ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

MICROALGAL BIOMASS AND OIL PRODUCTION USING LEACHATE

PhD THESIS

Zareen Taj KHANZADA

Department of Environmental Engineering

Environmental Biotechnology Programme

Thesis Advisor: Prof. Dr. Suleyman ÖVEZ

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ISTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

SIZINTI SUYU KULLANILARAK MİKROALG BİYOKÜTLESİ VE YAĞ/LİPİD ÜRETİMİ

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Zareen Taj KHANZADA, a Ph.D. student of ITU Graduate School of Science Engineering and Technology student ID 501102803, successfully defended the thesis/dissertation entitled "MICROALGAL BIOMASS AND OIL PRODUCTION USING LEACHATE", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To Allah, who has been with me throughout the completion of this thesis.

FOREWORD

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ABBREVIATIONS

LFL **:** Landfill leachate
TL **:** Ultra-membrane treated leachate
NH_4^+-N **:** Ammonium nitrogen
NO_3-N **:** Nitrate nitrogen

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MICROALGAL BIOMASS AND OIL PRODUCTION USING LEACHATE

SUMMARY

Landfill leachate secreted from compressed wastes in landfills overtime, is toxic to natural ecosystem and pose serious health issues for communities and surrounding environment. Since landfills continue to produce leachate (with high concentration of NH_4^+-N) through out its life span even after closure, this waste stream can be used as a nutrient resource for the growth of microalgae. The dual role of microalgae to remediate leachate (tertiary treatment) and simultaneously produce biomass make them one of the most sustainable renewable biological system to accumulate oils for future biodiesel conversion. For this purpose ultra-membrane treated landfill leachate TL was taken from Odayeri, Istanbul municipal landfill (Istanbul Büyükşehir Belediyesi) and evaluated in laboratory experiments and onsite pilot scale open raceway pond cultivation for the production of microalgal (Chlorella vulgaris and Chlamydomonas reinhardii) biomass and simultaneous removal of nutrients (mainly NH_4^+-N-NO_3). Firstly different dilutions of TL were screened in laboratory studies and then scaled up in onsite open raceway pond cultivation. Laboratory conditions were also altered to check its effect on growth, NH_4^+-N removal and oil production. Produced microalgal biomass was subjected to oil extraction process and oil content and productivity was also analysed. It was observed that increasing leachate concentration as in lower dilutions of TL (70-100%) had a negative effect on biomass growth and nutrient removal. 50% TL showed better biomass growth (~1.67 gL^{-1} dry biomass) and 10% TL (~50 mgL^{-1} NH_4^+-N) had 100% NH_4^+-N removal efficiency. Onsite raceway pond cultivation had reduced biomass growth and nutrient removal. NO_3-N removal was minimum from both the setups. In terms of oil content, microalgal cultures growing in 50% TL produced highest biomass (2.5 gL^{-1} dry weight) but accumulated the lowest cell oil content. 10% TL grown biomass produced the highest oil content (114.64 mg g^{-1} dry biomass) and lipid productivity (< 10 mgL^{-1} day^{-1}) which was still on the lowest scale according to literature survey (4 – 505 mgL^{-1} day^{-1}). The issues discussed in the thesis can be a focus point for growing biomass and nutrient removal from landfill leachate which in turn can improve the microalgal oil production for future biodiesel energy generation.

SIZINTI SUYU KULLANILARAK MİKROALG BİYOKÜTLESİ VE YAĞ/LİPİD ÜRETİMİ

ÖZET

Sızıntı suları, sıkıştırılmış katı atıkların düzenli depolanması sahalarından ortaya çıkan, doğal ekosisteme zehirli etkisi olan, toplumlara ve civarındaki çevreye ciddi sağlık problemleri yaratan bir atıksu türüdür. Düzenli depolama sahaları uzunca bir zaman sürekli yüksek konsantrasyonlu amonyak azotu (NH_4^+-N) içeren sızıntı suyunu katı atıkların depolanması ve depolanma sonrası kapatılma planı içerisinde de devam etmektedir. Bu önemli besi maddesi (Amonyak azotu) mikroalg üretiminde zengin bir kaynak olarak kullanılabilir. Sızıntı suyunun üçüncül arıtma sonrası kullanılarak üretilen mikroalgler hem atıksu problemini arıtarak ortadan kaldırmakta ve hemde mikroalg çoğaltılmasıyla sürdürülebilir ve yenilenebilir enerji üretimine en iyi kaynak temin edecek seçeneklerden birisi olan biyokütle (ve dolayısıyla yağ) sağlayarak iki amaca birden ulaşılmasında önemli rol oynamaktadır. Bu amaca ulaşmak için tasarlanan laboratuvar ve arazide kurulan pilot ölçekli mikroalg kültürü çoğaltma havuzu deneysel düzeneklerinde İstanbul Düzenli Depolama Tesisinin ikincil arıtımına uygulanan ultra filtrasyon (UF) sisteminin çıkışından alınan örnekler kullanılmıştır. Bu sistemin temel amacı UF sisteminden yüksek konsantrasyonlarda çıkan amonyak azotu (NH_4^+-N) ve Nitrat Azotunun (NO_3^--N) giderimini sağlarken aynı zamanda mikroalg biyokütlesi elde edilmesidir. Öncelikle arıtılmış sızıntı suyu numunelerinde değişik seyreltileri kullanılarak laboratuvar deney setlerinde mikroalg çoğaltma denemeleri yapılmış, daha sonra bu setlerden elde edilen optimum seyrelti ve koşullar düzenli depolama alanı içerisinde kurulan pilot tesise uyarlanarak mikroalg biyokütlesi çoğaltma çalışmalarında kullanılmıştır. Laboratuvar şartları altında yapılan deneysel setlerde çoğaltma koşulları değiştirilerek en iyi koşullar tespit edilmiş, tespit edilen bu koşulların kullanılan mikroalg türlerinin çoğalması üzerine olan etkileri ile amonyak azotunun giderimine ve yağ/lipid üretimine olan etkileri araştırılmıştır. Mikroalg biyokütlesinin çoğaltılması ile hücrelerin içerisinde depolanan yağ/lipid üretim miktarı ve verimliliğide ayrıca analiz edilmiştir. Yapılan deneysel çalışmalar arıtılmış sızıntı suyunun (UF çıkışı) besleme suyunun içindeki oranı arttıkça özellikle %70-100 seviyelerinde mikroalg çoğalma hızı ve nutriyent giderimi üzerine etkisi olumsuz olarak yansıdığı bulunmuştur. %50 seyreltisi kullanılan arıtılmış sızıntı suyu en iyi mikroalg çoğalma verimi görülen seyrelti olmuş ve yaklaşık 1.67 gr/L-kuru biyokütle elde edilmiştir. En iyi amonyak azotu giderimi % 10 arıtılmış sızıntı suyu kullanılan deney setinde (~50 mg/L-NH_4^+-N) tam giderim verimine (%100) ulaşılmıştır.

Pilot tesiste yapılan denemelerde ise mikroalg çoğalması ve besi maddesi (nutriyent) giderim verimi laboratuvar deneyi sonuçlarına göre düşmüştür. Özellikle nitrat (NO_3^-) hem laboratuvar deneylerinde hem de pilot sistemdeki çalışmalarda giderim verimi en az olan besi maddesi olarak bulunmuştur. Yağ içeriği olarak bakıldığında ise, en çok mikroalg çoğalmasının sağlandığı (2,5 gr/L-kuru biyokütle) %50 arıtılmış sızıntı suyunun kullanıldığı örneklerde mikroalg hücrelerinin çok düşük bir yağ üretim oranına (< 10 mg/L-gün) sahip olduğu tespit edilmiştir. Buna karşılık %10 arıtılmış

sızıntı suyu içeren seyreltik çözeltide yapılan denemelerde yağ üretim oranı (~ 4 mg/L-gün) daha düşük olmasına rağmen, mikroalg hücrelerinin (Chlorella vulgaris ve Chlamydomonas reinhardii) en yüksek miktarda yağ (114.64 mg/gr-kuru biyokütle) biriktirdiği bulunmuştur. Bunun yanında, yağ üretim oranları literatürde verilen değerlerle (4 – 505 mg/L-gün) karşılaştırıldığında en düşük oranda üretim yapıldığı görülmüştür.

Bu tez çalışması ile yapılan laboratuvar ve pilot sistem deneysel çalışmaları mikroalg çoğaltılmasında kullanılmasının mümkün olabileceği, sızıntı suyunun yapısında yüksek konsantrasyonlarda bulunan amonyak ve nitratın arıtımında kullanılabileceği ancak elde edilen biyokütle içeriğinin ve özellikle yağ/lipid miktarının sürdürülebilir ve yenilenebilir enerji üretimine mevcut koşullarda yeterli kaynak sağlamadığı görülmüştür. Bu durum farklı koşullar ve farklı mikroalg türleriyle değiştirilebilir, geliştirilebilir, hücrelerin yağ/lipid içeriği arttırılabilir ve sonuçta biyodizel yakıtı olarak kullanılabilecek daha verimli sürdürülebilir bir enerji üretim sistemine çevrilebilir.

1. INTRODUCTION

Landfills are widely employed for the disposal of municipal and industrial solid waste. Around 95% of total Municipal solid waste collected worldwide is disposed off in landfills. After landfilling, solid waste undergoes physico-chemical and biological changes. Upon degradation of the organic fraction of wastes, rainwater percolation, biochemical, chemical and physical reactions and inherent moisture content of the wastes, a highly contaminated liquid called "leachate" is generated (Kurniawan et al., 2006; Tsarpali et al., 2012).

Toxicity of landfill leachate

Landfill leachate LFL is a highly polluted effluent that consist of toxic xenobiotic organic compounds such as tanins, fatty acids, humic and falvic acids etc. High biochemical (BOD) and chemical oxygen demand (COD) (5000-20,000 mgL^{-1}), and low ratios of BOD_5/COD (less than 0.1) and BOD_5/NH_4^+-N which indicate the presence of a significant amount of biologically inert material. Inorganic macro component found in high concentrations in LFL is mainly ammoniacal nitrogen (3000-5000 mgL^{-1} NH_4^+-N or NH_3-N). Toxic heavy metals common in LFL are Ag, Hg, Cd, Mn, Cu and Zn etc. LFL have increased concentrations of dissolved inorganic nutrients leading to high ionic strength. Conductivity, total dissolved solids (TDS), ammonium nitrogen NH_4^+-N and respective BOD_5/NH_4^+-N could be used as a low cost effective tool to estimate leachate strength and toxicity (Regadío et al., 2012; Słomczyńska and Słomczyński, 2004; Tsarpali et al., 2012).

Environmental impact of LFL

The major potential environmental impact of LFL is pollution of soil, surface water and groundwater (Pan et al., 2017). Ammoniacal nitrogen (NH_4^+-N or NH_3-N) in LFL could cause various types of injury to agricultural plants including necrosis, growth reduction, growth stimulation and increased frost sensitivity (Zaman and Mangkoedihardjo, 2012). LFL discharged to surface waters, create oxygen depletion leading to fish kills and changes in the flora and fauna of receiving ecosystem (Jun et al., 2009; Kjeldsen et al., 2002; Lopes et al., 2012; Salem et al., 2008; Tsarpali et al., 2012). LFL gradually seeping from a landfill can enter the underlying groundwater, poses potentially serious hazards to the surrounding environment and to public health (Jun et al., 2009; Kurniawan et al., 2006; Li et al., 2012; Lopes et al., 2012). The presence of estrogens in LFL have been of concern since they can detrimentally

affect the reproduction of wildlife at very low concentrations of only a few parts per trillion (ng L^{-1}). The endocrine disrupting chemicals detected in some rivers and lakes were found to be sourced (leached) from a nearby refused dump (Zhang et al., 2009). The toxicants in LFL may accumulate in the biotic species and passed on to higher consumers and humans through food chain mechanism, causing bio-magnification over long term exposure (Zaman and Mangkoedihardjo, 2012).

Bioassays to monitor LFL quality

Bioassays using different test organisms are useful leachate quality monitoring tools and have provided essential information on the toxicological effects of LFL (Ghosh et al., 2017). Test organisms used in different studies included fish, zooplankton, aerobic luminescent bacteria and microalgae (Baun et al., 2004, 1999; Bernard et al., 1996; Cheung et al., 1993; Koshy et al., 2007; Osada et al., 2011; Słomczyńska and Słomczyński, 2004; Tsarpali et al., 2012). From bioassay studies, it can be inferred that of all the toxic compounds that remain in stabilized LFL, ammoniacal-N has been identified as one of the major toxicants to living organisms (Kjeldsen et al., 2002; Osada et al., 2011; Tsarpali et al., 2012). Ammoniacal-N in aqueous solution consists of two principal forms, the ammonium ion (NH_4^+) and un-ionised ammonia (NH_3), which are inter-convertable with relative concentrations being pH and temperature dependent (Körner et al., 2001; Li et al., 2012; Markou and Georgakakis, 2011) (Figure 1). The unionized form (ammonia NH_3) is more toxic due to the fact that it is uncharged and lipid soluble and thus traverses biological membranes more readily than the charged and hydrated ammonium ion (NH_4^+) (Körner et al., 2001; Osada et al., 2011).

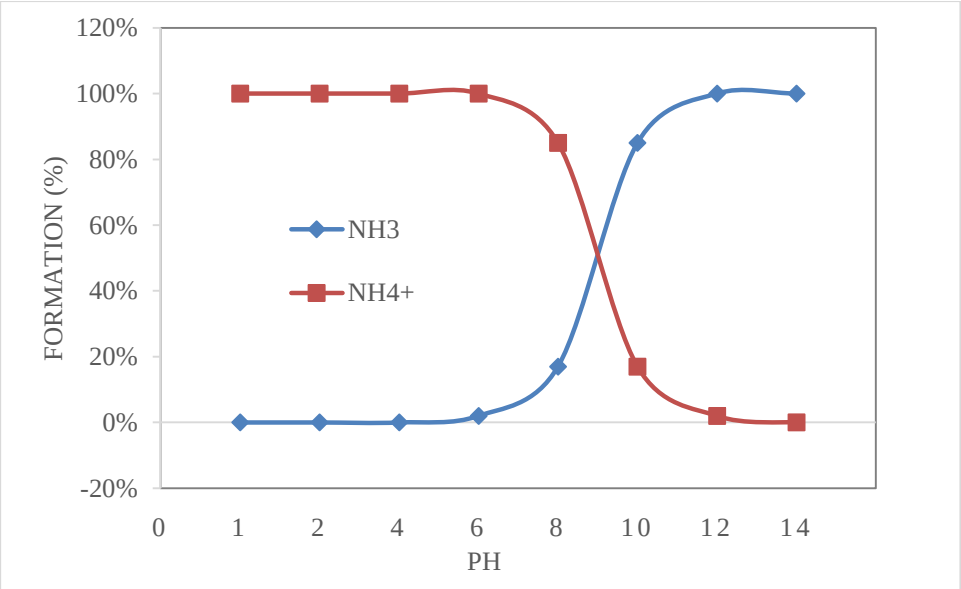


Figure 1 : Formation of ammonium (NH_4^+) and ammonia (NH_3) species as a function of pH (Markou and Georgakakis, 2011).

Dumped waste in landfills undergo successive aerobic, acetogenic, methanogenic, and final stabilization stages of organic waste degradation, in which its properties such as COD, BOD, BOD/COD ratio, NH_4^+-N and pH vary considerably. Content of dissolved organic matter decreases with time but the ammoniacal-N concentration does not follow the same decreasing trend since no mechanism for its removal exists under these conditions in a landfill, causing NH_4^+-N to accumulate in the leachate over time. In young landfills, NH_4^+-N is formed through the deamination of amino acids during degradation of organic compounds. Leachate from older landfills is rich in NH_4^+-N due to hydrolysis and fermentation of the nitrogenous fractions of biodegradable substrates (Kulikowska and Klimiuk, 2008). One of the main issues regarding management of closed landfills has been the disposal of leachate which still continues to be produced (with high concentrations of ammoniacal-N) for a long time even after the closure of the landfills (Kjeldsen et al., 2002; Kurniawan et al., 2006; Tsarpali et al., 2012).

Integrated treatment of LFL

Owing to the potential risks posed by the heavily polluted LFL to surrounding ecosystem, environmental regulatory agencies are being forced to implement increasingly stringent standards for direct discharge of LFL into receiving water channel. To meet these stricter quality standards, on-site LFL treatment becomes imperative and requires selection of an appropriate treatment technology (Ahmed and Lan, 2012; Kjeldsen et al., 2002). Biological LFL treatment processes are conventional activated sludge (CAS), aerated lagoons, suspended/attached growth, sequencing batch reactors (SBR) and upflow anaerobic sludge blanket (UASB) reactors. The physico-chemical treatment options include air-stripping, adsorption, coagulation–flocculation, chemical (or electrochemical) oxidation, chemical precipitation, and/or membrane separation using membrane (MBR) technologies (Ahmed and Lan, 2012; Ayati et al., 2012; El-gohary and Kamel, 2016; Iskander et al., 2017; Kurniawan et al., 2006; Mahmud et al., 2012; Pi et al., 2009). Membrane technology MBR have been demonstrated to offer great potentials in treating LFL, however, with progressively more strict discharge standards being required for COD and NH_4^+-N in most countries, the MBR effluents may still require post-treatment (Ahmed and Lan, 2012). Optimizing cost effective and energy efficient technologies for one-step tertiary treatment of different wastewater streams (including LFL) remain a problem for industries and municipalities (Choi and Lee, 2013; Christenson and Sims, 2011; Su et al., 2012; Zhou et al., 2011).

The variation of LFL pollutant parameters with time have important implications in its management. Due to this variability in both quantity and quality of toxic compounds, designing of a universal LFL treatment system becomes complicated. Successful LFL

treatment requires the integration of both the biological and physico-chemical processes to achieve a cost effective removal of toxicants.

Microalgae for tertiary treatment of LFL

Microalgae are considered as sustainable renewable producers of some value added bioactive macro-molecules that have the potential for commercial production of essential oils, proteins, enzymes, pigments as well as feed concentrate for animals/fish etc. and for third generation biofuel energy (Amaro et al., 2012; Chew et al., 2017; Christenson and Sims, 2011; Dassisti and Bari, 2015; Gong and You, 2015; Harun et al., 2010; Katiyar et al., 2017; Markou and Nerantzis, 2014; Mutanda et al., 2011; Prieto et al., 2017). Microalgae are aquatic relatives of plants and thrive in aerated, liquid cultures where they have sufficient access to sun light, carbon dioxide CO_2 and other primary nutrients (nitrogen N and phosphorus P) and micronutrients. If not already available in the water source, the addition of commercial fertilizers for providing these nutrients can significantly increase biomass production costs, making the price of microalgal large scale production cost prohibitive. Since atmospheric CO_2 provides a near infinite source of carbon, N and P therefore, are the two nutrients of most concern when analyzing a water source for potential microalgal production (Christenson and Sims, 2011; Olguín, 2012) N is the major constituent of proteins, hormones, energy transfer molecules (ATPs), building up of genetic material, chlorophyll and enzymes involved in photosynthesis. It accounts for 1-10% dry biomass and its availability affects the photosynthesis of microalgae (Jia and Yuan, 2016; Perez-Garcia et al., 2011).

