible. It has been found that only <1% of prokaryotic species from the environment are cultivable in laboratory conditions (Rastogi and Sani, 2011). Next generation sequencing now allows for the taxonomic characterisation of complex microbial assemblages of previously unknown species from a variety of environmental samples. Recent studies have described how to best approach the characterisation of environmental communities comprised of both eukaryotic and prokaryotic taxa (Krustok et al., 2015; Uyaguari-Diaz et al., 2016). While metagenomics provides useful information on community structures, the information on functionality is still difficult to obtain solely from DNA as the characterisation of DNA-derived communities does not distinguish between active and dormant organisms present in a system. Understanding metabolic functionality through metatranscriptomics and metaproteomics approaches using mRNA and proteins would help elucidate the genes involved in photosynthesis, ammonia oxidation, nitrogen fixation, denitrification, enzymes involved in specific carbon utilisation and others (Rastogi and Sani, 2011). However, data bases are still fragmentary and contain many genes/proteins for which functions have not been assigned.

6.5 Take home messages

- The microalgae strains isolated from polluted environments had better predispositions to successfully grow in landfill leachate. Chlorophytes especially appeared to be very adaptable.

- The highest nutrient removal was achieved with *Chlamydomonas* sp. SW15aRL, isolated from landfill leachate. Results showed its growth was phosphate limited in leachates. This strain was also able to grow in diluted landfill leachates from different sites and sampled at different times. To date, it seems to be the only strain having been maintained solely in leachate long term.

- The remediation of landfill leachate with microalgae was possible. However, treatment time was extensive (under the conditions such as those employed throughout this project) and dilution may be necessary.

- The effectiveness of the treatment is dependent on prior knowledge of the leachate composition and the adjustment of nutrients (P limitation).

- While microalgal growth inhibition can be caused by ammonia as well as other leachate constituents such as heavy metals, nutrient limitations were also affecting the growth of *Chlamydomonas* sp. SW15aRL in diluted landfill leachate. Distinguishing between growth limitation and inhibition is difficult due to the complex nature of landfill leachate. Limitations other than just phosphorus are possible and may be the causes of secondary nutrient limitations. The dilutions applied to reduce the toxicity of ammonia also reduced the levels of other inorganic constituents, possibly causing nutrient limitations.

- In addition to macronutrient reduction, it was demonstrated that the remediation of landfill leachate using microalgae also reduced its negative ecotoxicological impact. While considering material balance of the pollutant reduction, the results showed that contribution from other processes such as volatilisation and precipitation has to be taken into account.

6.6 References

Brennan, L., Owende, P., 2010. Biofuels from microalgae - A review of technologies for production, processing, and extractions of biofuels and co-products. Renewable and Sustainable Energy Reviews 14, 557–577. doi:10.1016/j.rser.2009.10.009.

Chen, C.Y., Yeh, K.L., Aisyah, R., Lee, D.J., Chang, J.S., 2011. Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review. Bioresource Technology 102, 71–81. doi:10.1016/j.biortech.2010.06.159.

Cheng, H.-X., Tian, G.-M., 2013. Preliminary Evaluation of a Newly Isolated Microalga *Scenedesmus* sp. CHX1 for Treating Landfill Leachate. 2013 Third Int. Conf. Intell. Syst. Des. Eng. Appl. 1057–1060. doi:10.1109/ISDEA.2012.249.

Chiu, S.Y., Kao, C.Y., Chen, T.Y., Chang, Y.B., Kuo, C.M., Lin, C.S., 2015. Cultivation of microalgal *Chlorella* for biomass and lipid production using wastewater as nutrient resource. Bioresource Technology 184, 179–189. doi: http://dx.doi.org/10.1016/j.biortech.2014.11.080.

Chu, L. M., Cheung, K. C., Wong, M. H., 1996. Algal purification of pretreated landfill Leachate. Toxicological and Environmental Chemistry, 53, 1-4.

Doblin, M.A., Blackburn, S.I., Hallegraeff, G.M., 1999. Growth and biomass stimulation of the toxic dinoflagellate *Gymnodinium catenatum* (Graham) by dissolved organic substances. Journal of Experimental Marine Biology and Ecology 236, 33–47.

Edmundson, S.J., Wilkie, A.C., 2013. Landfill leachate-a water and nutrient resource for algae-based biofuels. Environ. Technol. 34, 1849–57. doi:10.1080/09593330.2013.826256.

Gagnon, R., Levasseur, M., Weise, A.M., Fauchot, J., Campbell, P.G.C., Weissenboeck, B.J., Merzouk, A., Gosselin, M., 2005. Growth stimulation of *Alexandrium tamarense* (Dinophyceae) by humic substances from the Manicouagan river (Eastern Canada). J. Phycol. 41, 489–497. doi:10.1111/j.1529-8817.2005.00077.x.

Imai, A., Fukushima, T., Matsushige, K., 1999. Effects of iron limitation and aquatic humic substances on the growth of *Microcystis aeruginosa*. Canadian Journal of Fisheries and Aquatic Sciences 56 (10), 1929-1937. doi:10.1139/f99-131.

Kalis, E.J.J., Weng, L., Dousma, F., Temminghoff, E.J.M., Riemsdijk, W.H.V., 2006. Measuring Free Metal Ion Concentrations in Situ in Natural Waters Using the Donnan Membrane Technique. Environ. Sci. Technol. 40, 955-961. doi: 10.1021/es051435v.

Kropat, J., Hong-Hermesdorf, A., Casero, D., Ent, P., Castruita, M., Pellegrini, M., Merchant, S., Malasarn, D., 2011. A revised mineral nutrient supplement increases biomass and growth rate in *Chlamydomonas reinhardtii*. The Plant Journal, 66, 770–780. doi: 10.1111/j.1365-313X.2011.04537.x.

Krustok, I., Truu J., Odlare M., Truu M., Ligi T., Tiirik K., Nehrenheim E., 2015. Effect of lake water on algal biomass and microbial community structure in municipal wastewater based lab-scale photobioreactors. Applied Microbiology and Biotechnology 99 (15), 6537-49. doi: 10.1007/s00253-015-6580-7.

Krustok, I., 2016. Microbiological analysis of municipal wastewater treating photobioreactors, Thesis, School of Business, Society and Engineering, Mälardalen University, Västerås, Sweden.

Lin, L., Chan, G.Y.S., Jiang, B.L., Lan, C.Y., 2007. Use of ammoniacal nitrogen tolerant microalgae in landfill leachate treatment. Waste Management 27, 1376–1382. doi:10.1016/j.wasman.2006.09.001.

Murphy, F., Devlin, G., Deverell, R., McDonnell, K., 2013. Biofuel Production in Ireland - An Approach to 2020 Targets with a Focus on Algal Biomass. Energies 6, 6391-6412. doi:10.3390/en6126391.

Olguín, E.J., 2003. Phycoremediation : key issues for cost-effective nutrient removal processes. Biotechnol. Adv. 22, 81–91. doi:10.1016/j.biotechadv.2003.08.009.

Paskuliakova A., Tonry, S., Touzet, N., 2016a. Microalgae isolation and selection for the treatment of landfill leachate. WIT Transactions on Ecology and The Environment, 209, 65-78. © 2016 WIT Press. ISSN 1743-3541. doi:10.2495/WP160071.

Paskuliakova A., Tonry, S., Touzet, N., 2016b. Phycoremediation of landfill leachate with chlorophytes: Phosphate a limiting factor on ammonia nitrogen removal. Water Research 99, 180–187. http://dx.doi.org/10.1016/j.watres.2016.04.029.

Pires, J.C., Alvim-Ferraz, M.C., Martins, F.G., Simões, M., 2013. Wastewater treatment to enhance the economic viability of microalgae culture. Environ Sci Pollut Res Int. 20 (8), 5096-105. doi: 10.1007/s11356-013-1791-x.

Prakash, A., Rashid, M.A., Jensen, A., Subba Rao, D.V., 1973. Influence of humic substance on the growth of marine phytoplankton: Diatoms. Limnology and Oceanography, 18 (4), 516-524.

Rastogi, G., Sani, R.K., 2011. Chapter 2: Molecular Techniques to Assess Microbial Community Structure, Function, and Dynamics in the Environment. Microbes and Microbial Technology (Ahmad I Ahmad F Pichtel J, eds), 29–57. Springer, New York. Agricultural and Environmental Applications. doi: 10.1007/978-1-4419-7931-5_2.

Rensing, C., Maier, R.M., 2003. Issues underlying use of biosensors to measure metal bioavailability. Ecotoxicology and Environmental Safety 56, 140-147. https://doi.org/10.1016/S0147-6513(03)00057-5.

Richardson, J.W., Johnson, M.D., Zhang, X., Zemke, P., Chen, W., Hu, Q., 2014. A financial assessment of two alternative cultivation systems and their contributions to algae biofuel economic viability. Algal Research 4, 96–104. doi: http://dx.doi.org/10.1016/j.algal.2013.12.003.

Sforza, E., Al, M.K., Sharif, A., 2015. Exploitation of Urban Landfill Leachate as Nutrient Source for Microalgal Biomass Production 43.

Uyaguari-Diaz, M.I., Chan, M., Chaban, B.L., Croxen, M.A., Finke, J.F., Hill, J.E., Peabody, M.A., Rossum, T.V., Suttle, C.A., Brinkman, F.S.L., Isaac-Renton, J., Prystajecky, N.A., Tang, P., 2016. A comprehensive method for amplicon based and metagenomic characterization of viruses, bacteria, and eukaryotes in freshwater samples. Microbiome. 4 (20). doi: 10.1186/s40168-016-0166-1.

Wu, Z., He, M., Lin, C., 2011. In situ measurements of concentrations of Cd, Co, Fe and Mn. Environmental Pollution 159, 1123-1128. https://doi.org/10.1016/j.envpol.2011.02.015.

Zhang, X., Lu, Z., Wang, Y., Wensel, P., Sommerfeld, M., Hu, Q., 2016. Recycling Nannochloropsis oceanica culture media and growth inhibitors characterization. Algal Research 20, 282–290. http://dx.doi.org/10.1016/j.algal.2016.09.001.

Zhao, X., Zhou, Y., Huang, S., Qiu, D., Schideman, L., Chai, X., Zhao, Y., 2014. Characterization of microalgae-bacteria consortium cultured in landfill leachate for carbon fixation and lipid production. Bioresour. Technol. 156, 322–328. doi:10.1016/j.biortech.2013.12.112.