Use of wastewater as pond medium to grow microalgal biomass have been shown to significantly reduce not only the need for chemical fertilizers and associated life cycle burdens but also to reduce the use of fresh water during microalgae cultivation (Clarens et al., 2010; Olguín, 2012). Utilizing waste nutrients and wastewater resources alleviate economic constraints on large-scale microalgae cultivation (Maity et al., 2014; Pacheco et al., 2015; Pittman et al., 2011). Dual-use microalgae cultivation for wastewater treatment coupled with biofuel (biodiesel) generation has also been an attractive option in terms of reducing the energy cost and greenhouse gas GHG emissions.

Researchers around the world have evaluated LFL as a resource for growing microalgae coupled with nutrient removal (N and P), heavy metals and toxic organics etc (Kumari et al., 2016; Lin et al., 2007; Mustafa et al., 2012; Richards and Mullins, 2013; Sarunporn and Raymond, 2014; Sforza et al., 2015). Due to the variability in composition (nutrient load and toxicity etc.) and characteristics (age and structure) of dumped waste in different regions (Cheng et al., 2011), which alters the generated leachate accordingly, there is a wide margin for researchers to exploit this problematic mixture of waste stream further under their

respective native environmental conditions and sustainably remove contaminants and reuse its nutrients.

Odayeri- Istanbul municipal landfill (Istanbul Büyükşehir Belediyesi) has been in operation since 1995. Around 2000 m^3day^{-1} of leachate is collected and goes through biological nitrification and denitrification treatment. Biological treatment is followed by ultra-membrane filtration (treated leachate TL) and then nano-filtration to further remove COD, NH_4^+-N and heavy metals, thereby reducing the toxic impact on receiving environment (water channels). One of the reasons to select TL effluent for the experiments was because it contained high NH_4^+-N concentration (even after biological treatment) and other nutrients essential for microalgal growth. TL was collected and evaluated in laboratory experiments and onsite pilot scale raceway pond cultivation, to check its ability to support growth of indigenous microalgal species coupled with NH_4^+-N removal as a means of sustainable tertiary treatment, while also simultaneously facilitating oil production (for future biodiesel conversion).

1.1 Purpose of the Thesis

The objective of the thesis was to evaluate the feasibility of indigeneous microalgal cultures:

1. To produce biomass (while growing in leachate)
2. For the tertiary treatment of leachate (microalgae removing toxins from leachate) and
3. To extract oil from the biomass (for future biodiesel energy generation)

1.2 Thesis Outline

Chapter 2 represents biomass growth and removal of NH_4^+-N from TL via microalgae in laboratory scale batch screening. Growth conditions are altered to check the environmnetal effects on NH_4^+-N removal and biomass production.

Chapter 3 also represent biomass growth and removal of nutrients (mainly NH_4^+-N) from TL via microalgae in laboratory and onsite (Odayeri-Istanbul municipal landfill) pilot scale open raceway pond cultivation. The chapter discusses the removal dynamics of high and low concentraton of NH_4^+-N.

Chapter 4 focuses on oil extraction from biomass generated from the lab study. Lipid content and productivity was analysed to check the potential of microalgal oils to be considered as a raw material for biodiesel production.

1.3 Hypothesis

It was hypothesized for the thesis that the microalgal cultures/species would be:

1. Able to grow in landfill leachate (ultra-membrane filterated effluent TL) and produce biomass,
2. Able to remove nutrients (especially NH_4^+-N) from landfill leachate, thereby facilitating tertiary treatment and,
3. Able to accumulate enough oil in their biomass to be converted (economically) into biodiesel.

2. MICROALGAE AS A SUSTAINABLE BIOLOGICAL SYSTEM FOR IMPROVING LEACHATE QUALITY[1]

Utilizing waste nutrients and wastewater resources for microalgae cultivation alleviate economic constraints on large-scale algae cultivation (Maity et al., 2014; Pacheco et al., 2015; Pittman et al., 2011). Microalgal wastewater treatment retains useful nitrogenous or other waste compounds in the biomass whereas common nitrogen removal methods (such as bacterial nitrification/denitrification) remove the majority of the nitrogen as nitrogen gas N_2 (Wang et al., 2017). Also the biological sludge (bacterial biomass) generated during the conventional treatment processes requires regular disposal. Microalgae based treatment can potentially achieve removal of pollutants and toxic chemicals in an ecologically safer way with the added benefits of residual microalgal biomass resource recovery and recycling (Abdel-Raouf et al., 2012; Cabanelas et al., 2013; Choi and Lee, 2013; Christenson and Sims, 2011; Maity et al., 2014; Martinez et al., 2000). This dual role of leachate remediation and biomass production by microalgae make them one of the most sustainable biological systems for leachate quality improvement and a promising feed stock for commercial production of value added bio-products.

Microalgae are able to degrade wastewater contaminants either by directly transforming the pollutant or by synergistically enhancing the degradation potential of the microbial community present (DeBashan et al., 2004; Rawat et al., 2011; Unnithan et al., 2014). Mutual interaction between bacteria and microalgae often benefit each other as algal photosynthesis provides oxygen, a key electron acceptor often required by pollutant degrading autotrophic/ heterotrophic bacteria (Tandon and Jin, 2017; Unnithan et al., 2014). Nutrients such as N and P form complexes with organic compounds present in wastewater. In the absence of decomposers (bacterial) activity, N might not be available for microalgal assimilation (Danger et al., 2007). Kumari et al. (Kumari et al., 2016) observed that co-culturing bacteria and algae was more efficient in mineralizing organic cytotoxic and genotoxic compounds in LFL. Zhao et al. (Zhao et al., 2014) and Zhou et al. (Zhou et al., 2013) indicated the effectiveness of bacterial-algal consortium for treating contaminants mainly ammoniacal-N. Lin et al. (Lin et al., 2007) observed that Brassica chinensis seed germination was significantly enhanced (65%) when grown in microalgae (chlorella sp.) treated 50% LFL in comparison with algae-free LFL

[1] This chapter is based on the paper " Zareen T. Khanzada and Süleyman Övez, Microalgae as a sustainable biological system for improving leachate quality. Energy, 2017, 140, 757-765."

samples. Paskulikova et al. (Paskuliakova et al., 2016) recently suggested that screening of microalgal strains that can with hold fluctuating pollutant concentrations as in leachate, could be a focus point in future for an effective nutrient removal via phyco-remediation.

In an attempt to sustainably utilize LFL nutrients, TL was collected from Odayeri and evaluated in a preliminary trial to grow indigenous microalgal species. The tertiary treatment of TL via microalgae was hypothesized to improve leachate quality (by removing NH_4^+-N) coupled with microalgal biomass production.

2.1 Experimental

Mixed culture of fresh water indigenous microalgal species of Chlorella v*ulgaris* and Chlamydomonas *reinhardii* were obtained from marine department, Istanbul University. Ultra-membrane filtration effluent TL was kindly provided by Odayeri and stored at 4 ^{0}C in 20 L air tight plastic containers until use. Physico-chemical characteristics of TL is presented in Table 2.1.

Table 2.1 : Physico-chemical characteristics of TL.

TL Parameters	(mg L^{-1})
TOC	350
TKN	500
NH_4^+-N	485
TP	10
Ortho-PO_4	5,33
TDS	6117
Conductivity EC (mS/cm)	17,5
Salinity (ppt)	10
Alkalinity	1700
Chloride (Cl^-)	9000
pH	7

2.1.1 Materials

Inoculum was pre-cultured in a mixture of BG11 media (no nitrogen source) and TL to acclamatise microalgal cultures before starting the experiments. Exponentially growing inoculum at a volumetric ratio of 20% (v/v) measured at an absorbance of 680 nm (using spectrophotometer, model U-2001, HITACHI. Japan) was used to start the experiments and monitor growth of microalgal cultures through out the study.

Cultures were grown in 1L glass bottles (500 ml working volume) at room temperature of 23-25 ^{0}C. Continuous artificial irradience of 80 µmol $m^{-2}s^{-1}$ was provided through white fluorescent lamps (measured by a digital light meter-linkoln USA). Continuous air bubbling was supplied at a rate of 3 L min^{-1} (flow rate in each 500 ml bottle was ~ 0.31 ml min^{-1}). BG11 UTEX media (+ive control) consisted of the following nutrients: $MgSO_4$, KCl, $CaCl_2.2H_2O$, $NaHCO_3$,$NaNO_3$, KH_2PO_4, K_2HPO_4, Fe Ammonium citrate, Citric acid and trace elemental solution. Trace elemental solution included $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, $MnCl_2.4H_2O$, $CoCl_2.6H_2O$, $FeSO_4.7H_2O$, EDTA, H_3BO_3, $NaMO_4.2H_2O$.

2.1.2 Experimental setup

Microalgal growth and NH_4^+-N removal was evaluated in 3 lab experimental sets, conducted with different dilutions of TL (i.e., 10%, 30%, 50%, 70%, 90%, 100%), distilled water dw (as –ive control) and regular BG11 media (as +ive control). The only difference among the experimental sets was as follows:

Experimental set 1- Raw (unautoclaved) TL was used and pH was maintained at 6.5-7.5 by adding 0.5N $NaOH/H_2SO_4$.

Experimental set 2- Autoclaved TL (20 min, 1 atm at 121 ^{0}C) was used with pH maintained at 6.5-7.5 by adding 0.5N $NaOH/H_2SO_4$.

Experimental set 3- Autoclaved TL (20 min, 1 atm at 121 ^{0}C) was used with pH maintained at 6.5-7.5 by adding $NaHCO_3$ (saturated sol.)/H_2SO_4.

2.1.3 Analytical methods

Dry weight was measured by filtering the samples through whatman filter papers (0.7 pore size) at the start and end of each batch cultures. Filters were oven dried at 60^{0}C until constant weight (around 3 days) and then weighted on a measuring balance. Chloride ions Cl^- and NH_4^+-N was measured by an ion-selective electrode (Orion 95–12) with an Orion IonAnalyser 701A meter (Orion Research Inc., Boston, MA). Total organic carbon (TOC) was measured by open reflux method ISO 6060 (Rice et al., 2012), Total kjeldahl nitrogen (TKN) was measured by method SM 4500-N orgB (Rice et al., 2012), Total phosphorus (TP) was measured by following standard methods SM 4500- PBD (Rice et al., 2012). Conductivity, salinity and total dissolved solids (TDS) was measured by portable ISE meter (HACH HQ40d). Heavy metals were measured by Inductively Coupled Plasma (ICP) Optical Emission Spectrometer (OES) (Perkin-Elmer Optima 7000DV ICP-OES). Cultivation was carried out under batch mode in dublicates for 4 weeks. The data was statistically analysed by Student's t test comparing with the control at significance level $p \leq 0.05$. the values presented in the paper are averages of dublicate cultures.

2.2 Results and Discussion

2.2.1 Growth of microalgae in the 3 experimental sets

Microalgae grew well in all the dilutions of the 3 experimental sets. A lag phase of first 2-3 days was observed in lower dilutions (70%-100% TL), irrespective of adaptation time of 3-4 weeks given to microalgal cultures in TL before the start of experiments (Figures 2.1a, 2.2a, 2.3a). This lag phase could be due to high salinity (Cl^- 9000 mg L^{-1}) in TL (Table 2.1). High salinity and dark color of TL also affect microalgal growth due to the sensitivity of fresh water species to salinity and reduction of light penetration by the dark color (Zhao et al., 2014).

In 10% TL microalgae growth was minimal and stayed closer to negative control (dw) due to diminished nutrients, except in set 3 where intermitant bicarbonate addition significantly increased growth curve at $p<0.05$ (Figures 2.1a, 2.2a, 2.3a). Sodium bicarbonate was primarily added to buffer the acidifying leachate medium (explained in section 2.2.2) but this intermitant supply also provided an extra inorganic carbon C in set 3, in comparison to only air supplied (carbon dioxide as inorganic C source) in sets 1 and 2. Effect of bicarbonate addition was more pronounced in 10% TL, whereas in the rest of dilutions (30-100% TL) growth curves showed no significant difference among experimental sets 1, 2 and 3 (Figures 2.1a, 2.2a, 2.3a).

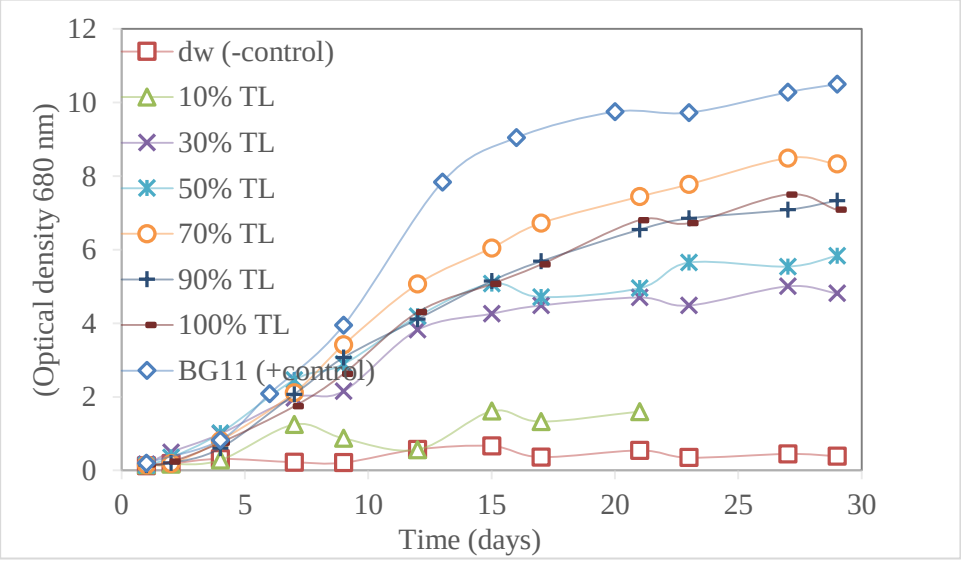


Figure 2.1a : Microalgal growth in different dilutions (10%-100%) of TL in set 1.

Growth in all the dilutions (10%-100% TL) was lesser than control nutrient media BG11 as shown by growth curves at 95% significance level (Figure 2.1a, 2.2a, 2.3a). Experimental sets 1, 2, 3 were not significantly different from each other in terms of biomass production (Table 2.2). Microalgal growth curves showed an onset of stationary phase near day 20th in all the lower dilutions (50-100% TL) (Figure 2.1a, 2.2a, 2.3a). Overall 50% and 70% TL showed

better growth curves and dry biomass in all the 3 experimental sets (Figures 2.1, 2.2, 2.3; Table 2.2). Experimental set 1 was not autoclaved and bacterial population in raw TL probably had some symbiotic relation with microalgae as explained later in section 2.2.3, which might have enhanced (but not significant) effect on growth. But leachate is also suggested to contain biota that would compete with any microalgal species introduced and requires prior disinfection, if fresh water species are to be used (Richards and Mullins, 2013). TL had sufficient nitrogen source to support microalgal growth but was limited in phosphate PO_4, which is an essential nutrient for microalgae growth (Rasoul-Amini et al., 2014; Roopnarain et al., 2014; Wu et al., 2013; Xin et al., 2010) Bacterial community with their low nitrogen to phosphorus (N:P) ratios and their differential recycling activity can change the relative quantity of nutrients available for algae. Thus in P limited conditions, as in the present study, bacteria may assimilate a considerable amount of P from surrounding ecosystems and decrease its availability for algae (Danger et al., 2007). This can explain to some extent non significant biomass and NH_4^+-N removal efficiency in set 1, when compared to sets 2 and 3, where microbial community had some stimulatory as well as inhibitory effect on microalgae.

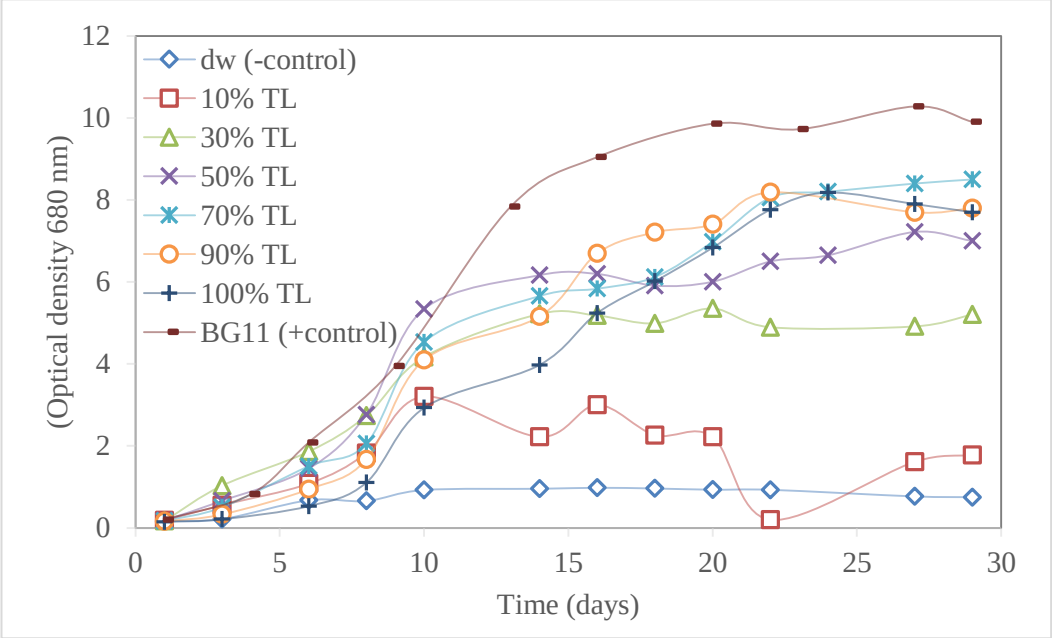


Figure 2.2a : Microalgal growth in different dilutions (10%-100%) of TL in set 2.

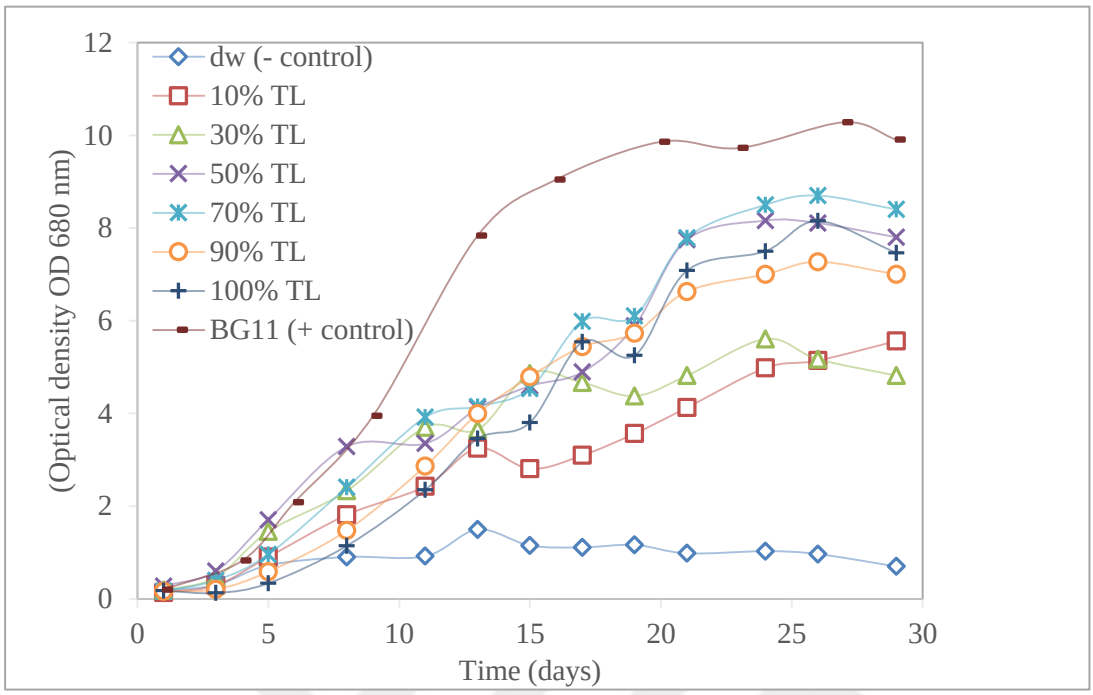


Figure 2.3a : Microalgal growth in different dilutions (10%-100%) of TL in set 2.

Table 2 : Microalgal dry biomass produced in the three experimental sets

Treatments ↓	Microalgal dry biomass (g/L)						
	dw	10% TL	30% TL	50% TL	70% TL	90% TL	100% TL
Experimental set 1	0,37 ± 0,02	0,68 ± 0,03	1,36 ± 0,04	1,67 ± 0,04	1,65 ± 0,04	1,62 ± 0,04	1,64 ± 0,04
Experimental set 2	0,4 ± 0,04	0,67 ± 0,03	1,33 ± 0,04	1,58 ± 0,03	1,57 ± 0,03	155 ± 0,04	1,53 ± 0,04
Experimental set 3	0,38 ± 0,04	1,33 ± 0,05	1,38 ± 0,04	1,62 ± 0,04	1,59 ± 0,05	1,56 ± 0,04	1,57 ± 0,03

2.2.2 pH dynamics in the three experimental sets

Neutral pH is usually optimum for microalgae cultivation (Lam and Lee, 2012a). In this study pH of the medium was manually maintained within 6.5-7.5 range by adding 0.5N $NaOH/H_2SO_4$ (in set 1 and 2) and saturated solution of $NaHCO_3$ (in set 3) on alternate days. A continuous decrease in the medium pH was observed in all the 3 experimental sets, reaching to as low as 3.5 in different TL dilutions (10%-100% TL) (Figures 2.1 b,c : 2.2 b,c: 2.3 b,c). 10% and 30% TL showed continuous pH fluctuations through out the study duration. In lower TL dilutions (50%-100% TL with increasing concentrations of NH_4^+-N) this pH decrease during the experiments was found to be an indicator of:

1) Microalgal growth- as on the onset of stationary phase (day 20^{th}), pH started to reach to neutral in all the 3 batch experiments; and

2) NH_4^+-N consumption- since NH_4^+-N assimilation by microalgae produces H^+ ions (as not all hygrogen from ammonium is required for biomass growth), which reduces medium pH (Markou and Georgakakis, 2011; Martínez, 2000; Perez-Garcia et al., 2011; Scherholz and Curtis, 2013; Su et al., 2012).

According to the simplified reaction (Goldman et al., 1982):

$$NH_4^+ \leftrightarrow NH_3 + H^+ \leftrightarrow org - N + H^+$$

Medium pH reduction was also observed by tam and wong (Tam and Wong, 1996) where unicellular green alga chlorella vulgaris was grown mixotrophically in diferent leachate concentrations for 21 days. They observed that algal growth and their removal of NH_3-N from media coincided with a decrease in pH values. Medium pH containing more than 50 mgL^{-1} NH_4^+-N decreased significantly throughout the cultivation period. They reviewed that medium became acidic in the logrithmic phase of growth and then changed to alkaline when the cells were in lag or stationary phase and in the absence of growth. Mustafa et al. (Mustafa et al., 2012) evaluated microalgal consortium in high rate algal pond HRAP and also observed a decrease in pond pH (reaching below 5) with different loading rates, but their pond was an open system with uncontrolled conditions, so this decreased pH cannot be attributed to only NH_4^+-N consumption. Leachate medium pH reduction in the present study is also in line with what Edmundson and Wilkie (Edmundson and Wilkie, 2013) observed in their study, where acidification of leachate medium was observed within 24 hours of culturing (when initial pH was adjusted to 7 by adding HCl). The acidifying media (pH reaching to 4 in 72 hours) also showed growth comparable to nutrient media (control). Cultures with no prior pH adjustment to 7, showed an increase in medium pH within 24 hours with no or stagnant growth until the end of study (72 hours).

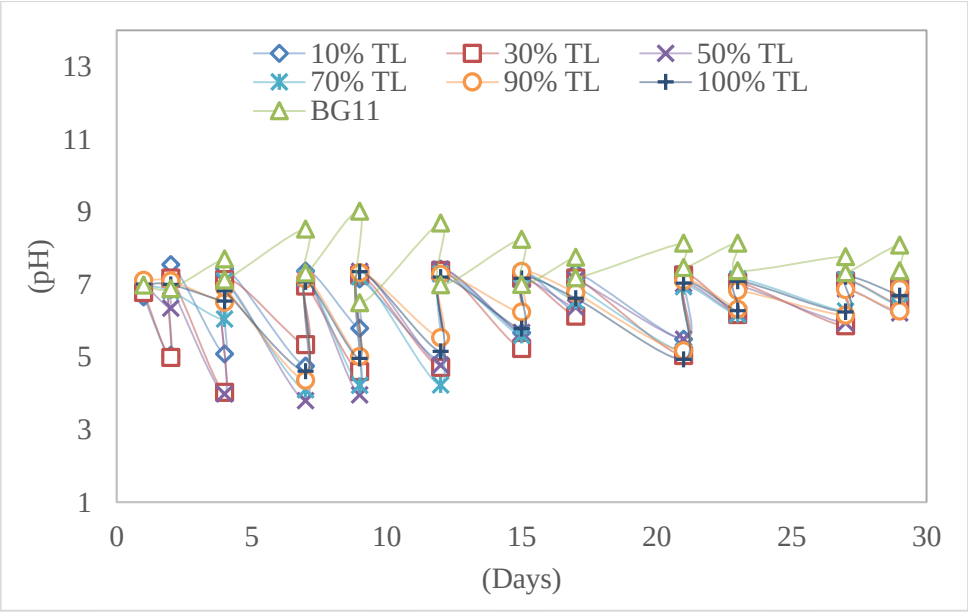


Figure 2.1b : pH dynamics in different dilutions of TL (10%-100%) in experimental set1.

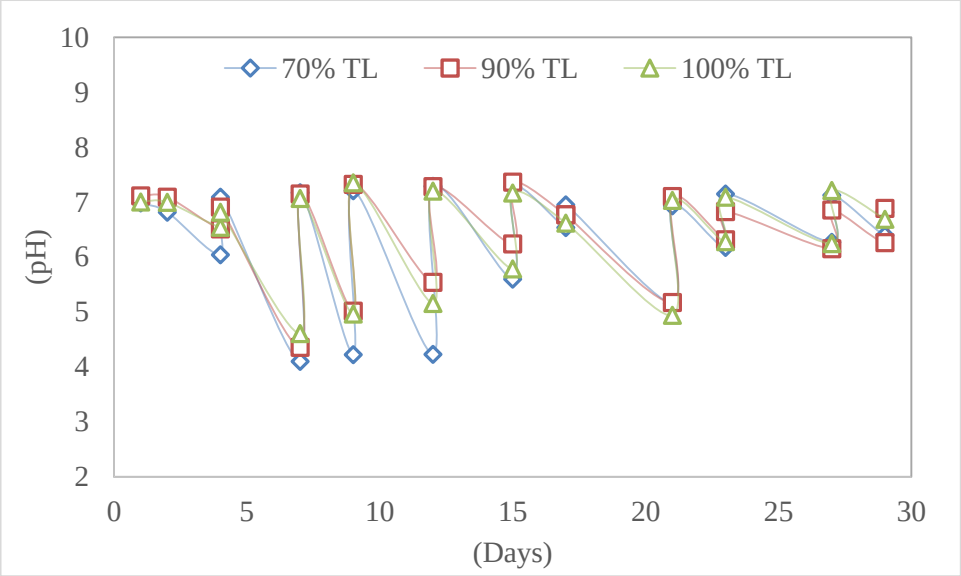


Figure 2.1c : pH dynamics in lower dilutions of TL (70%-100%) in experimental set1.

pH fluctuations have not been discussed in detail in literature, particularly when leachate is used as microalgal growth medium. In the present study acidification of the medium started within 24 hours for 10-50% TL dilutions, but in lower dilutions (70-100% TL) acidification started after 2-3 days (Figure 2.1c, 2.2c, 2.3c). During these 2-3 days no growth was observed and microalgae were in lag phase (Figure 2.1a, 2.2a, 2.3a). This lag phase phenomenon could explain rise/neutral pH during 72 hours study (with high initial ammonium concentration of 980 mg L^{-1} NH_4^+-N) of Edmundson and Wilkie (Edmundson and Wilkie, 2013). In their study adding HCl initially might have played some part in decreasing lag phase by some unknown factors. Lin et al. (Lin et al., 2007) also observed an increase in medium pH while using

leachate with high ammonium concentrations (1345 mg L^{-1} NH_4^+-N). Only 10% dilution (with 135 mg L^{-1} NH_4^+-N) supported microalgal growth during 12 days study period. Ammoniacal tolerant species (with standing 405 mg L^{-1} NH_4^+-N) were also identified.

Constantly low pH as observed in the present study reduces the chances of free ammonia (NH_3) formation (above pH 8 with aeration) which is toxic to microalgae (as described in section 1.2.1), but this acidic pH conditions also does not support optimum microalgal growth. Scherholz and Curtis (Scherholz and Curtis, 2013) observed cultures (chlorella vulgaris and chlamydomonas reinhardii) grown on ammonium (NH_4^+-N) levels of 9% or more, fell below a pH of 4 and stopped growing. Lam and Lee (Lam and Lee, 2012a) observed in their study that chlorella showed well adaptation to pH fluctuations, but below pH 4 growth was reverted and above pH 9 growth was significantly inhibited (biomass was stagnant to 0.33 g L^{-1}).

In contrast to continuous acidification of leachate medium, pH in BG11 nutrient media (+ control) was constantly increasing (Figure 2.1b, 2.2b, 2.3b). The removal of dissolved $CO_{2\,(aq)}$ (in the form of carbonic acid H_2CO_3) and bicarbonate (HCO_3^-) by photosynthetic microalgae leads to an increase in medium pH. When bicarbonate ion is consumed this pH increase is due to produced hydroxyl ions (OH-)(Kim et al., 2013; Lam and Lee, 2013; White et al., 2013).
According to simplified reaction (Alava et al., 1997; Hill, 2006):

$$HCO_3^- \leftrightarrow CO_{2\,aq} + OH^-$$

In set 3, intermitant sodium bicarbonate $NaHCO_3$ was added to buffer the acidifying media, but the buffering capacity seemed not enough to counter act H^+ ions produced in the system. Another explanation for this decreasing pH during microalgae cultivation in leachate could be due of bacterial nitrification. Nitrification (by autotrophic bacteria) also reduces medium pH by producing H^+ ions (Parkes et al., 2007; Summerfelt et al., 2015). Nitrification is a two-step autotrophic oxidation of ammonium ion (NH_4^+) to nitrate (NO_3^-) mediated by bacteria in the presence of light and air (Kim et al., 2006).
According to simplified reaction:

$$NH_4^+ + 2O_2 \longrightarrow 2H^+ + NO_3^- + H_2O$$

In the present study nitrifying bacterial community might be present in experimental set 1, since it was a not autoclaved (ultra-membrane filtrated effluent) and there are chances that despite of autoclaving (in sets 2 and 3) nitrification might have been going on along with microalgal consumption of NH_4^+-N. Also intermitant bicarbonate supply in experimental set 3, which was added to buffer the media against pH fluctuations, was adding alkalinity to the system, which inturn might have aided nitrification (Kim et al., 2006; Parkes et al., 2007).

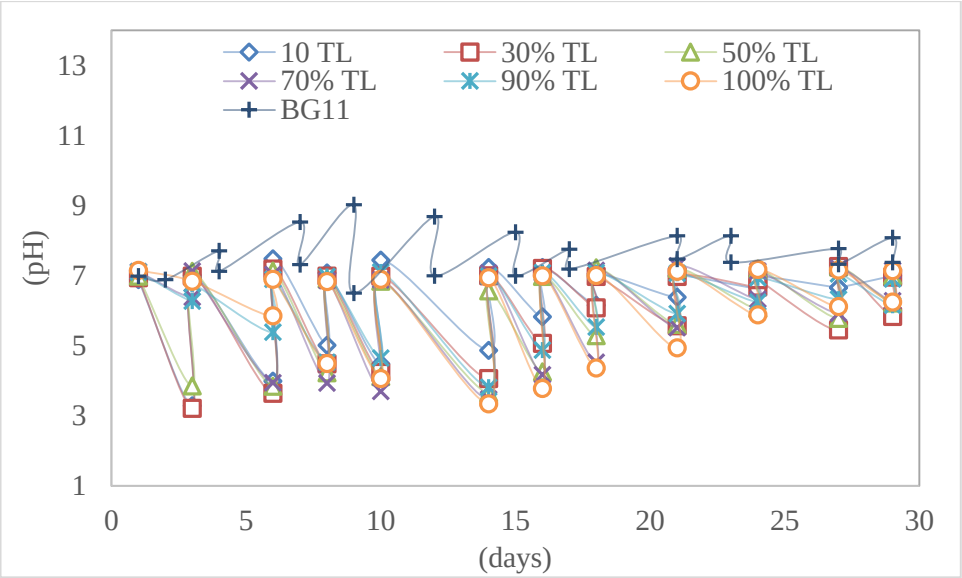


Figure 2.2b : pH dynamics in different dilutions of TL (10%-100%) in experimental set 2.

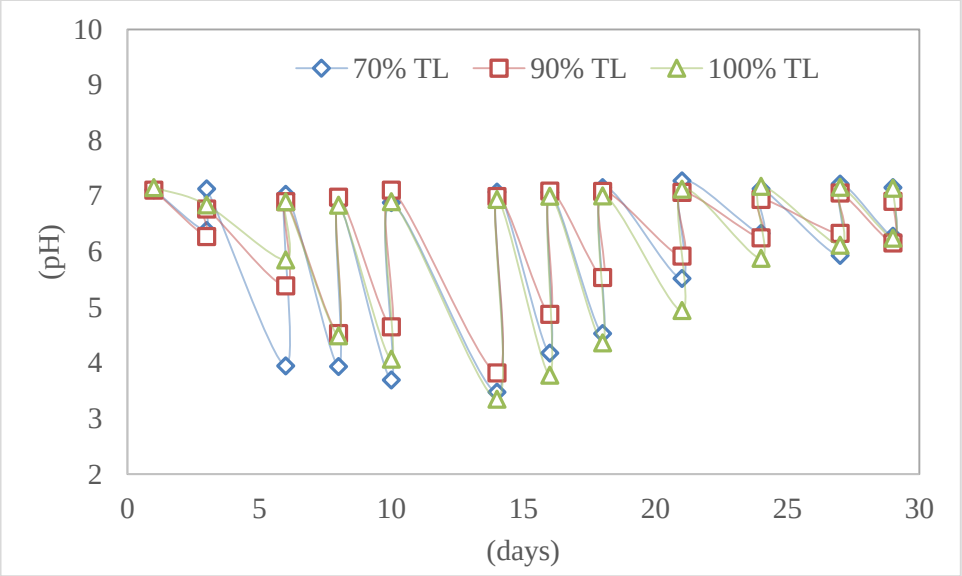


Figure 2.2c : pH dynamics in lower dilutions of TL (70%-100%) in experimental set 2.

Nitrifying organisms may assist algal cultures by removing high concentrations of NH_4^+-N that may be inhibitory to algal growth (by converting them into NO_3^--N, which is less toxic to microalgae). DeBashan et al. (DeBashan et al., 2004) observed an increase of nitrates in their study and concluded that natural resident microflora of wastewater converted NH_4^+-N to NO_3^--N. Nitrate was not checked in the present study. One of the reasons for not measuring NO_3^--N was that when NH_4^+-N is present, NO_3^--N assimilation by microalgae is inhibited. Upon depletion of ammonium, nitrate assimilatory pathway is resumed and nitrate transport into the cells can occur, which requires a net influx of hydrogen into the cell for reduction, which increases the medium pH (Scherholz and Curtis, 2013). There is also evidence that nitrifying autotrophic bacteria are inhibited by miroalgae. Choi et al. (Choi et al., 2010) observed the

growth of algae and cyanobacteria significantly inhibiting the maximum nitrification rate by a factor of 4 although the performance of the autotrophic reactor was not significantly affected. Nitrification inhibition was suggested to be due to the competition for common carbon source (i.e., carbon dioxide) and nitrogen source (mainly NH_4^+-N).

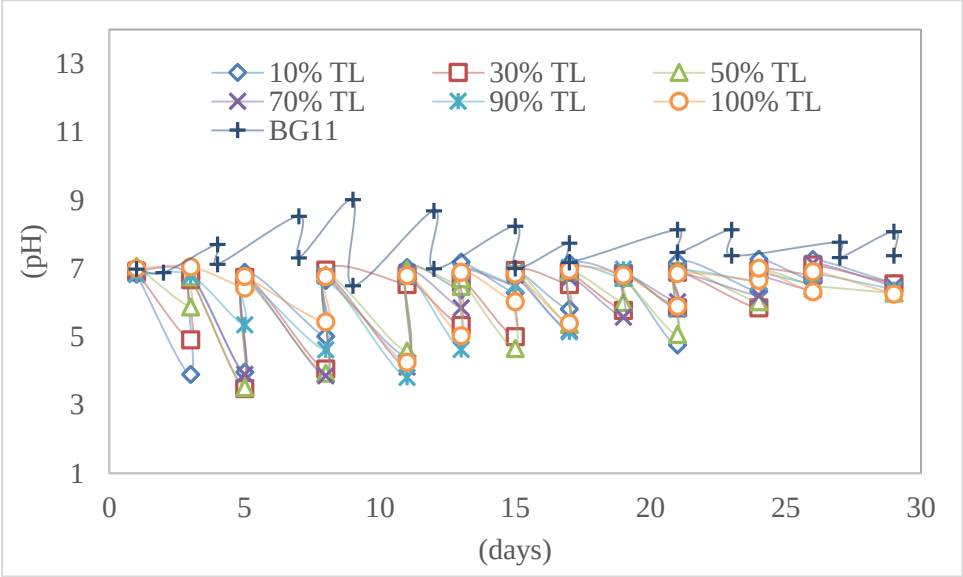


Figure 2.3b : pH dynamics in different dilutions of TL (10%-100%) in experimental set 3.