Appendix A: Chapter 2

Microalgae isolation and selection for the treatment of landfill leachate

Authors: Andrea Paskuliakova, Steve Tonry, Nicolas Touzet

Number of pages: 11

Figures:

Listings:

Tables:

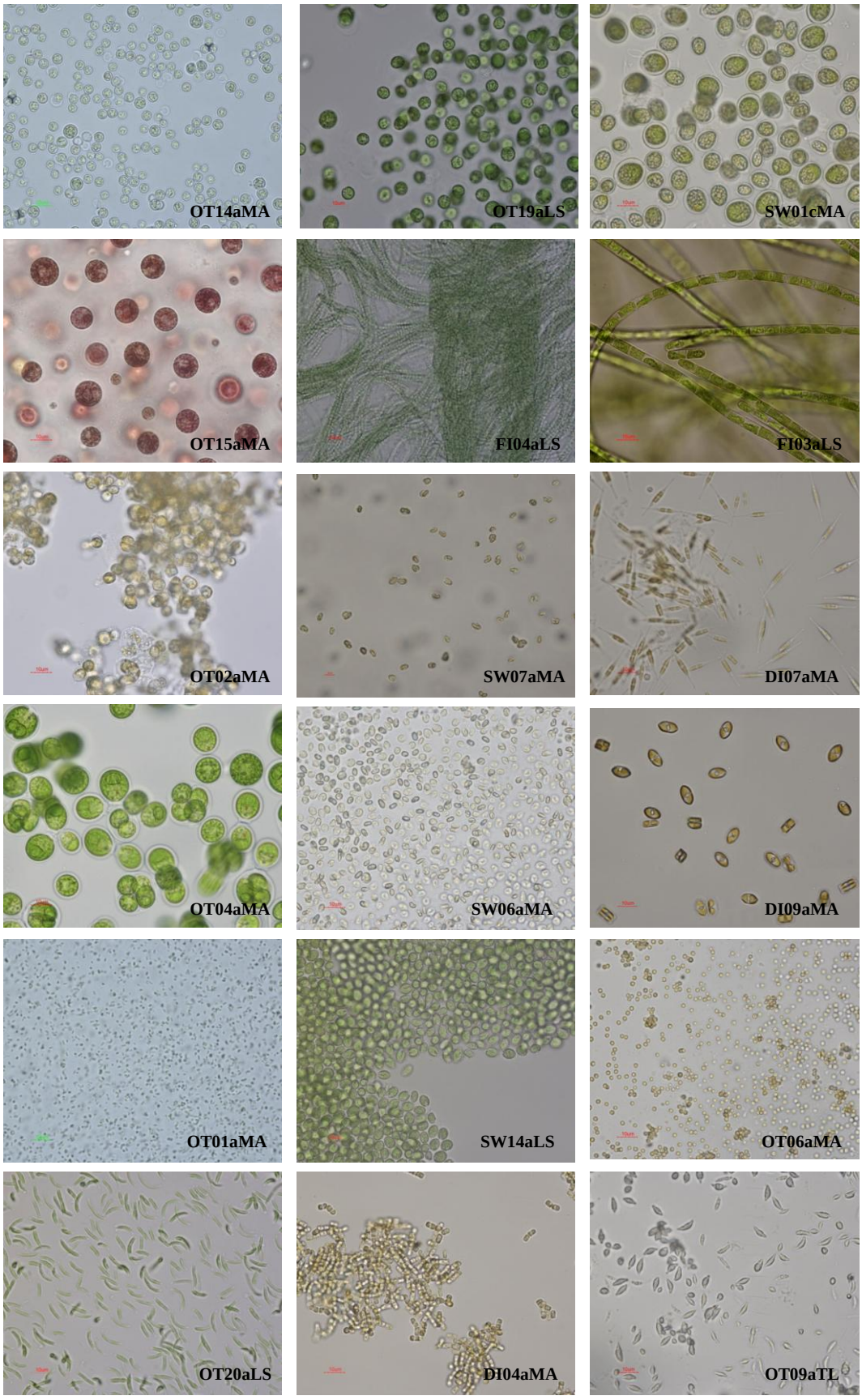


Figure S1. Overview of isolated microalgal species.

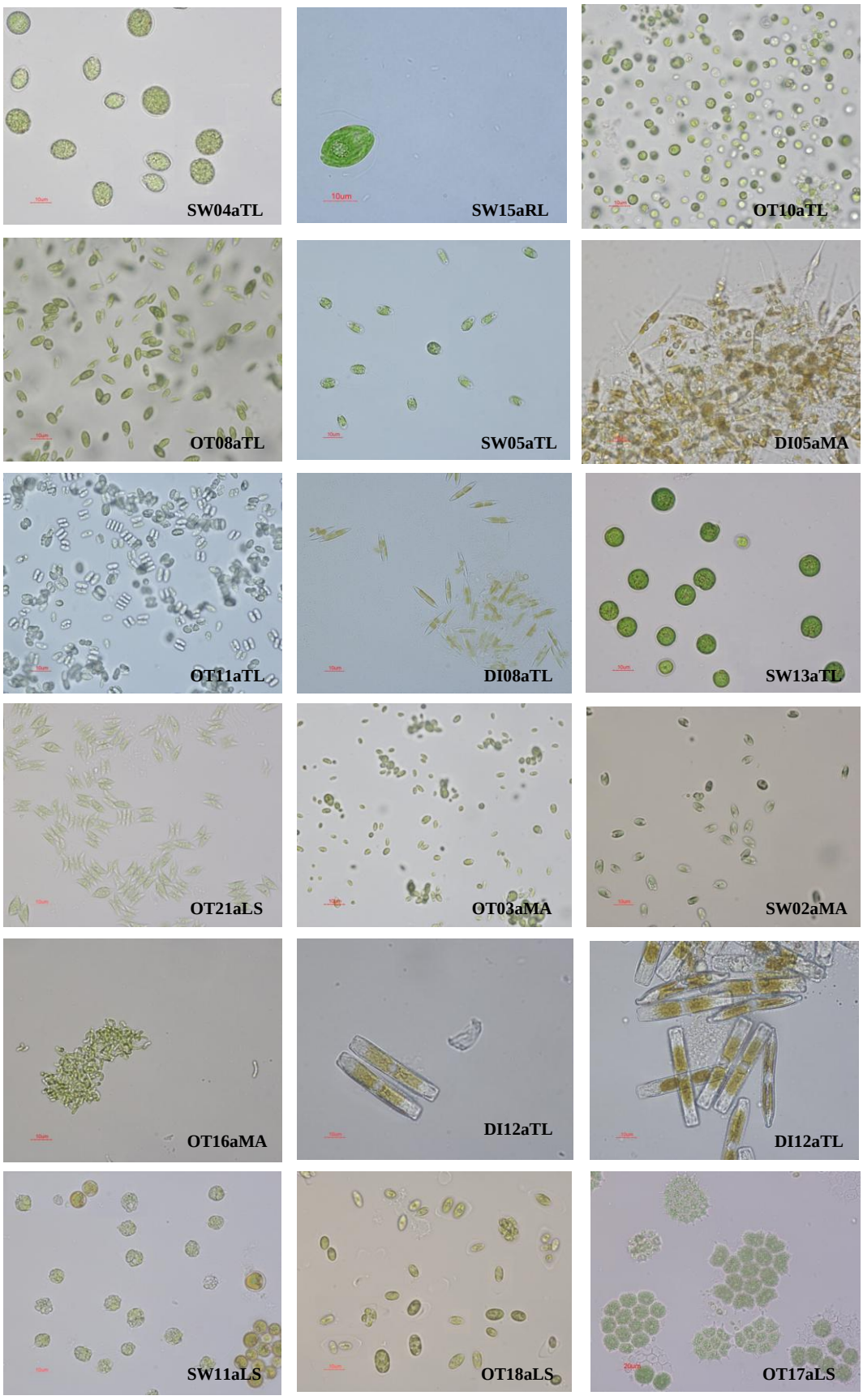


Figure S1. Overview of isolated microalgal species.

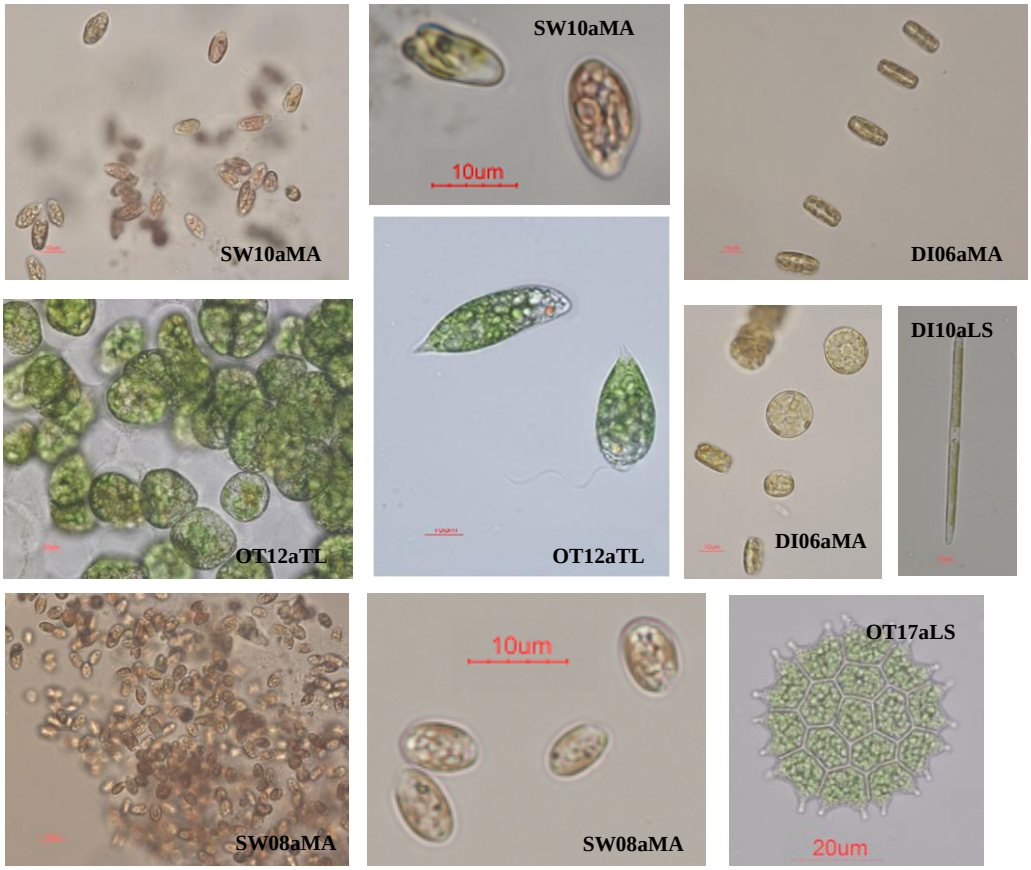


Figure S1. Overview of isolated microalgal species.

Figure S2. Palmelloids of *Chlamydomonas* sp. SW05aTL (left) and healthy fully developed cells (right).

The large subunit (28S) rDNA sequences (D1 – D2 region) of isolated strains

>DI08aTL

GACTAACCAGGATTCCCTCAGTAAGGGCGACTGAAGCGGGAAGAGCTCAGGATGTGAATCTGCGCCTTGTGCGC
CGAATTGTGGTCTGTCGACGGTGGCATTACTGGCCGGGCCAAGTCCTTTGGAACAAGGCAGCTGAGAGGGTGAG
ACTCCCGTCCGCCTGGCTGGGTGAGCCACTAGTCTACGAGTCGAGTTGTTTGGGATTGCAGCTCCAAGTGGGTGG
TAAATTCCATCTAAAGCTAAATATTGGTGGGAGACCGATAGCGTACAAGTACCGTGAGGGAAAGATGCAAAGAA
CTTTGAAAAGAGAGTTAAAGAGTACCTGAAATTGCTGAAAGGGAAGCGAAGGAAACCAGTGTCGGCTCAGTCAT
ATTTCCCTGTCCGCTTGCGGTTTGGGCGCTGTGTCTGGCCTGTGTCGTCCTTGGTTGGGTCTGCTGGAAGAGCGCA
GTGAGAGTTGATCTCTGTTGCTAGCAGCGGGTCTGACTGAGGAGGACGAAATGGTTTTCTTTACCCCGTCTTGAA
AAGGG

>SW04aTL

AGACTAACCAGGATTCCCCTAGTAACGGCGAGTGAAACGGGAATAGCCCAACTTGAAAATCTCTTCGGAGAATT
GTAGTCTATAGAAGCGCCCTCTGTAGCGGCGGGACCCCAAGTCTGGTGGAAGCCAGCGTCAGAGAGGGTGAGAA
CCCCGTCGGGTCTACGCTTAGCTGCTTCACGAGGTGCTTTCCACGAGTCGGGTTGTTTGGGAATGCAGCCCAAAA
TGGGAGGTAAATCCCTTCTAAGGCTAAATACTGGCGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAAGAT
GAAAAGAACTTTGAAAAGAGAGTTAAAAAGTGCTTGAAATTGTTGAGGGGGAAGCGTTTGGAAGACGTGGGTG
CGCCTAGGCTTACGCGTTTCTAACGATGCGCTGCATGTGCTAGGTGCTGGTCAGCATGGGTTCGTCGGCCGGGAT
AAACGCAGGGGTTGATACTCTGTCTATGCCGTCTGATGGACCAAGGTGTGAATGTCGCTCTGTCCTTCGGGAACT
GCGTCATCAAGATGCTGGCAGAAGTCTTCCAACCGGCCCGTCTTGAAAAGGG