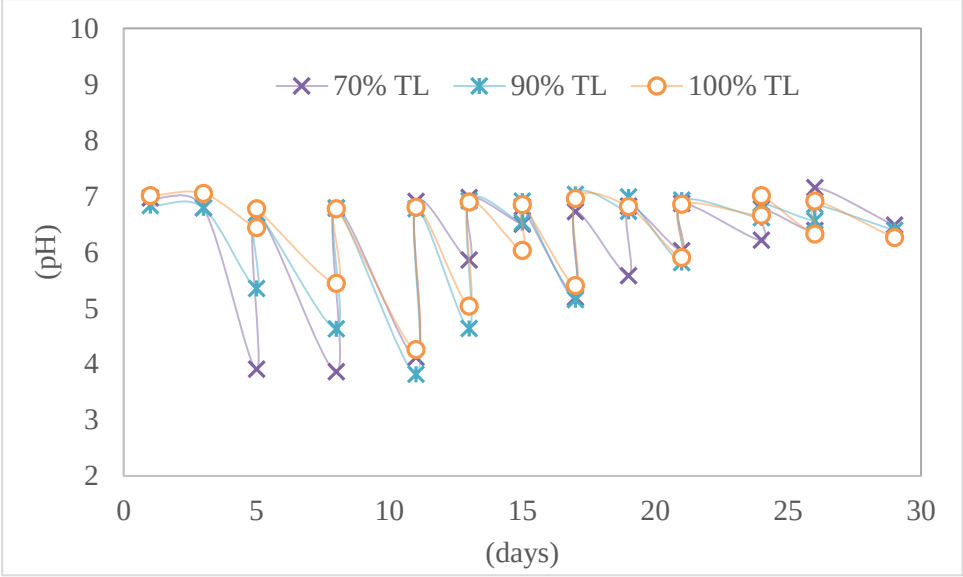


Figure 2.3c : pH dynamics in lower dilutions of TL (70%-100%) in experimental set 3.

2.2.3. Microalgal NH_4^+-N removal in the three experimental sets

Microalgae are able to assimilate a variety of nitrogenous compounds mainly in the order of: ammonium NH_4^+-N > nitrates NO_3^--N > urea etc. Ammonium is the preferred nitrogen source for algae because it is most energetically efficient source, since less energy is required for its

uptake (Perez-Garcia et al., 2011). Choi and Lee (Choi and Lee, 2013) observed that NH_4^+-N absorbed by chlorella vulgaris can be directly used, but the NO_3^--N cannot be used until it is doxidised to NH_4^+-N and the processes consume energy and reducing power. Therefore NH_4^+-N can be utilized rapidly at an early stage, which was in favour of chlorophyll synthesis (indirect biomass increase). But NH_4^+ can be inhibitory at high concentrations (as discussed in introduction section). Microalgae are sensitive to the combined effect of high NH_4^+-N and temperature. Excessive NH_4^+-N can damage photosynthesis organs (chlorophyll) and decrease photochemical efficiency (Choi and Lee, 2013).

In all the 3 experimental sets, maximum NH_4^+-N removal was observed in 10% TL (~ 50 mgL^{-1} NH_4^+-N) with 100% efficiency where in lack of nutrients also ceased microalgal growth (Figures 2.1a, 2.2a, 2.3a). After 10% TL, NH_4^+-N removal was systematically decreasing with increasing TL concentration (100 mgL^{-1} NH_4^+-N or more) (Figure 2.4). NH_4^+-N removal rates in the 3 experimental sets were higher in higher dilutions (10% and 30% TL) and lower in lower dilutions (50%-100% TL) which was also reported by lin et al. (Lin et al., 2007). In their study 10% leachate (135 mgL^{-1} NH_4^+-N) supported growth of three microalgal strains while higher concentrations suppressed their growth.

NH_4^+-N was not fully consumed by microalgae even after 29 days and showed a slow removal efficiency, which is the same as detected by Paskuliakova et al. (Paskuliakova et al., 2016) where 51% NH_4^+-N was removed after 24 days with initial NH_4^+-N concentration of 100 mgL^{-1}. This again implies leachate being a very complex mixture of compounds that needs time to be broken down into forms readily available for microalgal assimilation. Bacterial symbiotic relationship with microalgae plays an effective role in these situations as reviewed in introduction section 1.5. deBashan et al. (DeBashan et al., 2004) observed a significant increase in NH_4^+-N removal when chlorella species was co-immobilized with growth promoting bacteria with low initial ammonium concentration (0.1-4.26 mgL^{-1}). Zhao et al. (Zhao et al., 2014) evaluated microalgae-bacterial consortium grown in landfill leachate. Cultures grown in 10% leachate (183 mgL^{-1} NH_4^+-N) produced the highest dry biomass (1.58 gL^{-1}) with 90% NH_4^+-N removal in 12 days. Zhou et al. (Zhou et al., 2013) highlighted the importance of algal–bacterial consortiums for E2-Energy systems. Consortium of algae and bacteria grown in wastewater medium effectively removed both organics and nutrients. The lighted cultures had good removal of total soluble nitrogen TSN (86%) and NH_4^+-N (100%) respectively, with an initial NH_4^+-N concentration of ~100 mgL^{-1}. The dark controls had much lower nutrient removal efficiencies 18% for TSN, whereas NH_4^+-N actually increased by 31%. The increase in NH_4^+-N likely resulted from the breakdown of organic nitrogen into ammoniacal-N (ammonification). In dark cultures unused NH_4^+-N was found while in lighted cultures autotrophic algae continued to consume NH_4^+-N until it was gone at around 160 hours.

Autotrophic algal biomass production and total nutrient removal was enhanced by bacterial activity. In the present study, in set 1 where raw TL was used and bacterial presence did show a slight better effect (non significant visual observation) on growth curve but NH_4^+-N removal efficiency was not effected in all the dilutions (10%-100% TL) (Figures 2.1a, 2.4). In fact in set 1 NH_4^+-N removal was lower than in sets 2 and 3 (Figure 2.4). This could be due to the occuring of ammonification or phosphorus P limitation.

Primary source of carbon for autotrophic microalgal cells is carbon dioxide CO_2 from air, but they can also utilize bicarbonate ions (HCO_3^{-1}) as an inorganic carbon source for photosynthesis (Kim et al., 2013). Park et al. (Park et al., 2010) used anaerobic digestion effluent of livestock waste where inherent alkalinity (HCO_3^-) provided the necessary inorganic carbon source for microalgae. Scenedesmus cultures grew best in 100 mgL^{-1} NH_4^+-N (with alkalinity equivalent to 650-750 mgL^{-1} as $CaCO_3$) and increasing NH_4^+-N concentration from 200 to 500 mgL^{-1} decreased the cell density by 70%. Inorganic carbon (mostly HCO_3^{-1}) was finished within 2 days effecting growth (onset of stationary phase) and NH_4^+-N removal (13%). Supplying $NaHCO_3$ intermittently (as external inorganic carbon source) after day 2 steadily increased growth and NH_4^+-N removal (around 43%) in 8 days. Their results suggested that a certain level of alkalinity was necessary for a prolong ammonium removal. White et al. (White et al., 2013) observed a significant increase in nitrate utilization and growth of marine cultures of Nannochloropsis and Tetraselmis species by addition of bicarbonate salts $NaHCO_3$ (1 gL^{-1}). Their study suggested a species specific response to bicarbonate addition, with an optimum and threshold tolerance to bicarbonate addition above which cultures growth inhibition was recorded.

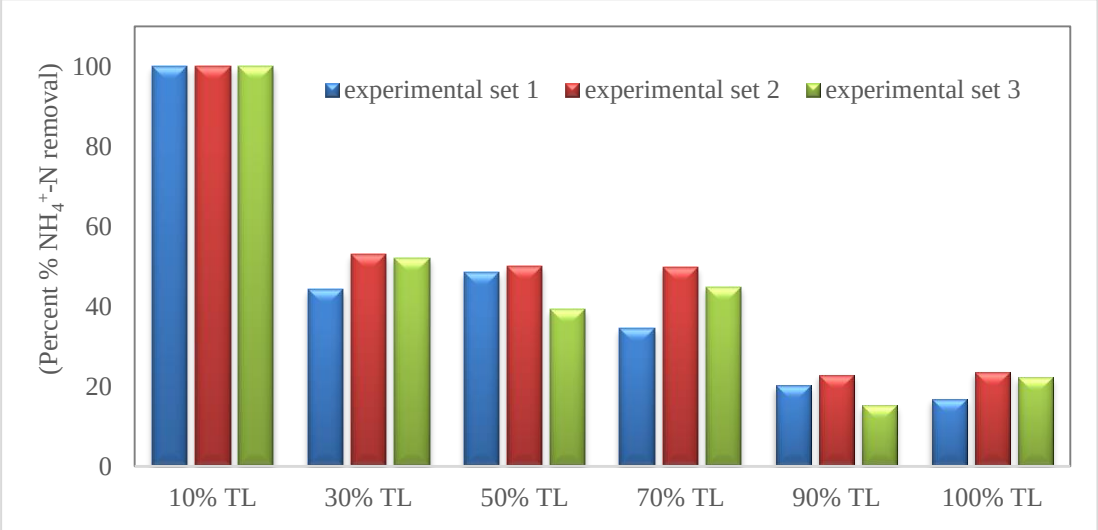


Figure 2.4 : Percent NH_4^+-N removal in the 3 experimental sets.

Intermitant addition of inorganic carbon in the form of $NaHCO_3$ in experimental set 3 slightly increased (not significant) the removal efficiency of NH_4^+-N in all the dilutions (Figure 2.4). High concentration of $NaHCO_3$ supplies increased levels of Na^+, which can inhibit fresh water species (Chi et al., 2014). This can explain to some extent, non significant biomass productivity and NH_4^+-N in set 3 when compared to sets 1 and 2, where high salinity was already putting pressure on microalgal cultures.

It can be inferred from the present study that NH_4^+-N removal among the 3 experimental sets was probably effected due to P limitation, where nitrogen N removal is said to be a function of P availability (Paskuliakova et al., 2016). Phosphate was recently found to be the rate limiting factor for NH_4^+-N removal from landfill leachate (Paskuliakova et al., 2016). The present study was conducted before the findings of Paskuliakova et al. (Paskuliakova et al., 2016) and also P was not added intentionally in the experimental sets to first check growth in leachate in its original form. The phosphorus limitation might have played a part in effecting NH_4^+-N removal efficiency in the present study, but even after addition of P in later experiments, it had some mixed effects on both growth and NH_4^+-N removal (data not shown). Since LFL is a very difficult wastewater stream to treat, lower dilutions with high concentrations of NH_4^+-N and other complex compounds often do not show a straight forward biological removal of pollutants.

2.3 Conclusion

In the present study irrespective of high salt concentrations (Cl^- 9000 mgL^{-1}), salinity (10 ppt) and dark color of leachate (pertaining to low light penetration), fresh water microalgae showed amoniacal tolerance and was able to grow and remove NH_4^+-N from TL in all the 3 experimental sets. 100% NH_4^+-N was removed in 10% TL (~ 50 mg/L NH_4^+-N) with removal efficiency decreasing with increasing leachate concentration. The different conditions provided in the 3 experimental sets had both inhibitory and stimulatory effect on microalgal growth and NH_4^+-N removal efficiency. Phophorus was expected to be the rate limiting nutrient for both microalgal growth and NH_4^+-N removal. Overall quality of TL was improved in terms of NH_4^+-N removal which is considered as one of the most persistant (long term) issue regarding leachate toxicity. Further screening for increasing microalgal biomass production and NH_4^+-N removal using leachate is required by closely monitoring NH_4^+-N and NO_3^--N removal dynamics.

3. GROWING FRESH WATER MICROALGAE IN HIGH AMMONIUM LANDFILL LEACHATE [1]

Wastewater treatment facilities have enough nutrient rich water available with excess of N and P in discharged wastewaters. Overloading of such nutrients may negatively impact receiving natural systems by creating nuisance algae (eutrophication), oxygen depletion and fish kills, undesirable pH shifts, and cyanotoxin production (Choi and Lee, 2013; Christenson and Sims, 2011). Conventional treatment of N from wastewater involves nitrification and denitrification via biological (bacterial) processes, which often lead to secondary contamination of the sludge byproduct, creating additional problems of safe disposal. The accepted standard nutrient removals still cannot be achieved and requires tertiary treatment (Gonçalves et al., 2017). Optimizing cost effective and energy efficient technologies for one-step tertiary treatment of wastewaters remain a problem for industries and municipalities (Choi and Lee, 2013; Christenson and Sims, 2011; Su et al., 2012).

Microalgal tertiary treatment (polishing) of secondary treatment effluent retains useful nitrogeneous or other waste compounds in the biomass and potentially achieve waste nutrient removal in an ecologically safer way with the added benefits of residual microalgal biomass resource recovery and recycling (Cabanelas et al., 2013; Choi and Lee, 2013; Christenson and Sims, 2011). Production of 1 ton of microalgal biomas requires approx. 40-100 kg N and 3-12 kg P and annual make up water volume for microalgae production is in the range of 11-13 ML/Ha/year, it is estimated that approx. 2500 m^3 wastewater can be treated to produce 1 ton of algal biomass (Lundquist et al., 2010). Successful implementation of combined microalgal wastewater treatment strategy and biomass production also allows for the minimizing of the use of freshwater, another precious resource especially for dry or populous regions (Christenson and Sims, 2011; Rawat et al., 2011).

Microalgae have been used in open ponds (raceways, natural lake, lagoon and artificial ponds etc.) and closed systems (fermenters, photobioreactors etc.) to treat different kinds of wastewaters

[1] This chapter is based on the paper " Zareen T.Khanzada and Süleyman Övez, Growing fresh water microalgae in high ammonium landfill leachate, American Journal of Mechanics and Applications, 2018, 6 (2), 50-61."

(Cabanelas et al., 2013; Christenson and Sims, 2011; González-fernández et al., 2011; Moheimani and Borowitzka, 2006; Mustafa et al., 2012; Nwoba et al., 2016; Olguín, 2012). Open pond system resemble natural aquatic ecosystem which rely on symbiotic relation between algae and bacteria to degrade environmental pollutants (Crane and Grover, 2010; Unnithan et al., 2014; Wang et al., 2014). Microalgae-bacterial consortia can break down various nitrogenous compounds from wastewater and show tremendous potential in treating wastewaters (Jia and Yuan, 2016). Current microalgal production is 90% from open ponds because of low energy requirements, minimal maintenance, few operating costs and little overheads when compared to photobioreactors (González-fernández et al., 2011; Rawat et al., 2011). Since open ponds present relatively low construction and operating costs, they can be constructed on degraded and marginal non-agricultural lands that avoid use of crop producing areas (Perez-Garcia et al., 2011). However, there are some limitations that pose challenges in mass open pond cultivation of competitive species, contamination by predators (grazers such as ciliates, rotifers and vorticellas etc) and heterotrophs (bacteria) are major disadvantage in these systems (Park et al., 2011). Dry biomass attained is usually low (0.25 - 1 gL^{-1}) when compared to photobioreactors (1.5 - 1.7 gL^{-1}) (Christenson and Sims, 2011). Since raceways require more water than photobioreactors to produce the same amount of biomass, it presents another economical benefit to use wastewater for open pond mass cultivation of microalgae (Park et al., 2010).

TL was collected in an attempt to use it as a nutrient resource and evaluated in laboratory and onsite pilot scale raceway pond cultivation, for its ability to support growth of indigenous microalgal species coupled with NH_4^+-N and NO_3-N removal as a means of sustainable tertiary treatment.

3.1 Experimental

Fresh water indigenous microalgal cultures of Chlorella vulgaris and Chlamydomonas reinhardii were obtained from Ege University, Izmir. 20% (v/v) exponentially growing inoculum measured at an absorbance of 680 nm (using spectrophotometer, model U-2001, HITACHI, Japan) was used to start and monitor microalgal growth in the lab study. Treated leachate TL (effluent of ultra-membrane filtration) was kindly provided by Odayeri and stored at 4 ^{0}C in 20 L air tight plastic containers in dark until use. Physico-chemical parameters of TL is presented in Table 1. 1L glass bottles with 500 ml working volume, continuous air bubbling supplied at a rate of 3 $Lmin^{-1}$ (flow rate in each 500 ml culture was ~0.31 ml min^{-1}), continuous artificial irradiance of 55 µmol photon $m^{-2}s^{-1}$ through white fluorescent lamps (measured by a digital quantum meter –Model MQ-200 Apogee, USA) and maintained at room temperature of 24-25 ^{0}C. pH was maintained between 6.5-7.5 manually on alternate days with 0.5N H_2SO_4/ NaOH. For lab study a set of different dilutions

of autoclaved TL (20 min at 121 ^{0}C) was made with distilled water dw, i.e., 10%, 30%, 50%, 70%, 90%, 100% TL; where dw was also set as negative control and regular BG11 UTEX media as positive control. The cultivation was carried out under batch mode in duplicate for 4-8 weeks.

3.1.1 Raceway pond cultivation

Raceway pond cultivation was carried out onsite (Odayeri landfill) near ultra-membrane filtration (UF) plant. Two ponds with 200 L capacity each, were single loop raceways mixed with paddle wheels operated by a gear motor. The raceway ponds were placed inside a glass house covered with a transparent corrugated acrylic roof following a greenhouse concept to reduce the chances of contamination by other microbes or indigenous algal species and environmental stresses (fluctuations in temperature, rainfall and winds etc.). Surface area of each pond was 13.8 m^2 with a culture depth of 15 cm. Initial inoculum was $0.5x10^5$ cells ml^{-1} (counted using haemocytometer). A total of five batch cultures were run for a year from September 2014 to september 2015 (Table 2). 50ml samples were taken twice a week from each pond after water evaporation correction (by adding tap water upto the marked level), and filtered through 0.45 μm glass fiber filters and stored at -4^{0}C for later analysis of NH_4^+-N, nitrate-nitrogen NO_3^--N, total organic carbon TOC and inorganic carbon IC. In addition dissolved oxygen DO, pH, temperature, conductivity, salinity, irradiance were monitored on the day of sampling (twice a week). For raceway pond cultivation P was externally added (K_2HPO_4 solution) to make N: P ratio 15 since TL was phosphorus limited (Table 3.1).