>OT08aTL

CTAACAGGATGCGCTTAGTAACGGCGAGCGAACCGCGCAAAGCCCAACTTGAAAATCTCCCTTGGAGAATTGTA
GTCTATAGAAGTACCCTCTGTAACGGGCGCAGCTCAAGTCTCCTGGAAAGGGGCGTCAGAGAGGGTGAGAACCC
CGTAAGCTGTTGCTTTGTTGCCTCACGAGGTGCTTTCAAAGAGTCGGGTTGCTTGGGAATGCAGCCCAAATTTGG
TGGTAAATCCCATCTAAGGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAAGATGAAA
AGAACTTTGAAAAGAGAGTTAAAAAGTGCTTGAAATTGTTGAGGGGGAAGCGTTTGAATGCTAGAGTGCGCCTA
GGCTTATGGGTTCTTAACGGAACTCTGTATGTGCTAGGTGCTGGCCAGCATGGGTTAGGCGAGCGGGATAAACG
CTGGGGTTGATACCCGGTCTATGCCGCTTGCTTGACCAAGGCGTGAAGACTGCCCTTGTCCTTCGGGAACTGCAG
TCTCAAGATGCTGGCAAAAGGCTTTCAACCGACCCGTCTTGAAAAGG

>OT09aTL

CAGGATGCGCTTAGTAACGGCGAGCGAACCGCGCAAAGCCCAACTTGAAAATCTCCCTTGGAGAATTGTAGTCT
ACAGAGGCATCCTCTGCAGCAGACGGAGCTCAAATCTTCTGGAAAGGAGTGTCAGAGAGGGTGAGAACCCCGTC
GGCTCTTGTCTTTGCTGCTCCACGAGGTGCTTTCAAAGAGTCGTGTTGCTTGGGAATGCAGCACTAATTGGGTGG
TAAATCCCATCTAAGGCTAAATACTGGCGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAAGATGAAAAGA
ACTTTGAAAAGAGAGTTAAAAAGTGCTTGAAATTGTTGAGGGGGAAGCGTTTGAATGCTCTGGGTGCGCCTAGG
CTTATGGGTCTCTAACGAGCCTCTGTATGTGCTAGGTGCTGGTCAGCATGGGTTAGGCCCGCAGGATAAACCCCG
AGATTGCTACTCGGTCTATACTGCAGGCCTGACCAAGGTGTGAAGAGCGCCCTTGTCCTTCGGGAACTGCGCTCT
CAAGATGCTGGCAGAAAGTATTCAACCGACCCGTCTTGAAACCGG

>OT10aTL

AACTAACCAGGATTCCCCTAGTAACGGCGAGCGAACCGGGAAGAGCCCAACTTGAAAATCTCCAGCCTCCGGCT
GGCGAATTGTAGTCTATAGAAGTGCTCTCTGTGGCCTGCTGGTTCCAAGTTCCCTGGAAAGGGACGTCAGAGAGG
GTGAGAACCCCGTTGGAACCGGTTGAAGCCACCCCACGAGACGCTTTCGAAGAGTCGGGTTGCTTGGGAATGCA
GCCCAAAGCAGGTGGTAAATCCCATCTAAGGCTAAATACTGACGGGAGACCGATAGCGAACAAGTACCGTGAG
GGAAAGATGAAAAGAACTTTGAAAAGAGAGTTAAAAAGTGCTTGAAATTGTTGAGAGGGAAGGGTTTGAAAGC
CATCGGTGCGCCCAGGCACACGCCCGCCTAACGGTTGGCTGAATGCGCTGGGTGCTGGTCAGCATGGGTTGAGG
GGGCGGGATAACCCCGGGGATTGCTACCCCGGCTATGCCGTTCCCTCGACCGAGGAACTAAGGGCGCTCTCTGA
GTCCTTCGGGAACTGCGTCCTCAGGATGCTGGCGGAAGGCTTTCAAACCGCCCGTCTTGAAAGG

>OT11aTL

TAACAGGATGCGCTTAGTAACGGCGAGCGAACCGCGCAAAGCCCAACTTGAAAATCTCCTTCGGAGAATTGTAG
TCTATAGAAGTACCCTCTGCAACGGGCTGAGCTAAAGTCTCCTGGAAAGGGGCGTCAGAGAGGGTGAGAACCCC
GTAAGCTCACGCTTAGTTGCCTCACGAGGTGCTTTCGAAGAGTCGGGTTGCTTGGGAATGCAGCCCAAATTTGGT
GGTAAATCCCATCTAAGGCTAAATACTGGCGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAAGATGAAAA
GAACTTTGAAAAGAGAGTTAAAAAGTGCTTGAAATTGTTGAGGGGGAAGCGTTTGAATGCTCGGGTGCGCTCAG
GCTTATAAGTTCTTAACGGAGCTTTGTATGTGCTGAGTGCTGGCCAGCATGGGTTAGGCTAGTGGGACAAAAGCG
TGGATTGATACCATGCCGCTGCCACTGGCTTGACCAAGGCGTGAAGACTGCCCTTGTCCTTCGGGAACTGCAGTC
TCAAGATGCTGGTGAAAGGCTTTCAACCGACCCGTCTTGAACGGAAA

>SW05aTL

CTAACAGGATTCCCCTAGTAACGGCGAGTGAAACGGGAATAGCCCAACTTGAAAATCTTCCTTTGAAGAATTGT
AGTCTAGAGAAGCGCCCTCTGCGGCGGCCGGAGCCCAAGTTCGCTGGAAGGCGGCGTCAGAGAGGGTGAGAAC
CCCGTCGGCTCTAGGTTTAGCTGCTCCACGAGGTGCTTTCGAAGAGTCGTATTGTTTGGGAATGCAGTACAAAAT
GGGAGGTAAATCCCTTCTAAGGCTAAATACTGACGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAAGATG
AAAAGAACTTTGAAAAGAGAGTTAAAAAGTGCTTGAAATTGTACACAGGGAAGCGTTTGAAAGGTGGGGTGCG
CTCAGGCTTATGCGTCTCTAACGAGCCGCTGTATGTGCTGAGTGCTGGTCAGCATGGATTTGTCTGGCGGGATAA
AAGCTAGGGTTGATACCTGGTCTATGCCGCCGGATGGATCAAGGTGTGAATGGCGCTCGGTCCTTTGGAACTGCG
CCATCAAGATGCTGGCAGAAGCCTTTCAACCGGCCCGTCTTGAAACCGG

>OT19aLS

ACAGGATAGCCCTAGTAACGGCGAGTGAAGCGGCTATAGCCCAACTTGAAAATCTCCCTCTGGAGAATTGTAGT
CTATAGAAGTGCCCTCTGCGTCGGACGGAGCTCAAGTCCACTGGAAAGTGGCGTCAGAGAGGGTGAGAACCCCG
TCGGCTCCTGTTTCGGCGCTCCACGAGGTACTTTCCACGAGTCGGGTTGTTTGGGAATGCAGCCCTAATTTGGTG
GTAAATCCCATCTAAGGCTAAATACTGGCGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAAGATGAAAAG
AACTTTGAAAAGAGAGTCAAAAAGTGCTTGAAATTGTTGAGGGGGAAGCCATTGGATCGCGTGTGTGCGCCCAG
GCTCAAGCGGTTCTAACGAGCCGTTGAATGTGCTGGGTGCTGGTCAGATCGGGTTGCTTTGGCGGGACAACCGTC
GGGAACGTAGGTAGCCCGGCCGATGCCGCCGAAGCGACCAAGGTGTAATGGTCGCTCCGTCCTTCGGGAACTGC
GTCCAAAAGGATCTGGCAGAAGCGTTTCAATGGGCCCGTCTTGAACGGGGA

>SW13aLS

ACAGGATTGGCCTAGTAACGGCGAGTGAAACGCCAAAAGCCCAACTTGAAAATCTTCCTTTGAAGAATTGTAGT
CTATAGAAGCGCCCTCTGCGGCGGTCGGGACCCAAGTACGCTGGAAGGCGTCGTCAGAGAGGGTGATAACCCCG
TCGGTCCTAGGCTTAGCTGCTCCACGAGGTGCTTTCGCAGAGTCGCATTGTTTGGGAATGCAGTGCAAAATGGGA
GGTAAATCCCTTCTAAGGCTAAATACTGACGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAAGATGAAAA
GAACTTTGAAAAGAGAGTTAAAAAGTGCTTGAAATTGTTGAAAGGGAAGCCATTGAAAGGTGTGGGTGCGCTCA
GGCTTATGTAGCTCTAACGATCTGCTGTATGTGCTGAGTGCTGGTCAGGATGGGTTCGTTTGGCGGGATAACAGC
GTGGGTTGATACCGCGCCTATGCCGCCGAGCGGACCGAGGTGTTGAACGTCGCTCGGTCCCTTCTGGAACTGCGT
CGTCAAGATCCTGGCAGAAGCCTTTCAATGGGCCCGTCTTGAACGGGGA

>OT17aLS

CAGGATTGCCCTAGTAACGGCGAGCGAACCGGCAAGAGCCCAACTTGAAAATCTCCCTTTGGAGAATTGTAGTC
TATAGAAGTACCCTCTGCAACGGGCGGAACCTAAGTCTCCTGGAAAGGAGCGTCAGAGAGGGTGAGAACCCCGT
CGGATCCTGCTTTGTTGCCTCACGAGGTGCTTTCGAAGAGTCGGGTTGCTTGGGAATGCAGCCCTAATTTGGTGG
TAAATCCCATCTAAGGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAAGATGAAAAGA
ACTTTGAAAAGAGAGTTAAAAAGTGCTTGAAATTGTTGAGGGGGAAGCGTTTGAATGCTCGGGTGCGCCTAGGC
TTATGCAGTCTTTACGGGCTGCTGTATGTGCTAGGTGCTGGCCAGCATGGGTTCGGTTGGCAGGATAAAAGTCGG
GATTGTTACCCGGCCTATACTGCCGACTGGACCAAGGCATGAAGACTGCCCTTGTCCTTCGGGAACTGCAGTCTC
AAGATGCTGGCGAAAAGCTTTCAACCGACCCGTCTTGAAACCGGGA

>OT18aLS

CAAGGATTCCCCTAGTAACGGCGAGCGAACCGGGAAAAGCCCAGTGTGAAAATCCGCAGCCTTTGGCTGCTGAA
TTGTAGTCTGGAGAATCGTCCTCTGAGCCGAGCTGTGCCCAAGTTCCCTGGAAGGGGACGTCGAAGAGGGTGAG
AACCCCGTCGGCGCAGTTGCCGGCTCTCCACGAGGCGGTTTCGCAGAGTCGGGTTGTTTGGGAATGCAGCCCAA
AGTAGGTGGTAAATCCCATCTAAGGCTAAATACTGACGGGAGACCGATAGCGAACAAGTACCGTGAGGGAAAG
ATGAAAAGAACTTTGAAAAGAGAGTCAAAAAGTGCTTGAAATTGTTGAGAGGGAAGGCATGGCAAGGTAGAGT
GTCCTCGGGCAAACGCTGTCCGCGAGGCCTGCTGAATGTTCCGAGGACTGGTCAAGTTGGATTGCTGAGGCTGTA
TAAGCTGCTCCTTTGTTGGTGTATCCTGCAGTCTTGGCGTTCGAGGAGTCAGGGCGCCCCGGTCTTTTGATCTGCG
TTCCGACGAACTTGACGGAAGCCTTGTCGTGCACCCGTCTTGAAACCGGAA

>FI03aLS

ACAGGATTCCCCTAGTAACGGCGAGCGAACCGGGAAGAGCCCAACTTGAAAATCTGACAGCTTCGCTGTCCGAA
TTGTAGTCTAAAGAAGCGTCCTCTGTAGCGGACCGGGCCCAAGTTCCCTGGAATGGGACGTCAGAGAGGGTGAG
AGCCCCGTCGACCCCGGACCCTGCTACTCCACGAGGCGCTGTCGCCGAGTCGGGTTGTTTGGGAATGCAGCCCTA
AATGGGTGGTAAATTCCATCTAAGGCTAAATACTGGCGAGAGACCGATAGCGAACAAGTACCGCGAGGGAAAG
ATGAAAAGAACTTTGAAAAGAGAGTTAAAAGTGCTTGAAATTGTTGAGGGGGAAGCGAATGGAAGCAGAGGTG
CGCCTCGGTTTTATGTGGGGGTTCGCGCCCCCGCCTATCAACGCGAGGCGCTGGTCAGCGTGGGTTAGCCTGGCG
GGAAAAAAGCAGGGGTTGTTACCCTGTCCATATCGCCGGGCTGACCGAGGTCTGAAGGGCGCGCTTCGGCGAGC
TTCGGCATCTGCGCCCTCAGGACGCTGGCTCAATGCTTCCATCCGGCCCGTCTTGAAAAGGG

>SW01cMA

AGACTAACTAGGATTCCCTTAGTAACGGCGAGTGAAACGGGATAAGCCCAACTTGAAAATCTGCAGGCCTTGTC
TGCCGAATTGTAGTCTATAGAAGCAACCTCTGAGGCGCGCCTGGATTAAGTTCCCTGGAAGGGGACGTCAGAGA
GGGTGAGAACCCCGTCATCTATGGCTGCCGTCTCTTCACGAGATGCTCTCGCAGAGTCGGGTTGGTTGAGAATCC
AGCCCTAATTGGGAGGTACATCCCTTCTAAGGCTAAATACCGGTAAGAGACCGATAGCGAACAAGTACCGCGAG
GGAAAGATGAAAAGGCCTTTGAAAAGAGAGTTAAAAAGTGCTTGAAATTGTTGAGGGGGAAGTGTATCAGAGG
CGTGTGTGCTCCTAGGCTGTATGTAGACTCTAACCGGTCTGCCCCTCAGTGCTAGGAGCTGGTCAGCATGGGTTA
GCTTAGTGGGACAAAAGCAGAGATTGCTACTTTGCCGATGCCACTTCGCTGTCCGAGGATTAAGAGCGCTTCGCC
TTCGGAGCTGCGTTCTCATGATGCTGGCAGAAGCACCTGATACAGCCCGTCTTGAAACCGGG

>SW02aMA

CCAGGATTCCCCTAGTAACGGCGAGTGAACCGGGAAGAGCTCAAGCTGAAAATCTTCGGAATTGTAGTCTATAG
GTGCGAACTCGTGCGAGGCCGGGTATAAATCCGTTCTTGGAACTGGGTGCCAGAGAGGGTTAGAGCCCCGTCTTT
GACTCGGAGTCTTGTGCGTGTCGAGTTTGTACCGGAGAGTCGGCTTGCTTGGGAATGCAGGCCAAAATGGGTGGT
AAACTTCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACTGTGAAGGAAAGATGGAAAGAA
CTTTGAAAAGAGAGTGAAAAAGGGCTTGAAATTGTTAAGAGGAGAAGTGATTGGAGCATATCATACGGCGTGGG
GTCCTAGAGCTCGGACTTGTCTGATCTTGCACCATCCCGCGCTGTTGGTCAGCATCGATTTTTGCTGGGGGAGAA
AGCGTGTGCTGTTGATGGCTTCGGCCTGATCGGGATGCGTGCAACCCTGGGGGGGATCGAGGCGGAAAGTCTGC
GCTTGGCGGCCGGCCCTCCGTGCGTGCGTTGCGTACTACCACACCCTTTTGGCGTCTGGTGGTGCGCGGCG

>OT01aMA

TAACCAGGATTCCCCTAGTAACGGCGAGCGAACCGGGAAAAGCCCAACGTGAAAATCTCCTTCCTTCGGAAGGC
GAATTGTAGTCTATAGACGCGTGCTCTGTGCCGCGTCGTGCCCAAGTCCTTTAGCACAAGGCGTCCGAGAGGGTG
AGAACCCCGTGGGGCACGACGTTCGGCACTCCACGAGCCGTTGTCGTCGAGTCGGGTTGATTGGGAATTCAGCC
CAAAGCAGGTGGTAAATCCCATCTAAGGCTAAATACTGACGGGAGACCGATAGCGAACAAGTACCGTGAGGGA
AAGATGAAAAGAACTTTGAAAAGAGAGTGAAAAGTACTTGAAATTGTTGAGAGGGAAGAGATGATGTCTCCGC
GGGTGCGTCCAGGTACACGGTTGCACTGGTTGTTGCCTGCATACACTGGACGCTGGTCAGCATGTGTTTGTCCCG
TCGGACAAGGGTCGGGGTTGTTACCCGGCTTATGCGACGGGACGGACAGAGGGAATCCCAGGCGCTCTTCGAGA
TCCGTTCGCGGAATCTGCGTCTGCCTGATGCTGGCGGAAGGGGCCTCATCTGCCCGTCTTGAAAAGGG

>OT03aMA

GACTAACCAGGATTCCCCTAGTAGCGGCGAGCGAAGCGGGAAGAGCCCAACTTGAAAATCTGCGGCCTTCGGCC
GTCGAATTGTAGTCTATAGAAGCTTTCTCTGCGGCCGGCCGTGCCCAAGTGCCCTGGAAGGGGCCGTCGAAGAG
GGTGAGAACCCCGTCGGCGCGTGCCTGTGTCGCTCCACGAGAGGCTCTCGCAGAGTCGGGTTGCTTGGGAATGC
AGCCCAAATCAGGTGGTAAATCCCATCTAAGGCTAAATACTGGCGGGAGACCGATAGCGAACAAGTACCGTGAG
GGAAAGATGAAAAGAACTTTGAAAAGAGAGTTAAAAAGTACTTGAAATTGTTGAGGGGGAAGGGAACGGAAGC
TGCGGGTGCGCCCAGGCTCACGCCGGCCTCACGGCCGGCTGCATGCTCTGGGTGCTGGTCAGCATCGAGCTGGC
GGGCGGGACAAGTGCAACGTTTGATACGTCGTCCCTGCCGCCCGACGGCTCGAGGCAGCAAAGGGTGCTCTTTG
AGTCCCCTCGGGGAACTGCACCCTCGCGATGCTGGCGGAAGGCTTCCGTTCTGCCCGTCTTGAAAAGGG

>DI06aMA

CAGGATTCCCCTAGTAACGGCGAGTGAAGCGGGATGAGCTCACCATGTGAATCTGCGTAACCTTCGGGTGCGCC
GAATTGTGGTCTGGAGTAGTATTGTCGGCCGAGTTTCCGGGCCAAGTCTCTTGGAAAAGGGCAGCTGAGAGGGT
GAAACTCCCGTTCTTGCCTGGAAACTTTGTGCTCTGGCACATGCTTACTATGAGTCGAGTTGCTTGGGATTGCAGC
TCAAATTTGGTGGTAAATTCCATCTAAAGCTAAATATTGGTGGGATACCGATAGTGCACAAGTACCGTGAGGGA
AAGATGCAAAGAACTTTGAAAAGAGAGTTAAAGAGTACCTGAAATTGTCGAAAGGGAAGCGAAGGAAACCAGT
GCCGAGGACTTGTCATATTTCTCTTGCCGCTTGCGGTAGGGGCGCTGTGGCCTGTCGTGGGTCAGCATCGGCTCTT
TGCCTGGGGTAAACCTTCGGTGGGTAGACGACCACATTATGTGGTGAGTGCCTGCTGTTGATATCCTGGGTTGGG
CTGAGGTCAGTCACTCTGTGCTCGTGATGCTGGCAAAATGGTTTTCTTTACCCCGTCTTGAAACCGGGA

>DI09aMA

CAGGATTCCCTAGTAACGGCGAGTGAAGCGGGATGAGCTCAGGATGTGAATCTGCGCTTTTTGCGCCGAGTTGTG
GTCTGTCGACGGTGACACTACCAGCCGGGCCAAGTTCCTTGGAAAAGGACAGCTGAGAGGGTGAGACTCCCGTC
CGCCCGGCAGGGTAAGTTGCCAGTCAACGAGTCGAGTTGTTTGGGATTGCAGCTCTAAGTGGGTGGTAAATTCCA
TCTAAAGCTAAATATTGGTGGGAGACCGATAGCGTACAAGTACCGTGAGGGAAAGATGCAAAGAACTTTGAAAA
GAGAGTTAAAGAGTACCTGAAATTGCTGAAAGGGAAGCGAAGGAAACCAGTGTTTGCCTGGTCATATTTCCCTG
GCCGCTTGCGGCTTGGGCGCTGTGTCCGGCTTGAGTTGTGCTTGGTTACCGGCCTTGGAAGAGCGCTGAAGGAGT
TGACTTCGGTTGCTAGCATTGGGCGGTGACTGAGCAGGACGAAATGGTTTTCTTTACCCCGTCTTGAAACCGGGA
A

>DI07aMA

GACTAACCAGGATTCCCTCAGTAAGGGCGACTGAAGCGGGAAGTGCTCAGGATGTGAATCTGCGCTATGCGCCG
AATTGTGGTCTGTAGACTGTGACATTATTGGCCGGGCCAAGTCCCTTGGAAAAGGGCAGCAGAGAGGGTGAGAC
TCCCGTCCGCCTGGCCGAATGAGTCGCTAGTCAACGAGTCGAGTTGTTTGGGATTGCAGCTCTAAGTGGGTGGTA
AATTCCATCTAAAGCTAAATATTGGTGGGAGACCGATAGCGTACAAGTACCGTGAGGGAAAGATGCAAAGAACT
TTGAAAAGAGAGTTAAAGAGTACCTGAAATTGCTGAAAGGGAAGCGAAGGAAACCAGTGTTGAATGTGTCATAC
TTCCTGATCCGCTTGCGGATTGGGCGCTGTGTCATGTTTTGGGTTGTCCTTGGTTGGTCGCGATGGAAGAGCGCTA
AAGGAGTTGACTTTGGTTGCTAGCATTGCTTCTGACTGAGGAGGACGAAATGGTTTTCTTTACCCCGTCTTGAAC
CGGGA

>DI04aMA
TAACCAGGATTCCCCTAGTAAGGGCGACTGAAGCGGGAAGAGCCCAACATGCGAATCTGTGCCTTGGCACCGAA
TTGTGGTCTGTTCGCGCTACGTGGCTCGGGCCAAGTCCCTTGGAAAAGGGCAGCTGAGAGGGTGAGACTCCCGTC
CGCCCGAGCCAGTGAGCGAGCATGTTGGAGTCGAGTTGTTTGGGATTGCAGCTCAAAGCGGGTGGTAAATTCCA
TCGAAGGCTAAATATTGGTGGGAGACCGATAGCGTACAAGTACCGTGAGGGAAAGATGCAAAGAACTTTGAAA
AGAGAGTTAAAGAGTACCTGAAATTGCTGAAAGGGAAGCGAAGGAAACCAGTGTTGCTGCGCAGTCATACTTCT
CCGTCCGCTTGCGGGCGGGGCGCTGTGTCTGCGCTCGGGTCAGGCTTGGTTGGGGGCGCCGCAAAGGCTCCTTTG
GGAGTTGACCGCTGGAGCTGGCGGTGGTCCCGACCGAGGTTGGCGAAATGGTTTTCTTTACCCCGTCTTGAAACC
GGG