Table 3.1: Physico-chemical characteristics of UF treated TL characteristics

Parameter	(mgL^{-1})	**Parameter**	(mgL^{-1})
BOD5	85	Potassium	2710
BOD5/COD	0,22	Mercury Hg	5,9
TOC	386	Lead Pb	5,15
Ortho-PO_4^-	5,42	Cadmium Cd	4,66
TKN	800	Zinc Zn	4,88
NH_4^+-N	760	Nickel Ni	4,42
alkalinity	700	Copper Cu	4,44
Chloride	9000	Cromium Cr	3,78
Sodium Na	1540	Sulphur S	17,54
pH	7	Calcium Ca	1,5
Salinity ‰	10	Magnesium Mg	3,26
Conductivity (EC) mS/cm	10	Iron Fe	11,09

3.1.2 Analytical methods

Dry weight was determined after oven drying the centrifuged microalgal paste at 60°C in oven until constant weight was reached. NH_4^+-N, NO_3^--N and chloride ions Cl^{-1} were measured using an ion-selective electrode (Orion 95–12) with an Orion IonAnalyser 701A meter (Orion Research Inc., Boston, MA). Ortho-Phosphate P-PO_4^- concentration was determined following standard methods SM 4500- PBD (Rice et al., 2012). TOC and IC were measured by Shimadzu TOC analyser-5000A and Carbon oxygen demand COD by open reflux method ISO 6060 (Rice et al., 2012) . DO, pH and temperature were measured by digital meter (Multi 3420- WTW Germany). Conductivity EC and salinity were measured by portable ISE meter (HACH HQ40d). Heavy metals were measured by Inductively Coupled Plasma (ICP) Optical Emission Spectrometer (Perkin-Elmer Optima 7000DV ICP-OES). The data was statistically analysed by Student's t-test comparing the treatments at $P \leq 0.05$ using microsoft excel. Averaged values are presented here.

3.2 Results and Discussion

3.2.1. Microalgal growth and NH_4^+-N removal dynamics in lab study

Microalgae are able to assimilate a variety of nitrogen sources from an aquatic environment, mainly ammonium (NH_4^+), nitrates (NO_3^-) and urea etc. NH_4^+-N is the most preferred nitrogen source for microalgae growth since it is in a reduced state and less energy is required for its metabolic uptake by microalgae (active transport at the plasma membrane), which also makes it energetically more efficient source (Perez-Garcia et al., 2011). NH_4^+-N is generally thought to directly assimilate into the glutamic acid (amino acid) pathway and release H^+ ions, which can reduce medium pH (Jia and Yuan, 2016; Scherholz and Curtis, 2013). In the lab study, microalgae were observed to be growing in all the dilutions of TL but growth was significantly reduced as compared to regular nutrient media BG11 (Figure 3.1). Microalgae growth showed a lag phase of atleast 2-3 days in the lower dilutions (50-100% TL) even after isolates were pre-acclimated to leachate medium for 3-4 weeks prior to starting the experiment. High salinity (~10 gL^{-1}), brown color and imbalance nutrient composition (C: N: P) of leachate seemed to have delayed the growth of cultures and increased lag phase in all the treatments (Figure 3.1; Table 3.1). Zhao et al. (Zhao et al., 2014) also observed a lag phase of 6 days for 20% leachate (338 mgL^{-1} NH_4^+-N) in their study.

Stimulatory effect on growth in higher dilutions (10-30% TL) was observed with respect to distilled water (negative control) and inhibitory effect was observed for lower dilutions (50-100%

TL) with respect to BG11 media (positive control). In higher dilutions (10-30% TL) growth curves showed an onset of stationary phase on reaching day 10, and for 10% TL growth curve stayed closer to negative control (dw) due to reduced or imbalanced nutrients (Figure 3.1). Lab experiment was terminated for higher dilutions (10-30% TL) on reaching day 30th after no further signs of growth and nutrient removal were observed. Overall microalgae in 50% TL (415 mgL^{-1} NH_4^+-N) showed better biomass production (1.5 gL^{-1} dry biomass) and clear growth curves (lag, log and stationary phases) (Figure 3.1; Table 3.2). In lower dilutions (70-100% TL) stationary phase was not obvious and microalgae seemed to be growing slowly and gradually as shown by the growth curves after reaching day 30th (Figure 3.1). The experiment was continued (for 50-100% TL) to further evaluate the dynamics of nutrient removal since cultures were still green which implied that leachate (with high NH_4^+-N ~760 mgL^{-1}) was not lethal to microalgae. Microalgae tolerated leachate and continued to sustain life until termination of experiment (on day 60^{th}) in the extended lower dilutions (50-100% TL) and no apparent rupture of cells was observed under microscope. Dry weights, growth rate and areal biomass productivity of lab study and raceway cultivation are presented in Table 3.2.

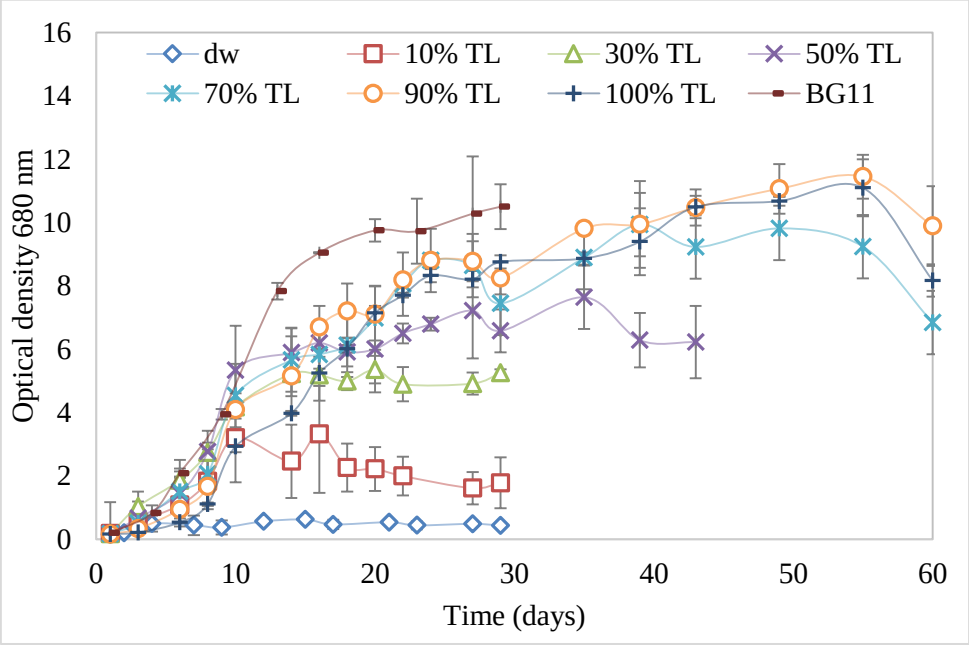


Figure 3.1 : Microalgal growth curve in the lab study (data shown is the mean ± SD, n=2).

Table 3.2 : Dry biomass produced in lab study and raceway cultivation.

Raceway pond cultivation	Biomass dry wt. (gL^{-1})	Areal productivity ($gm^{-2}d^{-1}$)
1st run (a) - (40 days)		
25th September, 2014 - 11th November, 2014	0,85	0,31
1st run (b) - (50 days)		
17th October, 2014 - 4th December, 2014	1,03	0,64
1st run (c) - (32 days)		
3rd November, 2014 - 4th December, 2014	0,93	0,5
2nd run (a) - (43 days)		
25th May, 2015 - 6th July, 2015	0,79	0,34
2nd run (b) - (54 days)		
28th July, 2015 - 19th September, 2015	0,68	0,231
Lab experiment (20th day for 10-30%, 30th day for 50-100% TL)	Biomass dry wt. (gL^{-1})	Growth rate per day (μ)
10% TL	0,46 ± 0,049	0,013
30% TL	0,67 ± 0,098	0,024
50% TL	1,5 ± 0,200	0,043
70% TL	1,43 ± 0,272	0,041
90% TL	1,14 ± 0,343	0,031
100% TL	0,91 ± 0,438	0,02

The slow upward growth curve observed in lower dilutions (70-100% TL) could also be due to nutrient stress with leachate being inconsistent in available nutrient composition (regarding C:N:P), high concentration of NH_4^+-N and ionic salts (like Cl^-, Na^+, K^+ etc.) or possible inhibition by some hidden element of leachate (heavy metal toxicity or xenobiotic toxicity) (Table 3.1). Tam and wong (Tam and Wong, 1996) also did not observe a proper stationary phase when NH_4^+-N concentration was increased from 125 to 1000 mgL^{-1}. Concentrations at which ammoniacal-nitrogen (NH_4^+-N-NH_3) toxicity becomes inhibitory varies greatly with individual algal species and culture conditions (Choi and Lee, 2013). Osada et al. (Osada et al., 2011) reviewed the specific toxicants masked by NH_3 toxicity. NH_3 toxicity contributed to 58.7% (by volume) and other toxicants to 41.3% of the total toxicity of the LFL. Lin et al. (Lin et al., 2007) observed optimum growth in only 10% leachate (135 mgL^{-1} NH_4^+-N) which was significantly lesser than control group in 12 days. More than 670 mgL^{-1} NH_4^+-N suppressed the growth of tested

microalgae. Choi and Lee (Choi and Lee, 2013) observed that at low N concentrations microalgal growth was limited in terms of chlorophyll synthesis, but in higher N concentrations chlorophyll was not increased after a threshold level.

Biomass produced in all the dilutions in the present study (Table 3.2) is similar to Edmundson and Wilkie (Edmundson and Wilkie, 2013) (0.55 g L^{-1} day). Dry weight observed in their study was 1.33 gL^{-1} similar to control group (BBM medium) 1.42 gL^{-1} in 4 days. They suggested leachate to have enough N source (980 mgL^{-1} NH_4^+-N) to support microalgal growth but limited total phosphorus content 13.2 mgL^{-1}. Sforza et al. (Sforza et al., 2015) observed highest dry biomass (1.5 gL^{-1}) in 10% leachate (216 mgL^{-1} NH_4^+-N) with 97% ammonia removal. Phosphorus (3.56 mgL^{-1}) was not externally added and was suggested to be the limiting nutrient for growth. Zhao et al. (Zhao et al., 2014) cultured bacteria-algal consortium in leachate. Highest biomass (1.58 gL^{-1}) was observed for 10% leachate (183 mgL^{-1} NH_4^+-N) under P starvation with 99% NH_4^+-N removal in 12 days. 52% of the removed ammonia was attributed to microalgal biological uptake based on nitrogen content of biomass. 20% leachate (338 mgL^{-1} NH_4^+-N) had minimum dry biomass production (0.94 gL^{-1}) but 95% NH_4^+-N removal. Ammonia stripping (volatilization) at high pH (↑ 8) and bacterial removal was suggested to be the cause for that ammonia removal. Cheng and Tian (Cheng and Tian, 2013) observed microalgal growth in leachate diluted to 10% (90.5 mgL^{-1} NH_4^+-N) with highest biomass and volumetric productivity (0.75 mgL^{-1} and 37 $mgL^{-1}day^{-1}$) in 20 days. Higher leachate concentrations were inhibitory. They observed no growth in 20% leachate but ammonia removal was 11.8%. Ammonia stripping under alkaline conditions (pH 8.5 and above) was suggested to be the reason for NH_4^+-N removal apart from microalgal assimilation.

In the present study NH_4^+-N removal was continued from media slowly and steadily until reaching day 20th in higher dilutions (10-30% TL) which was 10 days after reaching stationary phase (~ day 10th), which implied that NH_4^+-N was removed from the system even after growth (new cell formation) was ceased and used for culture's cell maintainance (Figure 3.2). In lower dilutions (50-100% TL) NH_4^+-N removal also continued 10 days further after reaching stationary phase (day 20th) and reached its maximum on day 30th (Figure 3.2). NH_4^+-N removal was below 70% in all the dilutions with 50% TL showing maximum removal 66.27% (Figure 3.2). NH_4^+-N was not fully removed from the system in all the dilutions tested and irrespective of enough nitrogen present in the medium, it was not taken up by miroalgae (Figure 3.2), which could be due to phosphorus P limitation, which is recently considered as rate limiting factor for NH_4^+-N removal from leachate (Paskuliakova et al., 2016). Tam and wong (Tam and Wong, 1996) observed the same trend where residual ammonia (50%) gradually increased in the medium with increase in initial nitrogen concentration from 80 mg L^{-1}. They observed that higher initial N had lower

removal efficiency and vice versa. Su et al. (Su et al., 2012) also observed the same pattern with high strength wastewater led to poor nutrient removal.

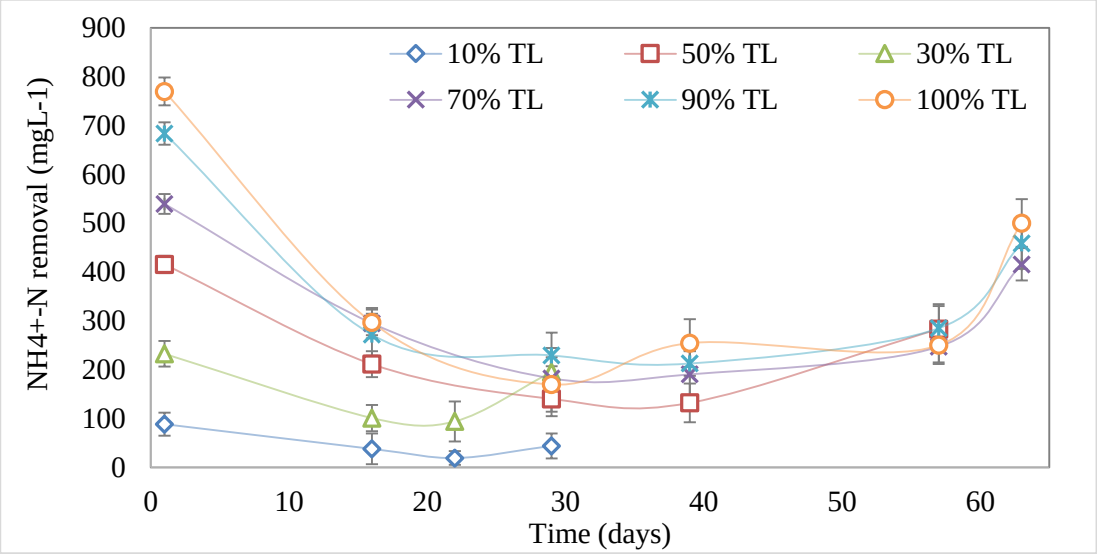


Figure 3.2 : NH_4^+-N removal in the lab study (data shown is the mean ± SD, n=2).

In the present study, lab experiment was not terminated even after cultures (in all the tested dilutions) reached stationary phase, since NH_4^+-N removal was continued, so experiment was prolonged until an interesting and obvious fact was observed. NH_4^+-N started to increase in all the dilutions (day 20^{th} onwards in 10-30%TL and day 30th onwards in 50-100% TL) (Figure 3.2). Zhou et al 2013 (Zhou et al., 2013) also observed increase in NH_4^+-N concentration by 31% in dark cultures. The increase was suggested to be the conversion of organic nitrogen into NH_4^+-N. He et al. (He et al., 2007) evaluated that the effluent NH_4^+-N concentration sometimes exceed the corresponding influent NH_4^+-N concentration, due to ammonification of organic nitrogenous compounds under anaerobic (low oxygen) condition. In the present study batch cultures were prolonged beyond cultures capacity to further grow (nutrient limitation) and remove nutrients from leachate medium (with already imbalanced nutrient concentration). Decomposers (bacteria) feeding on dead algal cells or ammonification of microalgal exudates (extra-polymeric substances EPS) might be increasing the NH_4^+-N concentration in the medium (Danger et al., 2007; Unnithan et al., 2014). Wang et al. (Wang et al., 2014) observed that microalgae produced more EPS containing proteins (in large proportions) when grown in high N wastewater.

3.2.2 NO_3-N removal dynamics in lab study

NO_3^--N is thermodynamically more stable (oxidized form) and is the more common form of inorganic nitrogen in aquatic environments. However Choi and Lee (Choi and Lee, 2013)

observed that NH_4^+-N assimilated by chlorella vulgaris can be directly used, but the NO_3^--N cannot be used until it is oxidised to NH_4^+-N and the process consume energy and reducing power. Therefore NH_4^+-N can be utilized rapidly at an early stage, which was in favour of chlorophyll synthesis (indirect biomass increase). In the present study NO_3^--N concentration was fluctuating during the course of the study but in the end remained more or less similar to initial concentration, which also suggested that microalgae tried to consume NH_4^+-N first and NO_3^--N was not utilized (Figure 3.3). Scherholz and Curtis (Scherholz and Curtis, 2013) observed that NO_3^- -N assimilation is inhibited by microalgae in the presence of NH_4^+-N and once NH_4^+-N is depleted, microalgal NO_3^- -N assimilation can occur. Since NH_4^+-N was already in excess and still present in the medium until the termination of experiment (~day 60), fluctuation in NO_3^- -N concentration might be only due to bacterial nitrification (conversion of NH_4^+-N into NO_3^- -N). Nitrification is a common process carried out by autotrophic bacteria which do not need organic carbon but consume a large amount of oxygen (González-fernández et al., 2011; Jia and Yuan, 2016).

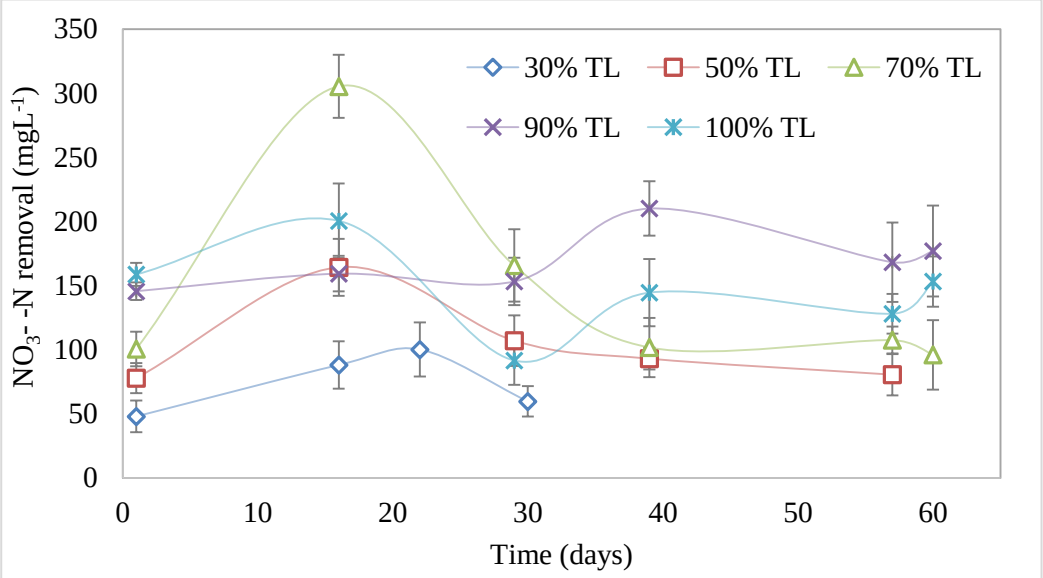


Figure 3.3 : NO_3^--N removal in lab study (data shown is the mean ± SD, n=2).