>OT04aMA
ATTCCCCTAGTAACGGCGAGTGAAACGGGAATGGCCCAACTTGAAAATCTTCCTTTGAAGAATTGTAGTCTACAG
AAGTGCCCTCTGATTCGTGCTAGACCTAAGTTCGCTGGAAGGCGGCGTCAAAGAGGGTGATAACCCCGTCGGTCT
ATGTATCGGGTCTCCACGAGGTGCTTTCGAAGAGTCGCATTGTTTGGGAATGCAGTGCAAATTGGGAGGTAAATC
CCTTCTACGGCTAAATACTGACGAGAGCCCGATAGCGAACAAGTACCGTGAGGGAAAGATGAAAAGAACTTTGA
AAAGAGAGTTAAAAAGTGCTTGAAATTGTTGAGAGGGAAGCGTTTGGGAGACTTAGGTGCGTTCAGGAAGATGC
TTATCTTAACGGATCGGCTGTATTCTCTGAGTGCTGGTCAGCATGGGTTAGCTTGACGGGATAACTGCAGGGGAT
GATACCTTGTCTATGCCGTCTCGTTGACCAAGGTGTGAATGGCGCTCGGTCCTCTGGAACTGCGTCATCAAGATG
CTGGCAGAAATCTCCCAACCGGCCCGTCTTGAAACACGGGAACCAAG

>SW10aMA
ACCAGGATTCCCCTAGTAACGGCGAGTGAACCGGGAAGAGCTCAAGCTGAAAATCTTCGGAATTGTAGTCTATA
GGTGCAGACTCGTGTGGACCCAGGTATAAGTCCGTTCTTGGAATTGGGTGCCAGAGAGGGTTAGAGCCCCGTCTT
TGACTTGGTGAGCCACACGTGATGAGTATGCACCAGAGAGTCGGCTTGCTTGGGAATGCAGGTCAAAATGGGTG
GTAAACTTCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACTGTGAAGGAAAGATGGAAAG
AACTTTGAAAAGAGAGTGAAAAAGGGCTTGAAATTGTTAAGAGGAGAAGTGATTGGAGCATATCATACCTTTTA
GGGAAAATGCTTGGGACTTGTCCTGGGAATGTATTCCTATTTGGTTGGTCAGCATCGGTTTGAGCTTGGGGAGAA
TACTTCTATTGTGAAACCTAGTGATCGGTAGTTGGAAAACCCGGGGTCAGACCGAGGCACAAAGGACGCGCATA
GCGACCAGCTCTAGCCCTTCTGCGTTGCAACGTAACACTCCTAGAGGGTTGCTTGTCTCGTGGTTGATGCAAAGA
GTGTAGGACTGCGTTCTGAAGATGCTGGCACAAATGGCTTCAATCAGCCCGTCTTGAAACCGG

>OT15aMA
ACCGGGATTCCCTCAGTAACGGCGAGTGAAGCGGGAAAAGCTCAACATGAAAACCTGGGAGCCTCGCTCTCCGG
ATTGTATTCTCTAGAGGCGTGTTCAGTGGCCGCTCGCGTATAAATCCTTTGGAAAAGGGTGTCATAGAGGGTGAG
ATCCCCGTATGTGACGCGATGTGGGCTGCGTTACGACGCGTTTTCGGCGAGTCGGGTTGCTTGAGATCGCAGCCC
TAATTGGGTGGTAAACTCCATCTAAAGCTAAATACTGACAAGAGACCGATAGCAAACAAGTACTGTGAAGGAAA
GATGCAAAGAACTTTGAAAAGAGAGTTAAAAGTACCTGAAACCGCTAAGGGGGAAGCAAAGGCCGCCAGTACG
CGCTAGGGAGTCTCCAGCGTCGCCTTGTGCGGCGTGTATCGACTTTTAAGCGGGTTCACGTGGACGTGGCGCGCG
GGATGGGCGGGGAGTGCTGATTAAGCGTCTCTGGCTACCGTGCGGCGTGTTGAGGCCTTTCCCCGCAAGGGTGA
GGACGTGGACGAAATGGCGATCTCTGACCCGTCTTGAAACAGGGAA

>SW11aLS
GAAGCACAGCGATTTCCTTAGTAGCGGCGAGCGAACCGGAATTAGCCCAACTTGAAAATCTGCCTTTGCAGAAT
TGTAGTCTATAGAAACGCCCTCTGTGGCGGCAGGAGCCTAAGTCCTCTGGAAAGGGGCGTCAGAGAGGGTGAGA
ACCCCGTCGGCTCTTCGCTTAGCCACTCCACGAGGTGTTTTCCACGAGTCGGGTTGTTTGAGAATGCAGCCCAAA
ATGGGAGGTAAATCCCTCCTAAGGCTAAATACTGACGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAAGA
TGAAAAGAACTTTGAAAAGAGAGTTAAAAAGTGCTTGAAATTGTTGAGAGGGAAGCGATTGGAGGCTGTGGGTG
CGCCTAGGCTTATGTCTCTCTGACGAGGGGCTGTATGTGCTAGGTGCTGGTCAGCATGGGTTAGTCTGGCGGGAT
AACAGCGGGGGTTGATACTCCGTCTATGCCGCCGGGCTGACCAAGGTGTGAACGGCGCTCTGTCCCTCGGGAAC
TGCGTTGTCAAGATGCTGGCAGAAGGCTTTCAATCGACCCGTCTTGAAACCGGGAA

>OT12aTL

AAGCACGGCGATTGCTCCAGTAATGGCGAATGAAATGGCATCCCATGGCTCTCCCTGAAGTGAGTTTCCCTGTTG
TTTGAAGGGCCCACCCTGCCAAAGGGTTAGGGTTGCGATCGGCTATCGTTGGATCTCGGCAAATGCTGTGGGCTA
TGGAGCCGTTGGCTGTGCCATCGGCGGTTGAGGCACCAGGAAACGCCCTGGAATGGGCTACCACAGAGGGCTAT
CGTCCCCTACACAGAGCAGAAGTGCCTCGACCGTCGGTGGTGTGGCCGGCGACATCCCTGAGTAGAGTGGCTTG
GGACTGCAGTTTGAAGTGTGAGGTGTATCCATCACAAGGAGGAATACAGACCTGGGACCGATAGAGCACAAGTA
GAGCGATCGAATGATGCAAAGGACTTTGCAAAGAGGGTTAAACGTCCCTGAAGCCTCGGCAGGAACGGCGGTG
AGTGGTGCTGTGGGAAACACCTAGTCAGGCAGTCTCCAAACCCTCTGGTCCCCAGTGAGTGGTGGACTGGATTGT
GAGGGGCTGACTGGGTCACTATCGGCTCTTCCAAGCGACCTTGGGCCAGCCCCAGGCTGGCCACTGAGCCCCTGC
CCCTTGGGGTGGGGATGCAGTCCTGGTAGCCCTTGGGGTGGCACCCTGCTCGTCAGGGCCCCAAGGCTCCGTCGT
TGAATGTGCGTTGAATCTCCAGACCAGAGGGGTGAGCTCGGCGTGTCGTCCCCACTCAGGGC

>SW15aRL

GGATTCCCCTAGTAACGGCGAGTGAACCGGGATCAGTCCAACTTGAAAATCTCCCTTTGGAGAATTGTAGTCTAT
AGAAGCGCCCTCTGTGGCGGCCGGAGCCCAAGTCCACTGGAAAGTGGCGTCAGAGAGGGTGAGAACCCCGTCG
GCTCTAGGCTTAGCTGCTCCACGAGGTGCTTTCCACGAGTCGGGTTGTTTGGGAATGCAGCCCAAAATGGGAGGT
AAATCCCTTCTAAGACTAAATATTGGCGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAAGATGAAAAGAA
CTTTGAAAAGAGAGTTAAAAAGTGCTTGAAATTGTTGAGAGGGAAGCGATTAGAAGCTGTGGGTGCGCCTAGGC
TTATGGGTTCCTAACGGGGCTCTGTATGTGCTAGGTGCTGGTCAGCATGGGTTAGCTTGGCGGGATAACAGCCGG
GGTTGATACCTGGCCTATGCCGCCGAGCTGATCAAGGAGTGAATGGCGCTCTGTCCCTCGGGAACTGCGCCATCA
AGATGCTGGCAGAAGGTTTCTAATCGGCCCGTCTTGAACCGGAA

>OT02aMA

ACTCAGTAAGGGCGACTGAAGCGGGAAGAGCTCGAACCGAAGAATTGTGGTGTGTTTGCATTATTGGTCCGGTC
GAAGTCCCTTGGAACAGGGCGGCCTAGAGGGTGAGACCCCCGTTTGGCTGGACCTTTGAGCTTACATAATAGAG
TCGAGTTGTTTGGGATTGCAGCTCAAAGTGGGTGGTAAATTCCATCGAAAGCTAAATATTGGTGGGAGACCGAT
AGCGTACAAGTACCGTGAGGGAAAGATGCAAAGAACTTTGAAAAGAGAGTTAAAGAGTACCTGAAATTGCTGA
AATGGAAGCGAAGGAAACCAGTGTCGTGCGTGTCATATTTCTCTACTTGCTTGCGAGTTGAGCGCTGTGGCTGCA
CTGGTCAAGGTTGGTTGGAGACGGCGTAATTCATGCTGTCTCTGACTGAGTTTTGACGAAATGGTTTTCTTTACCC
CGTCTTGAAACACGGGACCAAGGACCCGCTGAATTTAAG

> DI05aMA

CAGTAAGGGCGACTGAAGGGGGAAGAGCTCAACATGTGAATCTGCGCCTTGGCGCCGAATTGTGGTCTATCGAC
TCTGGTATTACTGGTCCTGCTAAGTCCCTTGGAACAGGGCAGCGTAGAGGGTGAGACTCCCGTTTGCACGACCTT
TTGAATCAGATGTCTTCGAGTCGAGTTGTTTGGGATTGCAGCTCAAAGCGGGTGGTAAATTCCATCTAAAGCTAA
ATATTGGTGGGAGACCGATAGCGTACAAGTACCGTGAGGGAAAGATGCAAAGAACTTTGAAAAGAGAGTTAAA
GAGTACCTGAAATTGCTGAAAGGGAAGCGAAGGAAATCAGTGCTTGCTGAGTCATATTTCTCACTCTGCTTGCAA
ATTGGGCGCTGTGTCTTGGCGTGTGTCAGGCTTGGTTGGGCTTGTCGGAATCGCGTAGTGAGAGTTGATCACTGC
ATGTCTGCGACGAGCTTGACTGAGGTTGACAAAATGGTTTTCTTTACCCCGTCTTGAAACACGGAACCAAGGACC
CGCTGAAT

Table S1. Sequences obtained from NCBI GeneBank and used in the construction of phylogenetic tree.

GenBank sequence	Organism
AY523030.2	*Euglena viridis* strain SAG1224-17d
AY130818.1	*Trachelomonas hispida* (UTEX) 1325
HQ287919.1	*Lepocinclis oxyuris*
HQ287925.1	*Phacus longicauda*
EF058257.1	*Glenodinium inaequale* strain ASW12003
FJ973367.1	*Diacronema* sp. strain CCMP
GU935638.1	*Chromulina* sp. SAG 17.97
JX946347.1	2.1 *Mallomonas* sp. 1 BYJ-2013 strain JJMCGRMSP
AB430621.1	2.2 *Melosira dubia* strain s0076
FN397580.1	*Navicula trivialis* strain HV25
gi|220980832:37-558	*Nitzschia palea* clone Nit B1
AB430632.1	2.3 *Asterionella formosa* strain: s0339
gi|370991562:44-566	*Nanofrustulum shiloi* strain: p194
AF289049.1	2.4 *Cylindrotheca closterium*
gi|370991608:46-567	*Psammodictyon constrictum* strain s0309
HM991674.1	*Thalassiosira concaviuscula*
AB430625.1	*Cyclotella meneghiniana* strain p567
AM710582.1	*Cymbella lanceolata* strain AT-194Gel07
AM710592.1	*Pinnularia substreptoraphe* strain AT-70.09
GQ406358.1	2.5 *Rhodochaete pulchella*
FJ973373.1	*Cyanoptyche gloeocystis* strain SAG 34.90
gi|602616324:14-672	*Rhodomonas* sp. strain CCAC 1630 B
HE820922.1	*Chroomonas mesostigmatica* strain NIES-1370
gi|195944118:1-577	*Chroomonas* sp. strain M1627
HE820908.1	*Cryptomonas tetrapyrenoidosa* strain M1800
AJ715455.1	*Cryptomonas tetrapyrenoidosa* strain NIES 279
HE610154.1	*Pyramimonas parkeae* strain CCMP726
HE861886.1	*Nephroselmis* sp. KGE8
AB491621.1	*Nephroselmis olivacea* strain NIES-483
gi|425874709:18-593	*Micractinium reisseri* strain KGE33
gi|408440882:15-593	*Chlorella variabilis* strain KGE26
AB506071.1	*Micractinium reisseri* clone 2
HE610125.1	*Stichococcus bacillaris* strain SAG 379-2
HF920670.1	*Draparnaldia glomerata* strain CCAP 418/2
AF183472.1	*Cylindrocapsa geminella* strain SAG 3.87
AF183447.1	*Ankistrodesmus stipitatus* strain SAG 202-5
KC145448.1	*Ankistrodesmus falcatus* strain UTEX 101
gi|425874707:17-580	*Acutodesmus obliquus* strain KGE31
gi|371804861:18-581	*Scenedesmus obliquus* isolate YSW009
AY779883.1	*Pseudopediastrum boryanum* var. *Cornutum* strain UTEX 470
AF395496.1	*Chlamydomonas peterfii* strain SAG 70.72
AF183457.1	*Carteria olivieri* strain UTEX LB 1032
DQ015735.1	*Chloromonas actinochloris* strain SAG 1.72
AF395510.1	*Pteromonas angulosa* strain SAG 64-3
AF183464.1	*Chlamydomonas noctigama* strain UTEX 406
DQ015734.1	*Microglena uva-maris* strain SAG 19.89 (*Chlamydomonas uva-maris*)
DQ015715.1	*Microglena monadina* strain SAG 8.87 (*Chlamydomonas monadina*)
AF183468.1	*Chlamydopodium vacuolatum* strain UTEX 2111 (*Characium vacuolatum*)
KC196734.1	*Haematococcus lacustris* isolate SAG 49.94
DQ015712.1	*Brachiomonas submarina* var. *pulsifera* strain UTEX 403
HE863713.1	*Chlamydomonas reinhardtii* strain YSW18
HE610134.1	*Pedinomonas tuberculata* strain SAG 42.84
HE610131.1	*Tetraselmis marina* strain CCMP898
HE610129.1	*Tetraselmis striata* strain SAG 41.85

Appendix B: Chapter 3

Phycoremediation of landfill leachate with chlorophytes: Phosphate a limiting factor on ammonia nitrogen removal.

Authors: Andrea Paskuliakova, Steve Tonry, Nicolas Touzet

Supplementary Information (SI):

Number of pages: 2
Number of tables: 2

Tables:

Table S1: Summary of the initial and final nutrient concentrations measured for the experiments carried out with the four microalgae strains with and without phosphate supplementation in 10% permeate (n=3, ±STD).

Table S2: Summary of the initial and final nutrient concentrations measured for the experiments carried out with the four microalgae strains with and without phosphate supplementation in 10% raw leachate (n=3, ±STD).

Table S1: Summary of the initial and final nutrient concentrations measured for the experiments carried out with the four microalgae strains with and without phosphate supplementation in 10% permeate (n=3, ±STD).

	TAN ($mg\ l^{-1}$)		PO_4^{3-}-P ($mg\ l^{-1}$)		TON ($mg\ l^{-1}$)		pH	
	Day 0	**Day 30**	**Day 0**	**Day 30**	**Day 0**	**Day 30**	**Day 0**	**Day 30**
SW13aLS	0.03 ± 0.02	2.60 ± 0.16	0.6 ± 0.0	ND	84.1 ± 7.5	49.2 ± 4.8	7.0	7.9
SW13aLS+P	0.01 ± 0.02	2.68 ± 0.92	11.6 ± 0.5	2.6 ± 0.1	87.0 ± 1.6	50.6 ± 2.3	7.0	7.9
SW15aRL	3.80 ± 0.07	0.04 ± 0.00	1.2 ± 0.1	0.8 ± 0.1	86.6 ± 2.7	80.8 ± 1.5	7.0	7.0
SW15aRL+P	3.94 ± 0.16	0.05 ± 0.00	11.7 ± 0.3	8.3 ± 0.8	87.7 ± 5.5	79.0 ± 2.2	7.0	7.0
OT08aTL	1.19 ± 0.01	0.02 ± 0.00	1.0 ± 0.0	0.1 ± 0.0	81.0 ± 5.5	65.8 ± 5.2	7.0	8.5
OT08aTL+P	1.10 ± 0.06	0.02 ± 0.01	11.3 ± 0.4	1.1 ± 0.6	77.5 ± 4.6	60.6 ± 5.9	7.0	8.8
OT11aTL	2.19 ± 0.14	0.02 ± 0.00	0.9 ± 0.1	ND	86.8 ± 1.3	64.2 ± 3.3	7.0	8.7
OT11aTL+P	2.23 ± 0.10	0.24 ± 0.32	11.8 ± 0.2	0.3 ± 0.1	86.8 ± 2.9	53.9 ± 5.8	7.0	9.5
CONTROL	0.03 ± 0.01	0.16 ± 0.16	0.5 ± 0.2	0.5 ± 0.1	84.6 ± 5.6	75.0 ± 1.9	7.0	7.0
CONTROL+P	0.02 ± 0.02	0.02 ± 0.01	10.1 ± 0.3	10.8 ± 0.5	82.6 ± 3.9	73.5 ± 2.0	7.0	7.0

*ND – not detected

Table S2: Summary of the initial and final nutrient concentrations measured for the experiments carried out with the four microalgae strains with and without phosphate supplementation in 10% raw leachate (n=3, ±STD).

	TAN (mg l^{-1})		PO_4^{3-}-P (mg l^{-1})		TON (mg l^{-1})		pH	
	Day 0	**Day 30**	**Day 0**	**Day 30**	**Day 0**	**Day 30**	**Day 0**	**Day 30**
SW13aLS	64.7 ± 8.0	64.7 ± 9.8	1.0 ± 0.0	0.9 ± 0.0	0.1 ± 0.1	ND	9.1	8.4
SW13aLS+P	65.6 ± 4.3	58.0 ± 4.4	2.1 ± 0.1	10.1 ± 0.6	ND	ND	9.1	8.4
SW15aRL	96.8 ± 2.3	47.0 ± 2.5	1.6 ± 0.0	0.1 ± 0.0	4.0 ± 0.1	4.9 ± 1.4	8.8	8.8
SW15aRL+P	97.0 ± 2.4	6.9 ± 3.0	6.7 ± 0.3	1.4 ± 0.2	3.8 ± 0.1	3.7 ± 0.0	8.8	10.0
OT08aTL	91.7 ± 4.7	55.7 ± 4.7	0.8 ± 0.1	0.2 ± 0.0	2.7 ± 0.2	2.8 ± 0.0	8.5	9.0
OT08aTL+P	92.2 ± 4.1	41.9 ± 4.6	8.8 ± 0.4	1.8 ± 0.4	2.7 ± 0.1	2.9 ± 0.1	8.5	8.9
OT11aTL	91.5 ± 2.2	53.9 ± 4.0	0.8 ± 0.0	0.1 ± 0.0	2.3 ± 0.1	2.8 ± 0.1	8.5	8.4
OT11aTL+P	93.1 ± 3.3	44.8 ± 1.7	7.8 ± 0.2	2.0 ± 0.7	2.6 ± 0.1	2.7 ± 0.0	8.5	8.5
CONTROL	87.5 ± 2.8	67.8 ± 5.5	1.0 ± 0.0	1.0 ± 0.0	0.1 ± 0.0	ND	8.5	8.5
CONTROL+P	88.8 ± 0.7	71.2 ± 3.0	10.6 ± 0.2	10.2 ± 0.0	ND	ND	8.5	8.5

*ND – not detected

Appendix C: Chapter 4

Microalgal bioremediation of nitrogenous compounds in landfill leachate –the importance of micronutrient balance in the treatment of leachates of variable composition

Authors: Andrea Paskuliakova, Ted McGowan, Steve Tonry, Nicolas Touzet

Supplementary Information (SI):

Number of pages: 9
Number of tables: 8
Number of figures: 1

Tables:

Table S1. Background information for landfill sites (leachate composition is indicative for the period over which this project was conducted).

Table S2: Overview of samples used in the experiments.

Table S3: Overview of nutrient composition of some common freshwater media (John et al., 2002; Kropat et al. 2011).

Table S4: Summary of the initial and final nutrient concentrations measured for the experiments carried out with the six different leachate samples with phosphate supplementation (n=3, ±STD).

Table S5: Summary of the initial and final nutrient concentrations measured for the experiments with different starting cell concentrations in leachate S1 (100%) with phosphate supplementation (molecular ratio N:P = 32:1) (n=3, ±STD).

Table S6. Summary of the initial and final nutrient concentrations measured for the experiments with 10% leachate S3 with and without minerals addition. Phosphate concentration was adjusted to mol N:P ~ 16:1. (n=3, ±STD).

Table S7. Summary of the initial and final nutrient concentrations measured for the experiments with 20% leachate S2 with and without minerals addition. Phosphate concentration was adjusted to mol N:P ~ 16:1. (n=3, ±STD).

Table S8. Summary of the initial and final nutrient concentrations measured for the experiments with 30% leachate S6 with and without minerals addition. Phosphate concentration was adjusted to mol N:P ~ 16:1 for all but one experiment as indicated in the table. (n=3, ±STD).

Figures:

Figure S1: TAN changes throughout duration of experiments in leachate S1 to S6. Phosphate concentration was adjusted to molecular ratio N:P ~16:1 at the start of the experiment. Values are an average of three replicates, error bars (± one standard deviation).

Table S1. Background information for landfill sites (leachate composition is indicative for the period over which this project was conducted).

Site information	LANDFILL			
	Site A	**Site B**	**Site C**	**Site D**
Started accepting waste	2002	1991	2008	1980
Type of waste accepted	· municipal solid non-hazardous (commercial & domestic) waste · industrial waste (non-hazardous de-watered sludge)	· general domestic · commercial waste · industrial wastes · sewage sludge · construction materials including those containing asbestos	· commercial, domestic and industrial non hazardous solid residual waste	· non hazardous waste excluding sewage sludge (after year 2000)
Amount of waste deposited	~ 350 000 tonnes	~500 000 m^3 capacity, which was not fully exhausted at the time of closure	~ 2 000 000 tonnes	~ 400 000 tonnes between year 1997 and 2012
Closure	2011	~2014	n/a	2012
Approximate amount of raw leachate produced	~ 12 000 tonnes $year^{-1}$ (= ~ 33 tonnes day^{-1})	~ 50-70 m^3 day^{-1}	~ 43 000 tonnes $year^{-1}$ (= ~ 118 tonnes day^{-1})	~ 24 000 – 38 000 $m^3 year^{-1}$ (= ~ 66 - 100 $m^3 day^{-1}$)
NH_4^+-N range (mg l^{-1})	up to ~ 500	580 – 1400	up to ~ 3400	up to ~ 140
Conductivity (mS cm^-)[1]	~ 2 - 20	9 – 21	up to ~ 17	0.9 - 3.5
pH	-	7.5 – 8.2	7.1 – 7.7	6.7 – 7.9
COD (mg l^{-1})	up to ~ 700	~ 1300 – 3300	~ 1700 – 10600	up to ~ 300
BOD (mg l^{-1})	up to ~ 100	~ 60 – 250	700 – 2400	up to ~ 80

Table S2. Overview of samples used in the experiments.

Sample ID	Type	Dilution used in the experiments	Site	Date sampled
S1	Raw leachate	100%	Site A	June 2015
S2	Treated leachate	20%	Site B	Feb/March 2015
S3	Raw leachate	10%	Site B	Feb/March 2015
S4	Raw leachate	10%	Site C	May 2015
S5	Raw leachate	100%	Site D	June 2015
S6	Raw leachate	30%	Site A	November 2015

Note: the leachates were samples either from holding tanks or in some cases directly from certain parts of landfill site

Table S3. Overview of nutrient composition of some common freshwater media (John et al., 2002; Kropat et al. 2011).