In the present study cultures were not axenic and continuous light and oxygen supply might have favoured nitrification to some extent but not to large extent since NH_4^+-N (which is the substrate for nitrification) was still present in the medium until the termination of experiment. The reason for this could be that nitrifiers (bacteria) are sensitive species and can wash out from system easily with fluctuating environmental conditions. Also bacteria have high compititive ability for P and when P and other nutrients become limited in a medium, nutrient removal dynamics by a phytoplankton community change (Crane and Grover, 2010; Danger et al., 2007).

3.2.3 Raceway pond cultivation and nutrient removal

In the present study, lab scale screening experiment was followed by onsite (Odayeri-Istanbul municipal landfill) open raceway pond (200L) micoalgae cultivation. In the lab study, irrespective of P limitation (inherent in TL medium) microalgae were able to grow in different dilutions of TL but for raceway cultivation, culture was continuously colapsing until P was externally added (N: P 15). The TL used in the present study was phosphorus deficient (~5 mgL^{-1} $P\text{-}PO_4$) as compared to nitrogen (~760 mgL^{-1} NH_4^+-N). Overall growth was reduced due to open cultivation where environmental conditions were not as controlled as in lab experiment (Figure 3.4; Table 3.2). Similar to lab study, high NH_4^+-N concentration was tolerated in raceway cultivation but growth was significantly reduced (Figure 4). Ayre et al. (Ayre et al., 2017) observed a 21% reduction in open pond biomass productivity when doubling the NH_4^+-N load from 800 to 1600 mgL^{-1}. Nwoba et al. (Nwoba et al., 2016) observed a decline in cell density for 2 days after inoculating the pond, but the cultures started to grow until reaching stationary phase on day 16 (Chlorella density reached to 51.8 $x10^6$ cells ml^{-1}).

The present raceway study was conducted with batch cultures (5 separate leachate batches delivered directly from ultra-membrane filteration UF plant). Research studies show that continuous (or semi-continuous) culturing mode is more economical for microalgal mass cultivation but could face some limitations because of repeated culturing. Moheimani et al. (Moheimani and Borowitzka, 2006) suggested that outdoor raceway pond can operate for at least 10 months on semi-continuous mode. Optimization in this manner can reduce economic burden, but some microalgal species can be maintained in continuous mode but others cannot. Monocultures cannot be maintained and the failure of open pond culturing is mainly because of contamination by bacteria, protozoa and other algae. Ayre et al. (Ayre et al., 2017) observed appearance of pennate diatom towards the end of semi-continuous growth of mixed species, which was not in batch mode. Nwoba et al. (Nwoba et al., 2016) also observed an increase in cyanobacteria contamination in semi-continuous mode. Godos et al. (Godos et al., 2009) observed a fluctuating dominance of algal species while treating high organic carbon wastewater (~2000-7000 mgL^{-1}) in continuous mode HRAP.

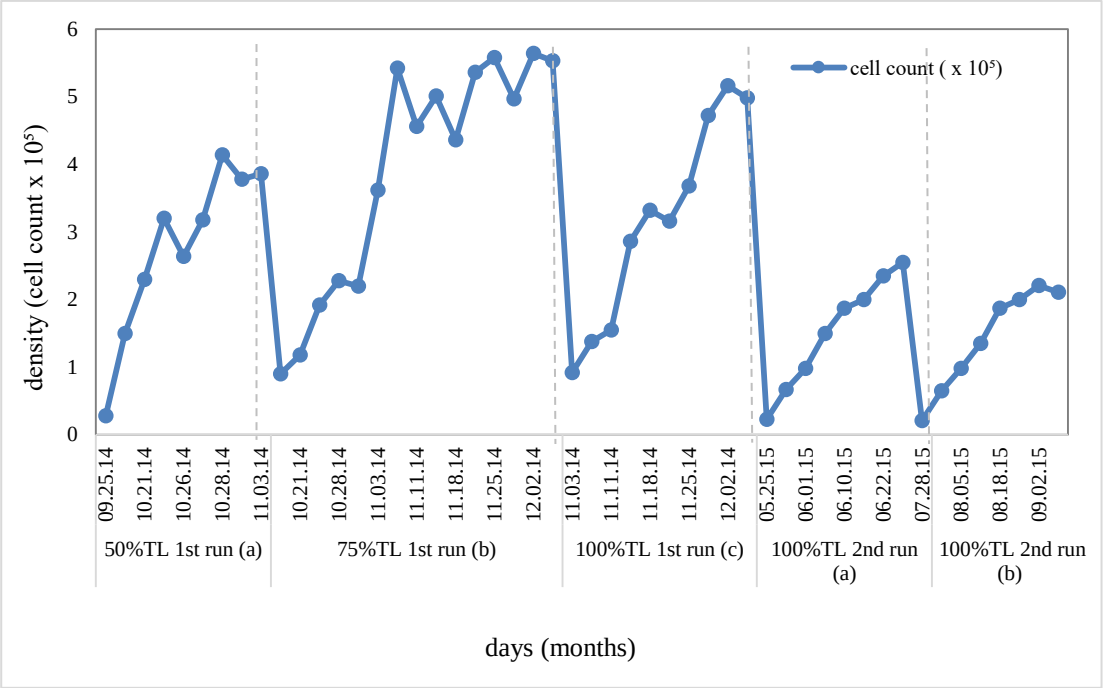


Figure 3.4 : Microalgal growth in raceway pond during five batches from Sept, 2014 to Sept. 2015.

Another issue with continuous culturing is that as the microalgal colonies age and mature, they secrete more organic matter (extra-cellular polymeric substances EPS) which can accumulate and favour heterotrophic bacteria and start competition and contamination, reducing microalgal dry biomass production and nutrient removal (Danger et al., 2007). EPS are negatively influenced by nutrient availability and microalgae growing in P depleted, high N media release EPS (containing polysaccharides) (Danger et al., 2007; Unnithan et al., 2014; Wang et al., 2014). Excess oganic matter accumulation (such as in continuous mode) can also induce autoinhibition of monocultures (Moheimani and Borowitzka, 2006). Present study had no incidences of significant protozoa or other foreign microalgae contamination, probably due of glass room coverage but high salinity and alkalinity of TL also kept the contamination in check (Moheimani and Borowitzka, 2006).

In the present study NH_4^+-N concentration in the open raceway cultivation kept on fluctuating in all the five batches (Figure 3.5) which was not observed in the lab study, where after reaching certain minimum value NH_4^+-N concentration successively increased (Figure 3.2). Fluctuation of NO_3^--N was not as pronounced as NH_4^+-N (Figure 3.2b) and no significant NH_4^+-N removal was observed in the raceway cultivation. Fluctuating NH_4^+-N concentration (between 0.98-14 mgL^{-1}) was also observed by Mustafa et al. (Mustafa et al., 2012) in out door high rate algal pond HRAP via consortium. The biomass productivity was significantly different in the tested two ponds following semi-continuous cultivation but nutrient reduction was not significant. Molinuevo-Salces et al. (Molinuevo-salces et al., 2010) observed a drop in NH_4^+-N removal by increasing

NH_4^+-N load in open pond treating swine slurry. The observed fluctuating NH_4^+-N concentration in the present study could be because of nitrification process as He et al. (He et al., 2007) also observed fluctuating NH_4^+-N concentrations in sequential anaerobic–aerobic process of landfill leachate biological (bacterial) nitrogen removal.

Nitrogen recovered from wastewater into algal biomass (assimilation) can be utilized and recycled but this advantage is frequently over-estimated since NH_3 stripping (volatilization at high temperature and alkalinity) together with nitrification/ denitrification has also been described as a possible N removal mechanism from open pond systems. Gonzalez-Fernandez et al. (González-fernández et al., 2011) evaluated that high N removal from the medium does not always correspond to high N recovery. They observed 6-7 fold increase in NH_3 volatilization by increasing NH_4^+-N loading rate in the pond and ponds fed with fresh pig slurry had higher extent of NH_3 volatilization. High pH of the system mediated high NH_3 volatilization. Open ponds operated in real condition presented lower NH_4^+-N removal. Main cause of NH_4^+-N removal from fresh pig slurry was denitrification. Ayre et al. (Ayre et al., 2017) compared NH_3 loss from culture media against algal biomass production and observed significant amount of N being lost from the system without being assimilated into algal biomass. Nitrification/denitrification and NH_3 stripping was suggetsed to be the cause of NH_4^+-N removal. Molinuevo-Salces et al. (Molinuevo-salces et al., 2010) observed NH_3 stripping to be the main cause for NH_4^+-N removal followed by nitrification when treating high NH_4^+-N (1600 mgL^{-1}) anaerobically digested swine slurry in open ponds. Godos et al. (Godos et al., 2009) observed 88% TKN (total kjeldahl nitrogen) removal from HRAP treating pig slurry in continuous mode. NH_4^+-N was continuously eliminating from the system but NO_3^--N remained high (33-46 mgL^{-1}) than the initial concentration and further increased in later stages of continuous mode. NO_3^--N fluctuation during last stages of cultivation was more pronounced where bacterial contamination increased from 625 mg to 1180 mg $VSSL^{-1}$. Nitrification was suggested to be the main cause of NH_4^+-N removal from the system. According to mass balance measurements 22% of influent N was assimilated into algal biomass. In the present raceway pond study, water temperature in the pond was below 30 ^0C in all the batch runs and pH was constantly decreasing below 7 (Figure 3.7), since NH_3 stripping occurs at high pH (above 8) so no NH_3 stripping could have been occured. Dissolved oxygen DO was above 8 mgL^{-1} O_2 (Figure 8) so denitrification process (which requires no or less than 0.2 mgL^{-1} O_2) was also ruled out as well. The only N removal mechanism apart from microalgal biomass assimilation was nitrification as raceway cultivation conditions were ideal for nitrification (excess of O_2 and NH_4^+-N). But since there was NH_4^+-N still present in the ponds by the end of each batch, it can be inferred that nitrification was not carried out at its fullest (as described earlier in section 3.2.3).

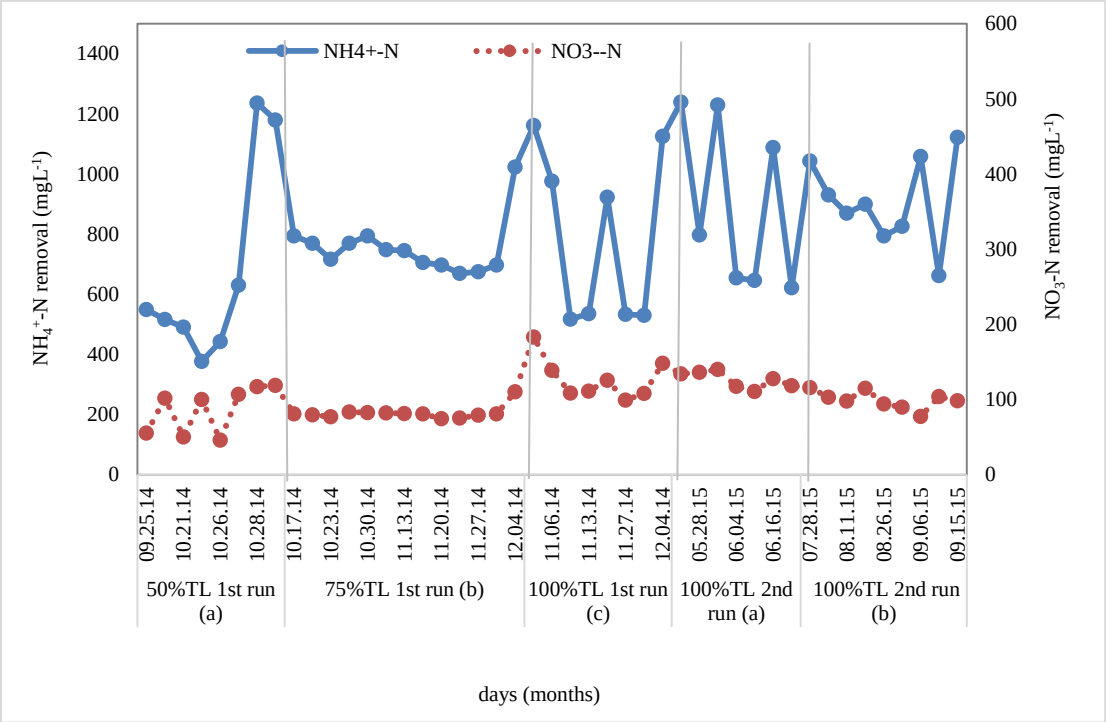


Figure 3.5 : NH_4^+-N and NO_3^--N removal dynamics in raceway pond cultivation.

3.2.4 Total organic carbon TOC and inorganic carbon IC removal in raceway cultivation

In raceway pond cultivation inorganic carbon IC remained minimum and unchanged throughout the 5 batches, but total organic carbon TOC concentrations were fluctuating and almost always higher than initial concentration by the end of each batch (Figure 3.6). Organic carbon in TL was mostly inert and recalcitrant based on BOD_5/COD ratio (Table 3.1) and not available for microalgal biomass assimilation. Microalgal and bacterial EPS could be the cause of the observed increasing TOC concentration. Prolonged batch culturing (more than 30 days) might have been a reason for TOC (or EPS) increase in the medium (Figure 3.6). Microbial EPS can reach over 40 gL^{-1} under biotic and abiotic stresses while adapting to extreme environments (Donot et al., 2012). Wang et al. (Wang et al., 2014) observed no soluble COD removal in a 15 days experiment using a mixture of sludge centrate and primary effluent (anaerobically digested sludge). Mustafa et al. (Mustafa et al., 2012) observed a fluctuating COD content in their semi-continuous HRAP pond cultivation treating leachate. High COD (150 mgL^{-1}) still remained in the system. Molinuevo-Salces et al. (Molinuevo-salces et al., 2010) observed more than 50% COD removals in open pond semi-continuous cultivation treating swine manure. But COD removal was concomitantly decreasing with increasing NH_4^+-N load. Godos et al. (Godos et al., 2009) observed a COD removal of 76% in continuous HRAP operated for 9 months. But this removal was not only from algal assimilation but also from other COD removal mechanisms such as aggregation, sedimentation of particulate organic matter together with algal bacterial flocs. Organic matter

removal is enhanced at high temperatures (↑30^{0}C) (Riano et al., 2011) but since temperature in the current raceway study was not high so this could also be a reason for no significant reduction in TOC (Figure 3.6). The only inorganic C source for microalgae in the present study was CO_2 (from air) and inherent alkalinity of TL used (Table 3.1). Nwoba et al. (Nwoba et al., 2016) did not observe any increase in biomass after the addition of inorganic carbon ($NaHCO_3$) in open raceway pond when compared with biocoil reactor.

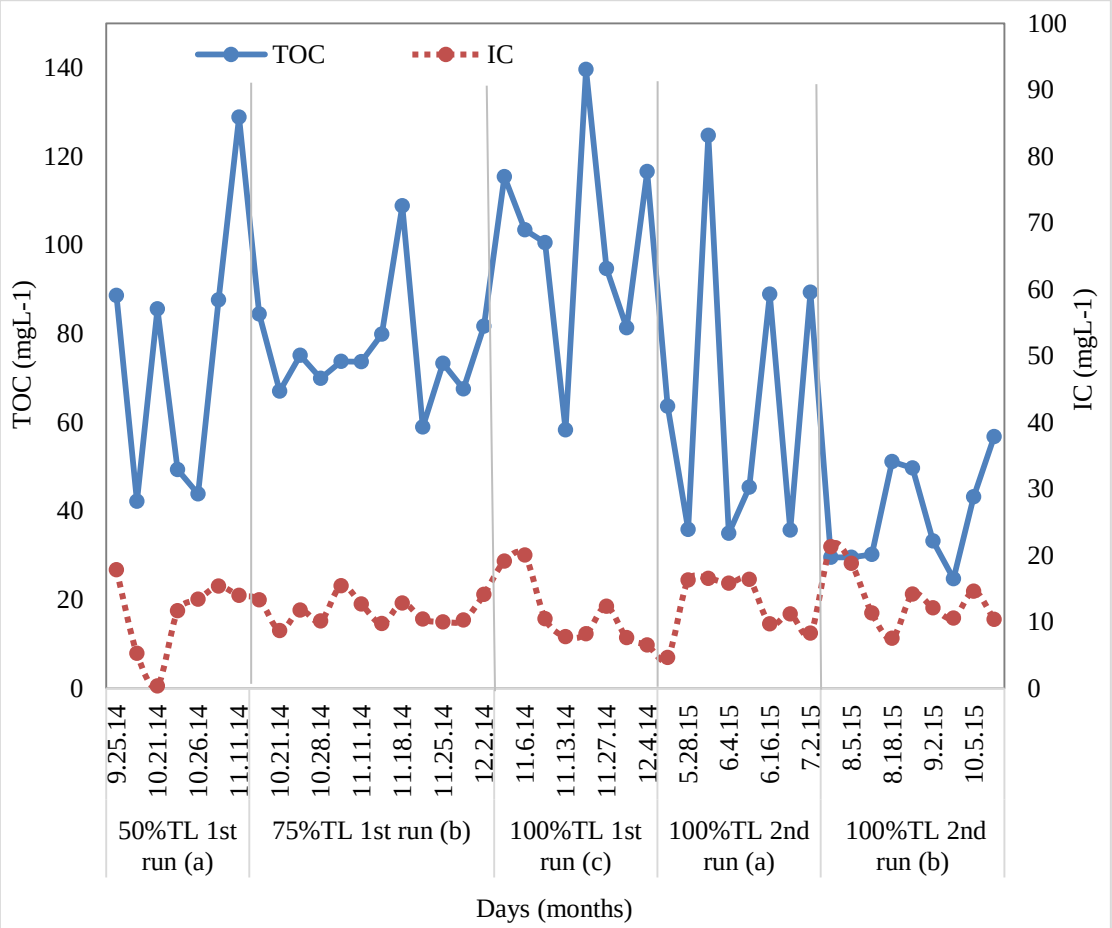


Figure 3.6 : TOC and IC removal dynamics during raceway cultivation.

3.2.5 Environmental data of other parameters for raceway pond cultivation

Environmental factors such as light, temperature and pH have been described as the main parameters affecting affluent treatment and biomass productivity in open ponds (Gonçalves et al., 2017; González-fernández et al., 2011). Light penetration within culture broth is of extreme importance for microalgae photosynthetic activities. Microalgal species are found ubiquitous in nature and cultivation temperatures are species specific. Optimal temperature range for microalgae is 15-30 ^{0}C. Lower temperatures result in low metabolic kinetics while higher temperatures hamper the microbial oxidative stress. In addition some other side effects such as

salt precipitation or reduction of gases solubillity (O_2 and CO_2) at increasing temperature should be kept in mind when operating open ponds (González-fernández et al., 2011). Solar irradience and raceway pond medium temperature (taken during 9-10 am twice weekly) for 1st run 2014 batches (a,b,c) and 2nd run 2015 batches (a,b) are presented in Figure 3.7.

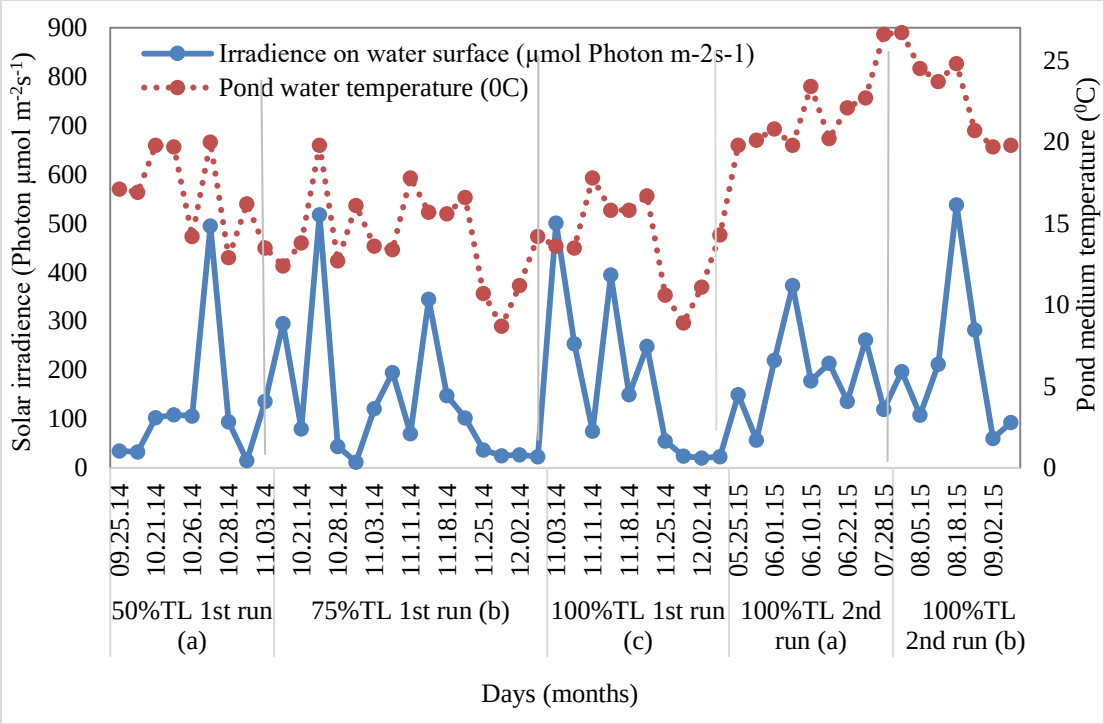


Figure 3.7 : Solar irradiance and pond water temperature.

Optimum pH range is between 7-9 but pH tolerances vary among different species (Jia and Yuan, 2016). Extreme pH may cause disruption of many cellular processes which could lead to culture collapse. pH determines CO_2 solubility in the culture medium and influences effective nutrient removal. Additionally pH values are responsible for NH_3 stripping and P precipitation (Gonçalves et al., 2017). In the present raceway study pH was found to be constantly decreasing (Figure 8) and manually corrected (by adding 6M NaOH) before taking samples twice a week. This decreasing pH (reaching to 5.7) was also observed by mustafa et al. (Mustafa et al., 2012) in their raceway cultivation with high loading rate of NH_4^+-N (4%) using leachate. Liang et al. (Liang et al., 2015) also observed a decrease in pH from 7 to 3.5 in removing NH_4^+-N via algae-bacteria system. Adjusting pH to neutral increased the chlorophyll content (biomass) and NH_4^+-N removal. Nwabo et al. (Nwoba et al., 2016) also observed a decreasing pH below 7 in their semi-continuous raceway cultivation treating high NH_4^+-N medium. The possible reasons for the acidic medium pH are explained in the authors recent work (Khanzada and Övez, 2017).

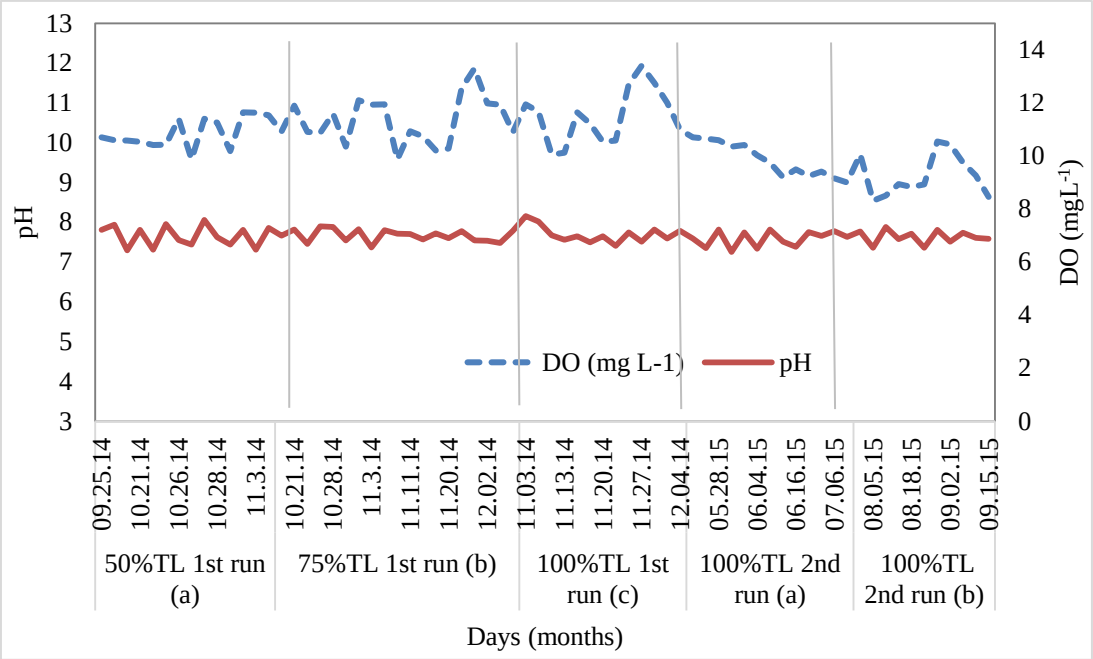


Figure 3.8 : pH and DO concentration during raceway pond cultivation.

Production of algal biomass results in more oxygen (photosynthetic product) being produced (Jia and Yuan, 2016). Dissolved oxygen DO favours nitrification as a means of NH_4^+-N removal (González-fernández et al., 2011). Nitrifiers (bacteria) require 4.57 g O_2 to oxidise per gram of NH_4^+ to NO_3^- (Jia and Yuan, 2016). In the present study DO concentration was between 8.5 – 12 mgL^{-1} throughout the raceway cultivation, measured during morning hours between 9-10 am. During 1st run 2014 (Figure 3.7 a,b,c) DO was reaching to highest concentration, i.e., 12 mgL^{-1} (Figure 3.8). 1st run was carried out in the colder months of 2014, .i.e., September to December. 2nd run of raceway cultivation, during summer months of May- September, 2015 (Figure 3.7 a,b), DO was reaching to its lowest value, .i.e., 8.5 mgL^{-1} (Figure 3.8). Godos et al. (Godos et al., 2009) observed a successively decreasing DO from 9 to 5.7 mgL^{-1} in continuous mode HRAP operating from January to May. The high values of DO (upto 12 mgL^{-1}) were recorded for lowest temperatures (~0 during Jan - Feb). Mustafa et al. (Mustafa et al., 2012) observed an increase of pH, DO and microalgal cell number during first 20 days, in raceway pond TL cultivation at 4% NH_4^+-N loading rate in semi continuous mode. DO at 4% NH_4^+-N loading rate was reaching to 12 and 8, similar to present study. Nwabo et al. (Nwoba et al., 2016) observed an increase in DO a little after inoculation and then a decrease and remained constant below 6 mgL^{-1} in raceway cultivation. Moheimani et al. (Moheimani and Borowitzka, 2006) observed DO concentration between 18-24 mgL^{-1} O_2 with actively photosynthesizing microalgae during Australian summer months of November to April (temperature between 25-45^{o}C).

In the present study both the Electrical conductivity EC and salinity of the TL medium remained same for a couple of weeks but nearing end of batch cultures, EC and salinity increased (Figure 9). Prolonged batch culturing was probably inducing more secretions (EPS) by surviving microalgae and bacteria into the liquid medium and increasing dissolved solids over time. Kumari et al. (Kumari et al., 2016) observed a reduction of 30% in EC while treating leachate with bacto-algal system in lab for 10 days.

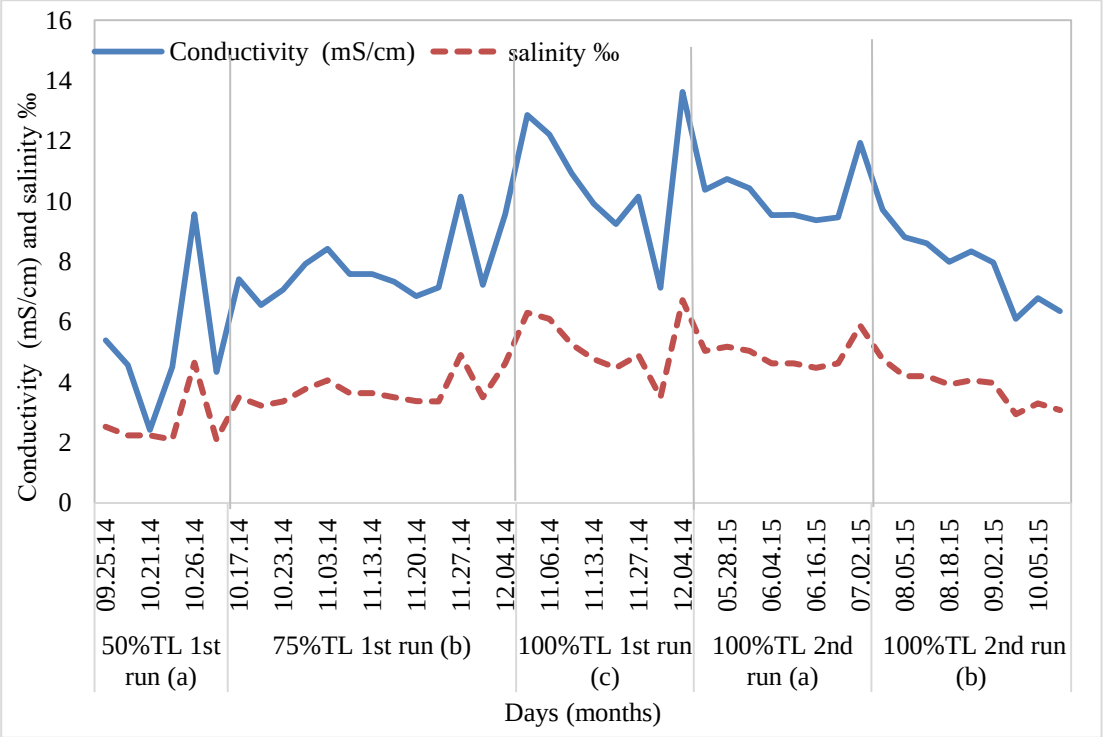


Figure 3.9 : Electrical conductivity EC and salinity data for raceway cultivation.

3.3 Conclusion

Indigenous fresh water microalgal species were able to grow in high NH_4^+-N (~760 mgL^{-1}) and saline (10,000 mgL^{-1} chloride), slightly dark colored ultra-membrane treated leachate TL and were able to utilize nutrients in the lab study, but onsite pilot scale raceway pond cultivation reduced the biomass growth and nutrient removal. Extending the duration of batch culturing was not efficient in removing NH_4^+-N in the lab study as well as raceway pond cultivation. Prolonged batch cultures released NH_4^+-N in the medium (lab study) while no significant NH_4^+-N removal was observed by the end of raceway cultivation with NH_4^+-N concentration fluctuating in raceway pond system. No significant NO_3-N removal was observed for both the studies. Further research in terms of monitoring nutrient removal, EPS measurement and growth optimization is required for microalgal cultivation in landfill leachate with effective waste removal and biomass

production.

4. LEACHATE FOR PRODUCING 3RD GENERATION MICROALGAL OILS [1]

Microalgal biomass — A sustainable alternative feedstock for biodiesel

The investigation on microalgae as a sustainable alternative energy source for transportation fuel is not new but the prevailing oil crisis in the oil producing regions, fast depleting fossil oil reserves and environmental pollution concerns (release of green house gases GHG etc.) have made it imperative for organizations and countries to invest more time and efforts into research on sustainable, renewable, environmental friendly-carbon neutral feedstock for biodiesel such as microalgae (Christenson and Sims, 2011; Martins et al., 2010; Palligarnai et al., 2010; Rittmann, 2008; Rodolfi et al., 2009; Schenk et al., 2008). The renewed interest in microalgae for producing oils is due in part to the high lipid content of some species, 10-30% of dry weight. Lipids and fatty acids form a major part of a microalgal cell as membrane components, metabolites and storage products (Princen, 1982). Microalgae contain storage lipids (triglycerides TAG) suitable for transesterification for biodiesel conversion (Rodolfi et al., 2009)(Miao and Wu, 2004). In fact paleobotanical evidence has also suggested that microalgae are responsible for major sources of hydrocarbon (fossil fuels) in a variety of oil-rich deposits dating from the Ordovician period to the present (Borrego et al., 1996; Moldowan and Seifert, 1980; Schenk et al., 2008). Eukaryotic algae contain a diverse composition of acyl lipids and their fatty acids. Even within divisions, individual algae contain a bewildering array of lipid compositions (such as saturated fatty acids, polyunsaturated fatty acids, glycolipids or phospholipids). Their lipid content differ from strain to strain and can also be adjusted through altering nutrient (C, N, P ratios) and growth conditions (temp, light intensity or pH etc.) (Huang et al., 2010; Khozin-goldberg and Cohen, 2006; Rawat et al., 2011; Rodolfi et al., 2009).

Utilizing wastewater resources for growing microalgae

The statement made by Chisti (Chisti, 2007) in his research article that microalgae produce 15-300 times more oil for biodiesel production than traditional crops on an area basis, further intensified the interest in research and development of 3rd generation microalgal biofuels (Christenson and Sims, 2011; Martins et al., 2010; Rittmann, 2008; Schenk et al., 2008) Some researchers suggested that the claim made by Chisti (Chisti, 2007) was not realistic with the then present technology and available strains, and the only way for microalgae to compete with terrestrial crops in the race of 2nd generation biofuels was to use wastewater (Clarens et al., 2010;

[1] This chapter is based on paper " Zareen T. Khanzada and Süleyman Övez, Leachate for producing 3rd generation microalgal oils, Modern Environmental Science and Engineering, 2017, 3 (9), 614-624."

Pittman et al., 2011; Rodolfi et al., 2009). If nutrient requirement for large scale microalgal cultivation is provided by chemical fertilizers then it will provide an immense upstream burden to the life cycle analysis LCA of microalgae biodiesel production system (Alam et al., 2012; Slade and Bauen, 2013; Sturm and Lamer, 2011). The processing stages of microalgae biofuels need to be simplified with virtually zero energy input for long-term sustainability and environmental benefits [20]. Zhou et al. (Zhou et al., 2013) evaluated that using waste organic biosolids from only three major sources (municipal wastewater, livestock manure and food waste) could potentially support enough bio-crude oil production to completely replace the US demand for petroleum imports.

LFL to produce microalgal biomass-to-bioenergy generation

Use of LFL as a source of fertilizer and water for microalgal oil production, bring an added value which otherwise would be considered as waste needing treatment. Depending on constantly varying characteristics of leachate, researchers around the world have investigated leachate in particular as a growth medium for microalgae for the treatment of toxic heavy metals, ammonia and organics etc. (as mentioned in Introduction section). But literature on leachate treatment coupled with microalgal lipid production is scarce (Zhao et al., 2014). The objective of the present study was to evaluate the potential of ultra-membrane treated landfill leachate TL, taken from Istanbul municipal landfill (Odayeri Istaç-Istanbul Buyukşehir Belediyesi) to sustainably grow native microalgal cultures and to screen lipids in microalgal cells for supplying raw material for future biodiesel conversion.

4.1 Experimental

4.1.1 Materials

Mixed culture of fresh water microalgal species of Chlorella v*ulgaris* and Chlamydomonas *reinhardii* were obtained from Bioenergy department, Ege University. Ultra-membrane treated leachate TL was kindly provided by Oda yeri and stored at 4 ^{0}C in 20 L air tight plastic containers in dark until use. Physico-chemical characteristics of autoclaved treated leachate is presented in (Table 4.1).

Table 4.1 : Characteristics of ultra-membrane treated leachate TL

Parameter	(mg L^{-1})
TOC	135,6
NH_4^+-N	485
ortho-PO_4	5
TDS	18,74
conductivity mS/cm	26,7
salinity ppt	23,9
Chloride Cl	9060
pH	7,5

Exponentially growing inoculum at a volumetric ratio of 20% (v/v) measured at an absorbance of 680 nm (using spectrophotometer, model U-2001, HITACHI, Japan) was used to start and monitor microalgal growth in the 3 experimental sets. BG11 UTEX medium used in the experiments consisted of the following nutrients: $NaNO_3$, $MgSO_4$, KCl, $CaCl_2$. $2H_2O$, $NaHCO_3$, $NaNO_3$, KH_2PO_4, K_2HPO_4, Fe Ammonium citrate, Citric acid, and trace elemental solution. Trace elemental solution includes $CuSO_4$. $5H_2O$, $ZnSO_4$. $7H_2O$, $MnCl_2$. $4H_2O$, $CoCl_2$. $6H_2O$, $FeSO_4$. $7H_2O$, EDTA, H_3BO_3, $NaMO_4$. $2H_2O$.

Cultures were put in 1L glass bottles with 500 ml working volume, continuous air bubbling was supplied at a rate of 3 L/min (flow rate in each bottle was around 0.31 ml min^{-1}), continuous artificial irradience of 60 µmol $m^{-2}s^{-1}$ was provided through white fluorescent lamps (measured by a digital light meter -linkoln USA) and maintained at room temperature of 25±1 ^{0}C. Cultivation was carried out under batch mode in dublicate for 27 days. The data was statistically analysed by Student's t test comparing the control at $p < 0.05$. Values presented in the results section are averages of dublicate cultures.