Parameter	Units	Media				
		BG-11	BBM	TAP/Kropat trace elements	HSM/Kropat trace elements	Sager-Granick
PO_4^{3-}-P	mg dm^{-3}	5.4	53.3	83.6	419.9	22.1
NO_3^--N	mg dm^{-3}	246.5	41.2	#N/A	#N/A	78.7
NH_4^+-N	mg dm^{-3}	0.0	#N/A	98.0	130.9	78.7
Tot N	mg dm^{-3}	246.6	41.2	98.0	130.9	157.5
Tot P	mg dm^{-3}	5.4	53.3	83.6	419.9	22.1
mol N:P	mol/mol	101:1	1.7:1	2.6:1	0.7:1	16.1
Cl	mg dm^{-3}	18.0	27.8	283.6	339.9	29.6
SO_4^{2-}	mg dm^{-3}	0.1	1825.2	97.0	25.0	117.4
CO_3^-	mg dm^{-3}	11.3	#N/A	#N/A	#N/A	#N/A
BO_3^-	mg dm^{-3}	2.7	10.9	10.8	10.8	1.0
Na	mg dm^{-3}	8.9	77.5	6.2	6.2	117.3
Ca	mg dm^{-3}	9.8	6.8	18.0	2.7	14.4
Mg	mg dm^{-3}	7.5	7.4	21.4	2.0	29.6
K	mg dm^{-3}	13.7	106.0	170.1	853.3	40.9
Fe	µg dm^{-3}	276.4	999.6	1837.3	1837.3	2065.7
Mn	µg dm^{-3}	502.7	399.9	2197.5	2197.5	73.9
Zn	µg dm^{-3}	50.0	2007.5	8893.0	8893.0	227.4
Co	µg dm^{-3}	10.0	99.0	724.9	724.9	49.5
Cu	µg dm^{-3}	20.1	399.7	635.5	635.5	15.9
Mo	µg dm^{-3}	154.5	473.0	623.2	623.2	79.3

Table S4. Summary of the initial and final nutrient concentrations measured for the experiments carried out with the six different leachate samples with phosphate supplementation (n=3, ±STD).

Substrate		TAN ($mg\ l^{-1}$)		TON ($mg\ l^{-1}$)		PO_4^{3-}-P ($mg\ l^{-1}$)		pH	
		day 0	day n	day 0	day n	day 0	day n	day 0	day n
S1 100%	**SW15aRL**	149.8 ± 4.5	16.6 ± 12.4	5.4 ± 0.5	4.4 ± 1.1	13.7 ± 0.6	1.4 ± 1.3	8.5	9.7
S1 100%	**CONTROL**	162.6 ± 0.4	118.3 ± 5.2	ND	0.3 ± 0.4	17.6 ± 0.5	2.7 ± 0.1	8.5	8.8
S2 20%	**SW15aRL**	29.5 ± 0.0	1.2 ± 0.7	267.2 ± 6.0	245.9 ± 7.4	41.1 ± 1.5	28.1 ± 14.8	7.6	7.6
S2 20%	**CONTROL**	26.0 ± 0.2	24.9 ± 0.5	238.8 ± 1.9	235.8 ± 4.3	39.0 ± 0.9	39.2 ± 0.6	7.6	7.9
S3 10%	**SW15aRL**	142.8 ± 2.9	97.2 ± 1.1	6.9 ± 0.3	5.6 ± 0.3	22.5 ± 0.1	15.7 ± 0.5	8.2	9.1
S3 10%	**CONTROL**	151.9 ± 4.8	129.7 ± 1.2	ND	ND	22.1 ± 0.1	21.5 ± 0.1	8.2	8.8
S4 10%	**SW15aRL**	224.7 ± 13.6	63.5 ± 6.8	3.4 ± 0.2	2.3 ± 0.1	35.3 ± 0.8	22.5 ± 0.9	8.5	9.2
S4 10%	**CONTROL**	225.0 ± 2.7	125.5 ± 5.3	ND	ND	35.3 ± 0.6	27.9 ± 0.4	8.5	8.8
S5 100%	**SW15aRL**	106.4 ± 5.9	89.6 ± 2.5	6.7 ± 0.4	6.6 ± 0.4	9.1 ± 0.5	1.7 ± 0.1	8.2	9.1
S5 100%	**CONTROL**	118.0 ± 1.8	97.8 ± 0.4	ND	ND	12.0 ± 0.2	1.6 ± 0.1	8.2	8.8
S6 30%	**SW15aRL**	129.6 ± 3.9	0.6 ± 0.2	ND	ND	19.0 ± 0.7	1.0 ± 0.4	8.5	10.0
S6 30%	**CONTROL**	128.3 ± 0.6	98.2 ± 9.1	ND	9.6 ± 11.6	19.8 ± 0.1	14.7 ± 0.6	8.5	8.4

n = 40 days (S1, S2, S4 and S6); n = 11 days (S3 and S5); ND = not detected

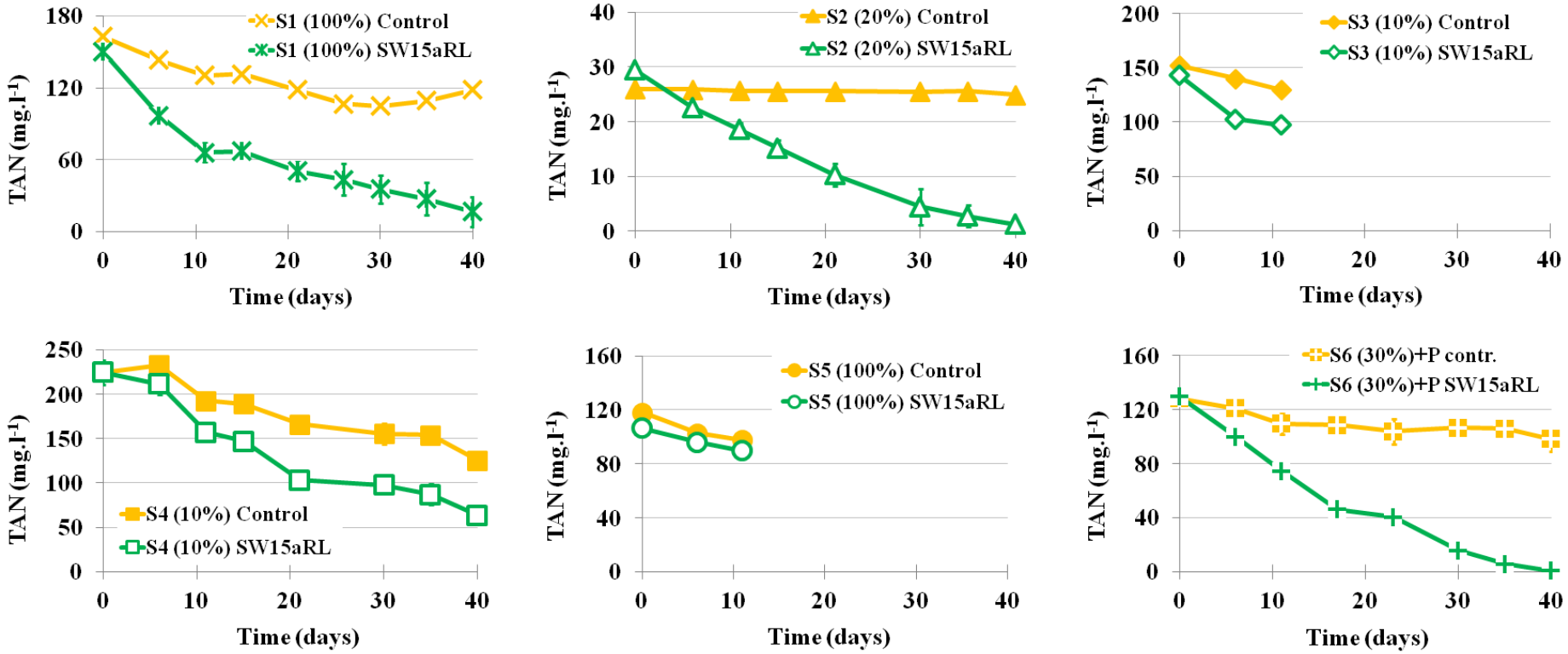


Figure S1. TAN changes throughout duration of experiments in leachate S1 to S6. Phosphate concentration was adjusted to molecular ration N:P ~16:1 at the start of the experiment. Values are an average of three replicates, error bars (± one standard deviation).

Table S5. Summary of the initial and final nutrient concentrations measured for the experiments with different starting cell concentrations in leachate S1 (100%) with phosphate supplementation (mol N:P = 32:1) (n=3, ±STD).

Experiment	TAN ($mg\ l^{-1}$)		TON ($mg\ l^{-1}$)		PO_4^{3-}-P ($mg\ l^{-1}$)		pH	
	day 0	day n	day 0	day n	day 0	day n	day 0	day n
~100 000 cell ml^{-1}	172.0 ± 2.4	24.6 ± 4.3	0.7 ± 0.3	0.3 ± 0.0	9.8 ± 0.1	ND	8.5	10.0
~250 000 cell ml^{-1}	171.8 ± 0.8	27.2 ± 4.0	0.7 ± 0.0	0.6 ± 0.0	9.5 ± 0.2	ND	8.5	10.0
~500 000 cell ml^{-1}	175.2 ± 1.1	17.9 ± 2.9	1.6 ± 0.1	1.1 ± 0.2	9.6 ± 0.2	ND	8.5	10.0
CONTROL	174.5 ± 5.8	124.2 ± 4.3	ND	ND	9.7 ± 0.1	2.3 ± 0.1	8.5	8.8

n=40; ND = not detected

Table S6. Summary of the initial and final nutrient concentrations measured for the experiments with 10% leachate S3 with and without minerals addition. Phosphate concentration was adjusted to mol N:P ~ 16:1. (n=3, ±STD).

Experiment	TAN (mg l^{-1})		PO_4^{3-}-P (mg l^{-1})		TON (mg l^{-1})		pH	
	day 0	day n	day 0	day n	day 0	day n	day 0	day n
S3 (10%) SW15aRL	164.0 ± 3.7	75.8 ± 7.4	22.0 ± 0.6	8.7 ± 3.6	0.1 ± 0.0	ND	8.5	8.8
S3 (10%) CONTROL	158.2 ± 4.7	131.2 ± 3.6	22*	22.2 ± 0.5	ND	ND	8.5	8.8
S3 (10%) + min SW15aRL	160.8 ± 2.0	36.7 ± 7.6	21.8 ± 0.2	3.7 ± 0.2	0.1 ± 0.0	ND	8.5	9.7
S3 (10%) + min CONTROL	149.7 ± 11.5	140.5 ± 5.8	22*	18.7 ± 1.7	ND	ND	8.5	8.8

n=40; *nominal value of added phosphate; ND = not detected

Table S7. Summary of the initial and final nutrient concentrations measured for the experiments with 20% leachate S2 with and without minerals addition. Phosphate concentration was adjusted to mol N:P ~ 16:1. (n=3, ±STD).

Experiment	TAN ($mg\ l^{-1}$)		PO_4^{3-}-P ($mg\ l^{-1}$)		TON ($mg\ l^{-1}$)		pH	
	day 0	day n	day 0	day n	day 0	day n	day 0	day n
S2 (20%) SW15aRL	26.0 ± 0.2	0.2 ± 0.0	39.4 ± 0.7	29.1 ± 4.7	232.8 ± 8.2	217.5 ± 11.5	7.0	8.2
S2 (20%) CONTROL	19.7 ± 0.3	19.3 ± 0.3	34.1 ± 1.5	38.9 ± 1.1	234.5 ± 7.3	241.3 ± 7.8	7.0	7.0
S2 (20%) + min SW15aRL	26.9 ± 0.4	0.4 ± 0.3	39.3 ± 0.2	18.5 ± 3.7	231.0 ±2.2	202.7 ± 5.9	7.0	8.0
S2 (20%) + min CONTROL	20.0 ± 0.4	18.7 ± 0.8	40.5 ± 2.2	38.7 ± 0.2	225.6 ± 4.4	239.2 ± 13.5	7.0	7.0

n=40

Table S8. Summary of the initial and final nutrient concentrations measured for the experiments with 30% leachate S6 with and without minerals addition. Phosphate concentration was adjusted to mol N:P ~ 16:1 for all but one experiment as indicated in the table. (n=3, ±STD).

Experiment	TAN ($mg\ l^{-1}$)		PO_4^{3-}-P ($mg\ l^{-1}$)		TON ($mg\ l^{-1}$)		pH	
	day 0	day n	day 0	day n	day 0	day n	day 0	day n
S6 (30%) no P added SW15aRL	123.6 ± 1.9	96.5 ± 4.6	0.5 ± 0.0	ND	ND	0.1 ± 0.0	8.5	8.8
S6 (30%) no P added CONTROL	126.7 ± 0.9	106.7 ± 1.6	0.2 ± 0.1	0.4 ± 0.1	ND	0.3 ± 0.2	8.5	8.8
S6 (30%) +P SW15aRL	129.6 ± 3.9	0.6 ± 0.2	19.0 ± 0.7	1.0 ± 0.4	ND	ND	8.5	10.0
S6 (30%) + P CONTROL	128.3 ± 0.6	98.2 ± 9.1	19.8 ± 0.1	14.7 ± 0.6	ND	9.6 ± 11.6	8.5	8.4
S6 (30%) +P+Fe SW15aRL	132.3 ± 5.1	1.2 ± 1.3	19.2 ± 0.1	2.2 ± 1.5	0.1 ± 0.0	ND	8.5	9.6
S6 (30%) +P+Fe CONTROL	129.1 ± 0.9	102.8 ± 5.8	19.2 ± 0.1	14.9 ± 0.2	0.1 ± 0.1	4.9 ± 6.8	8.5	8.5
S6 (30%) +P+0.5min SW15aRL	124.1 ± 6.1	1.2 ± 1.0	19.2 ± 0.4	1.2 ± 0.7	ND	ND	8.5	9.9
S6 (30%) +P+0.5min CONTROL	127.0 ± 7.0	90.8 ± 26.9	19.3 ± 0.6	11.8 ± 4.1	ND	26.8 ± 38.6	8.5	8.5
S6 (30%) +P+1min SW15aRL	130.3 ± 1.4	0.4 ± 0.0	19.0 ± 0.3	0.4 ± 0.0	ND	ND	8.5	9.9
S6 (30%) +P+1min CONTROL	130.3 ± 2.3	86.9 ± 17.2	18.6 ± 0.8	8.7 ± 4.6	ND	18.7 ± 22.5	8.5	8.5
S6 (30%) +P+2min SW15aRL	130.3 ± 4.2	0.2 ± 0.1	17.6 ± 0.6	0.4 ± 0.0	ND	ND	8.5	9.4
S6 (30%) +P+2min CONTROL	130.3 ± 1.4	94.6 ± 4.7	18.4 ± 0.5	3.4 ± 0.2	ND	6.0 ± 3.6	8.5	8.5

n=40 for all except the experiment with no phosphate added where n=23; ND = not detected

Appendix D: Chapter 5

Phycoremediation of landfill leachate with the Chlorophyte *Chlamydomonas* sp. SW15aRL and evaluation of toxicity pre and post treatment

Authors: Andrea Paskuliakova, Ted McGowan, Steve Tonry, Nicolas Touzet

Supplementary Information (SI):
Number of pages: 4
Number of tables: 6
Number of figures: 1

Tables:

Figures:

Table S1. Overview of the growth rates and biomass reached in the individual treatments as well as the amount of precipitate in the corresponding controls.

Treatment	**Growth rate ± std (day^{-1})**	**TSS ± std /SW15aRL ($g\ l^{-1}$)**	**TSS ± std /Control ($g\ l^{-1}$)**
S7 (30%) K_2HPO_4	0.08 ± 0.01	1.07 ± 0.09	0.02 ± 0.00
S7 (30%) H_3PO_4	0.10 ± 0.01	1.23 ± 0.03	0.02 ± 0.01

Table S2. Summary of the initial and final nutrient concentrations measured for the experiments with 30% leachate S7 with two different compounds as phosphorus source. Phosphate concentration was adjusted to mol N:P ~ 16:1. (n=3, ±STD).

Experiment	**TAN ($mg\ l^{-1}$)**		**PO_4^{3-}-P ($mg\ l^{-1}$)**		**TON ($mg\ l^{-1}$)**		**pH**	
	day 0	**day n**	**day 0**	**day n**	**day 0**	**day n**	**day 0**	**day n**
S7 (30%) K_2HPO_4 SW15aRL	100.8 ± 4.5	1.4 ± 1.7	17.7 ± 0.0	1.0 ± 0.5	27.1 ± 0.0	25.7 ± 1.8	8.2	9.7
S7 (30%) K_2HPO_4 CONTR.	101.0 ± 2.7	69.0 ± 10.1	18.0*	16.0 ± 0.6	26.7 ± 0.3	52.6 ± 13.6	8.2	8.0
S7 (30%) H_3PO_4 SW15aRL	101.9 ± 2.9	0.3 ± 0.2	19.1 ± 0.4	0.8 ± 0.4	27.2 ± 0.7	24.4 ± 1.0	7.9	9.6
S7 (30%) H_3PO_4 CONTR.	101.1 ± 3.4	72.2 ± 12.5	18.0*	17.6 ± 0.9	26.5 ± 1.7	42.3 ± 34.9	7.9	8.2

n=40; *concentration as calculated addition at the start of the experiment

Table S3. Summary of the initial and final nutrient concentrations measured for the experiments with 60% leachate S8. Phosphate concentration was adjusted to mol N:P ~ 16:1. (n=6, ±STD).

Experiment	TAN ($mg\ l^{-1}$)		PO_4^{3-}-P ($mg\ l^{-1}$)		TON ($mg\ l^{-1}$)		pH	
	day 0	day n	day 0	day n	day 0	day n	day 0	day n
S8 (60%) H_3PO_4 SW15aRL	158.7 ± 4.7	27.2 ± 4.5	23.4 ± 0.3	1.6 ± 0.5	0.1 ± 0.0	0.0 ± 0.0	8.2	9.5
S8 (60%) H_3PO_4 CONTROL	158.1 ± 2.7	140.0 ± 7.3	22.9 ± 0.2	8.5 ± 0.3	0.1 ± 0.1	2.6 ± 1.6	8.2	8.7

n=35

Table S4. Elemental composition of biomass as TSS from individual experiments.

Biomass as TSS from experiment	N (%DW)	C (%DW)	H (%DW)
S7 (30%) K_2HPO_4	6.0 ± 0.2	44.2 ± 0.0	6.7 ± 0.1
S7 (30%) H_3PO_4	8.1 ± 0.1	47.4 ± 0.5	6.9 ± 0.2
S8 (60%) H_3PO_4	5.8 ± 0.4	43.4 ± 1.5	6.3 ± 0.1

Table S5. Nitrogen balances overview: redistribution of N in the remediation experiment with SW15aRL and the experimental control. TAN changes in controls also included.

	TSS/ SW15aRL	N in TSS/ SW15aRL		TAN day 0	TAN reduction with SW15aRL		TAN volatilised with SW15aRL			TAN reduction in control	
Experiment	($g\ l^{-1}$)	(%DW)	($mg\ l^{-1}$)	($mg\ l^{-1}$)	($mg\ l^{-1}$)	%	($mg\ l^{-1}$)	% tot	% SW15 reduced	($mg\ l^{-1}$)	%
S7 (30%) K_2HPO_4	1.07 ± 0.09	6.0 ± 0.2	64	101	99	98	35	35	35	32	32
S7 (30%) H_3PO_4	1.23 ± 0.03	8.1 ± 0.1	98	102	102	100	4	4	4	29	28
S8 (60%) H_3PO_4	1.21 ± 0.07	5.8 ± 0.4	70	158	131	83	61	39	47	18	11

Table S6. Overview of growth rates obtained in microalgae growth inhibition test

	Control OECD	S8 (60%) raw leachate	S8 (60%)+P Control	S8 (60%)+P SW15aRL
Average growth rate over 72 h (day^{-1})	1.09 ± 0.01	1.20 ± 0.02	1.38 ± 0.03	1.47 ± 0.04
Growth rate increase in comparison to OECD control (%)	–	10	27	35

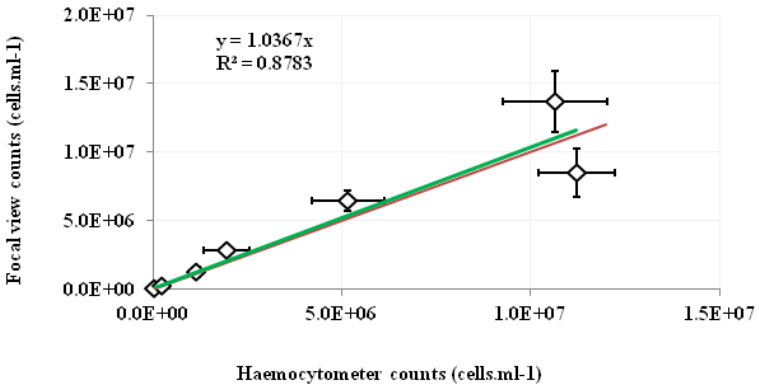


Figure S1. Correlation between focal view counts and Heamocytometer for *Chlorella* sp. OT10aTL.

Appendix E: General Information

Table E1. Overview of calibration data

Analyte	**Method**	**Method calibration range**	**R^2**
NH_4^+-N	Spectrophotometric	0 – 2.0 mg l^{-1} which can be extended up to 20 mg· l^{-1}	Calibration accepted if $R^2 \geq 0.999$
TON	Spectrophotometric	0 – 10.0 mg l^{-1} which can be extended up to 100 mg· l^{-1}	Calibration accepted if $R^2 \geq 0.996$
PO_4^{3-}-P	Spectrophotometric	0 – 2.0 mg l^{-1} which can be extended up to 20 mg· l^{-1}	Calibration accepted if $R^2 \geq 0.999$
Cl^-	Spectrophotometric	20 – 100 mg l^{-1}	0.999921
SO_4^{3-}	Spectrophotometric	20 – 100 mg l^{-1}	0.999984
Ca	Flame photometry	5 – 50 mg l^{-1}	0.9945
Mg	F-AAS	0.5 – 10.0 mg l^{-1}	0.9992
Na	Flame photometry	2.5 – 15.0 mg l^{-1}	0.9979
K	Flame photometry	2.5 – 25.0 mg l^{-1}	0.9985
Fe	ICP-MS	5 – 200 µg l^{-1}	0.9971; 0.9951
Mn	ICP-MS	5 – 100 µg l^{-1}	0.9999; 0.9998
Zn	ICP-MS	5 – 200 µg l^{-1}	0.9995; 0.9993
Co	ICP-MS	5 – 100 µg l^{-1}	1; 0.9999
Cu	ICP-MS	5 – 200 µg l^{-1}	0.9997; 0.9995
Mo	ICP-MS	5 – 200 µg l^{-1}	0.9999; 0.9996
Al	ICP-MS	5 – 200 µg l^{-1}	0.9997; 0.999
Cr	ICP-MS	5 -100 µg l^{-1}	0.9999; 0.9999
Ni	ICP-MS	5 – 200 µg l^{-1}	0.9997; 0.9992
Cd	ICP-MS	0.5 – 20.0 µg l^{-1}	0.9999; 0.9998
Pb	ICP-MS	0.5 – 20.0 µg l^{-1}	0.9905; 0.99
Lipids	Sulpho-phospho-vanillin method	20 – 100 µg per reaction volume	0.9936