4.1.2 Lab experimental setup

A set of 3 experiments were conducted with same cultivation conditions as mentioned in section 2.1. Different dilutions of autoclaved (20 min at 1 atm, 121 ^{0}C) treated leachate TL (i.e., 10%, 30%, 50%, 70%, 90%, 100%) with distilled water (dw) as –ive control and regular BG11 media as +ive control, were formulated to evaluate microalgal growth and lipid content. 0.5N $NaOH/H_2SO_4$ was added manually on alternate days for pH control. The only difference in the experiments was as follows:

In experimental set 1, pH was maintained within a range of 6.5-8.5.

In experimental set 2, pH was maintained within a range of 6.5-7.5 and

In experimental set 3, pH was maintained at a range of 6.5-7.5 with phosphate addition (same as BG11 media with N/P ratio 40:1)

4.1.3 Microalgal biomass dry weight and lipid extraction and staining

Biomass dry weight was measured by filtering the microalgal samples through whatman filter papers (0.7 pore size, GF/F) at the start and end of each batch cultures. Filters were oven dried at 60^0C until constant weight (~ 3 days) and then weighted on a measuring balance. For oil content determination dried algal biomass was homogenized in mortar and pestle and 500 mg from each experimental set and was subjected to lipid extraction following folch method (Folch et al., 1957) with sonication. 10 ml mixture of chloroform and methanol (2:1) was added to dried biomass in a glass tube and undergone sonication (50 Hz) for 20 min. The extract was equilibrated with 1/4th its volume of a saline solution and vortexed. Then the tubes were centrifuged at 2000 g for 10 min for the separation of two layers. Lower chloroform layer containing lipids was carefully transferred to pre-weighted glass vials and dried under fume hood at 80^0 C. The dried lipids were then gravimetrically measured.

Nile red (9-diethylamino-5H-benzo [α] phenoxazine-5-one, $C_{20}H_{18}N_2O_2$) staining of microalgal intact cells with neutral lipids was carried out using fluorescence microscopy (OLYMPUS BX50 microscope with attached camera) (Storms et al., 2014). Oven dried microalgal biomass was homogenized in pestal and mortar. Washed with phosphate buffer and centrifuged twice and then 10µm was spread on glass slide with 10µm nile red stain and 30% ethanol solution. Slides were visualized under florescence microscopy.

4.2 Results and Discussion

4.2.1 Microalgal biomass growth in the three experimental sets

Microalgae usually accumulate lipids as part of their grown up cells, thats why growth curve was first carefully monitored to check if TL was supporting microagal biomass growth. The high nitrogen content of TL makes it attractive for microalgae cultivation as green algae demand more nitrogen and phosphorus than do many other plant species but the same ammonium nitrogen N-NH_4^+ can become toxic in higher concentrations. Excessive N-NH_4^+ can damage photosynthesis organs (chloroplast) and decrease photochemical efficiency (Choi and Lee, 2013).

In the present study both stimulatory and inhibitory effects of TL was observed on miroalgal growth in the 3 experimental sets. Irrespective of a month adaptation time given to microalgae before the start of experiments, cultures still showed a lag phase of around 3-5 days in all the formulations (10%-100% TL) due primarily to high total dissolved solids (susceptable for fresh water species) or by some possible inhibition and inconsistency of nutrient compositions in leachate (Figure 4.1, 4.2, 4.3; Table 1). In experimental sets 1 and 2 microalgal growth in all the

dilutions (10%-100% TL) was significantly lower than regular nutrient media BG11 as shown by growth curves (Figures 4.1, 4.2). Experimental sets 1 and 2 were only different in pH ranges, which was established to check its effect on biomass growth. pH above 8.5 can start formation of free ammonia gas, which can leave the liquid medium and could no longer be available for microalgae to assimilate as a nitrogen source for its growth. But the growth curves did not show any significant effect of varying pH ranges on the growth curves of microalgae, which could imply that nitrogen was not lost from the medium in experimental set 2 in the form of ammonia gas (Figures 4.1, 4.2).

In general all the dilutions supported microalgal growth in a bell shaped curve, with 50% TL supporting the highest biomass growth in all the 3 experimental sets and the rest of dilutions showing decrease in successive order (Figure 4.4). In 10% TL, nutrients seemed insufficient due to dilution and the growth curve stayed closer to negative control (dw) and very low dry biomass was observed (Figures 4.1, 4.2, 4.4). In higher formulations (70%-100% TL) growth was slow and steady in all the 3 experimental sets, with growth curves not showing onset of proper stationary phase, but after day 20th there was an upward increase in growth curve albeit at a very slow pace.

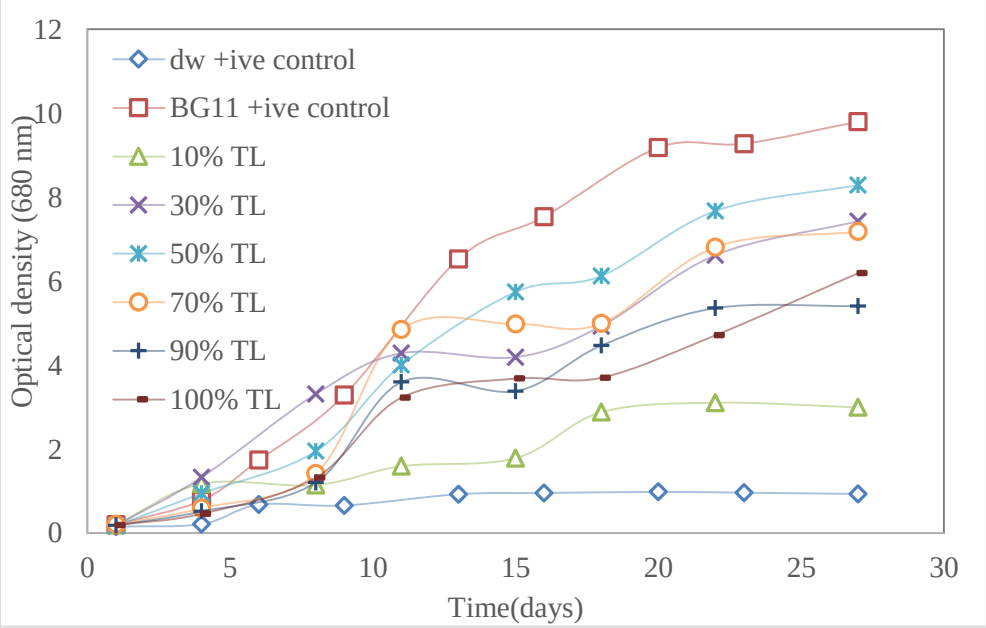


Figure 4.1 : Experimental set 1- Biomass growth curve of microalgae in different dilutions of TL.

TL had sufficient nitrogen source in the form of N-NH_4^+ to support microalgal growth but was limited in phosphorus P, which is an essential nutrient for growth (Paskuliakova et al., 2016; Rasoul-Amini et al., 2014; Xin et al., 2010). Experimental set 3 was supplemented with phosphate

$P\text{-}PO_4^-$. Since at pH 8 or more phosphate can precipitate (Li et al., 2011), in experimental set 3, pH was kept within 6.5-7.5 range to make sure any phosphate elimination from the medium was from metabolic uptake by microalgae. All the TL dilutions after $P\text{-}PO_4^-$ addition showed better growth curves and dry biomass than previous experimental sets 1 and 2 (Figures 4.3, 4.4). After $P\text{-}PO_4^-$ addition, 50% TL showed the highest microalgal growth almost equal to BG11 media with 2.50 g L^{-1} dry biomass (Figures 4.3, 4.4, 4.6b).

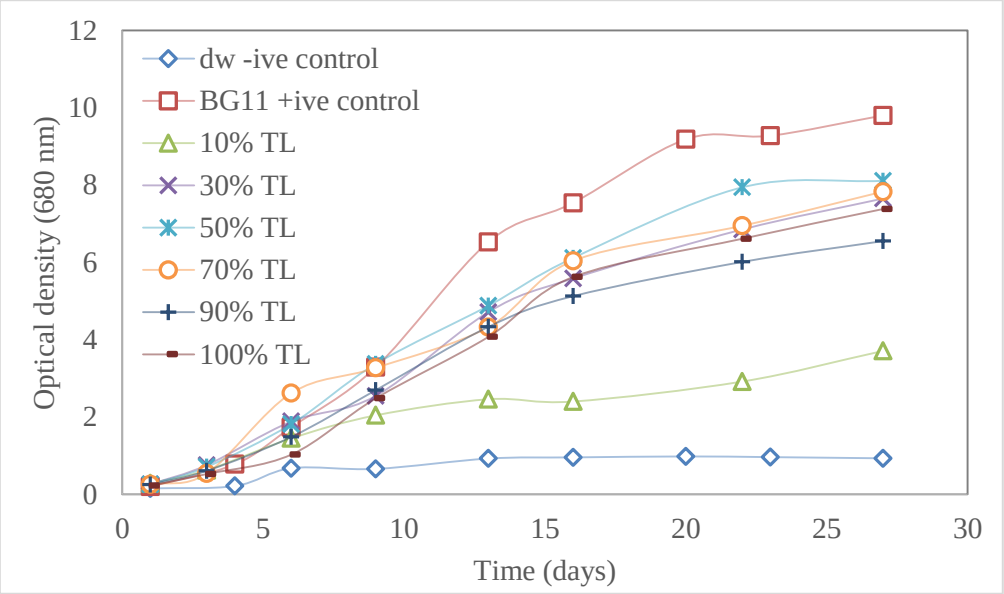


Figure 4.2 : Experimental set 2- Biomass growth curve of microalgae in different dilutions of TL

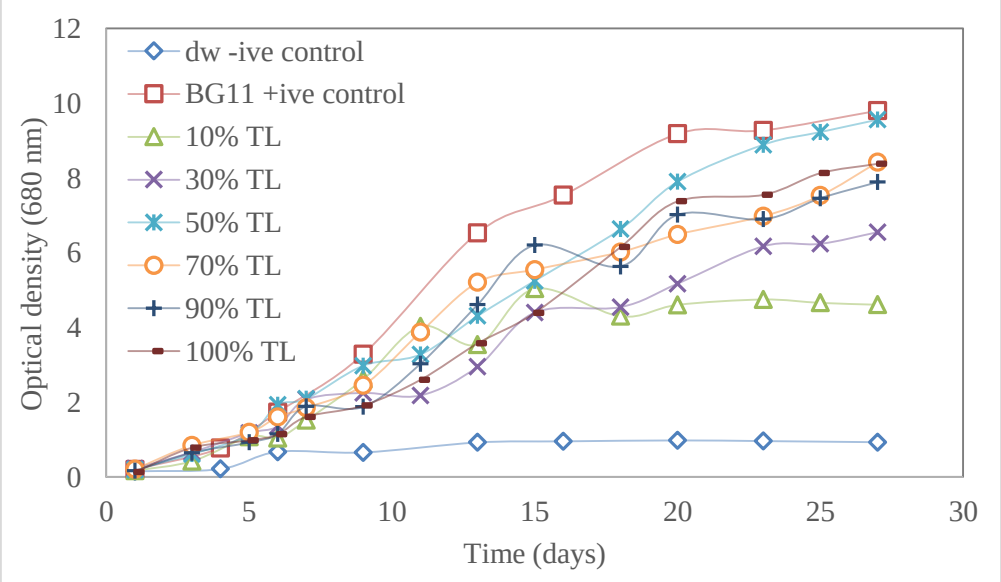


Figure 4.3 : Experimental set 3- Biomass growth curve of microalgae in different dilutions of TL.

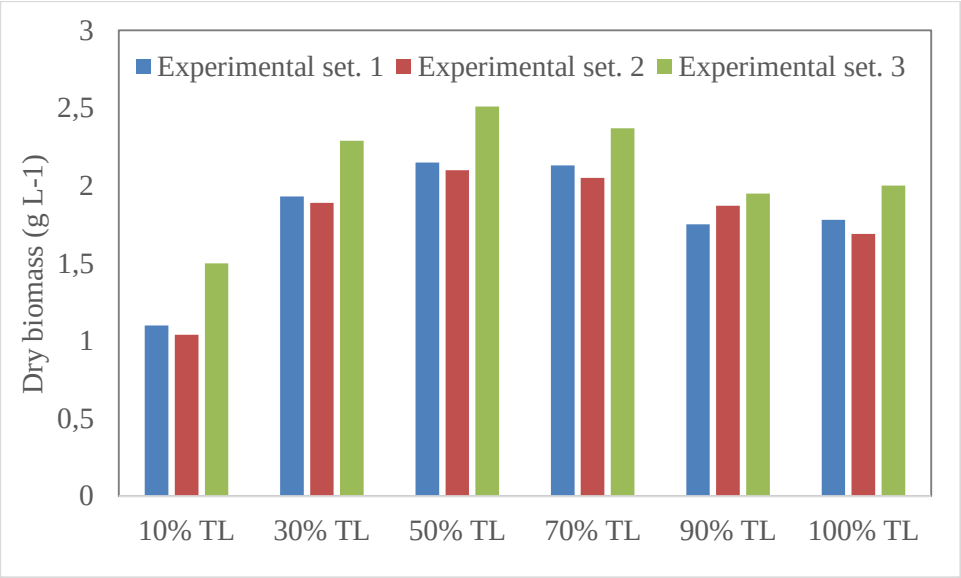


Figure 4.4 : Microalgal dry biomass produced in different dilutions of TL in the three experimental sets.

4.2.2 Microalgal lipid content in the three experimental sets

In any process aimed at oil production by photosynthesis, the key objective is a high photosynthetic efficiency of lipid production. According to literature survey environmental stress conditions particularly decreased nitrogen in the medium, induces oil production in microalgal cells. Rodolfi *et al.* (Rodolfi et al., 2009) evaluated that when nitrogen deprivation is imposed upon a culture exposed to suitable irradiences, photosynthesis continues, albeit at a slow rate and the fixed carbon flow is diverted from protein to either lipid or carbohydrate synthesis. The major limitation of this approach is that despite the fraction of lipids may increase, biomass productivity of the microalgal cells is often very low and so overall lipid productivity will not be high. Growth conditions that focus on providing high biomass productivity instead may ultimately be more economical and may be a more efficient means of increasing total lipid productivity (Cho et al., 2013; Rawat et al., 2011; Rodolfi et al., 2009). Similar trend was observed in the present study where 10% TL (~ 50 mg L^{-1} $N\text{-}NH_4^+$) had the lowest biomas yield, but it produced highest lipid content (107.24-114.64 mg g^{-1} dry biomass) (Figures 4.4, 4.5). 50% TL (~ 248 mg L^{-1} $N\text{-}NH_4^+$) showed highest microalgal growth interms of dry biomass in all the 3 experimental sets but lipid content was almost half when compared with 10% TL (Figures 4.5, 4.7bd). Microalgae growing in 50% TL and 100% TL produced almost same lipid content, which implied that TL can be used without dilution in further studies to optimize lipid yield.

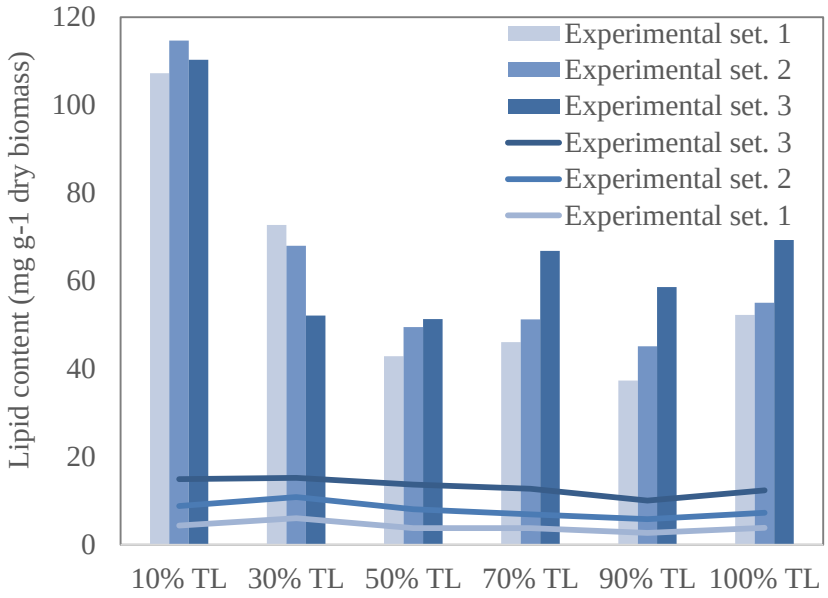


Figure 4.5 : Microalgal total cell lipid content (bar graph) and lipid productivity (stacked line) in different dilutions of TL for the 3 experimental sets.

4.2.3 Effect of phosphate on lipid content

To enhance oil yield of algae cultures the cell lipid content should be increased over the basal value without significant losses of productivity. The high biomass productivities (in some cases high lipid productivities) of the wastewater-grown microalgae suggests that there is real potential in the utilisation of these high nutrient resources for cost-effective biofuel generation and production of sustainable and renewable energy (Cho et al., 2013). Although culture composition and growth conditions may be less managable in municiple wastewater and most microalgae have relatively low total lipid content per cell under wastewater conditions, ranging from low (<10% dry biomass) to moderate (25–30% dry biomass) lipid content, the high biomass productivity potentially can translate to significant total lipid productivity (Christenson and Sims, 2011; Rawat et al., 2011).

In the present study Phosphate P-PO_4^- was added in experimental set 3 to enhance the growth of microalgae which could further increase the cells lipid content and productivity. Phosphate P-PO_4^- addition significantly enhanced the biomass growth curves and dry biomass of microalgae in higher dilutions (10%-50% TL) but the lipid content did not show any significant difference when compared with experimental sets 1 and 2 (Figures 4.3, 4.4, 4.5). Phosphate addition significantly increased lipid content and productivity in lower dilutions (70%-100% TL) (Figures 4.5, 4.6a). Chu *et al.* (Chu et al., 2013) pointed out that phosphorus plays a significant role in lipid production under nitrogen deficiency. In their study excess phosphate (35 mgL^{-1}) in nitrogen starvation conditions achieved highest lipid productivity (58.39 $mgL^{-1}day^{-1}$) but lipid content remained the same after 14 days of cultivation. While in

the present study, it seemed that phosphate P-PO_4^- was mainly metabolically uptaken for growth purposes and was not sufficient enough to induce lipids production in microalgal cells in higher dilutions (10%-50% TL) but in lower dilutions (70%-100% TL) P-PO_4^- induced significant lipid production (content) with no enhanced growth when compared to experimental sets 1 and 2 (Figures 4.4, 4.5).

Study by Xin *et al.* (Xin et al., 2010) suggested high lipid content (53%) and productivity (0.075 gL^{-1}) under phosphorus limitation. However under nitrogen limitation lipid content was enhanced (30%) but productivity per unit volume of the culture medium was rather low because the algal biomass was also very low (0.05 g L^{-1}). In the present study, cultures in 10% TL produced the highest lipid content irrrespective of phosphate limitation (sets 1 and 2) or addition (set 3) (Figure 4.5). The cultures in 10% TL turned pale green after around 10 days and remained so until the end of each batch cultures (Figure 4.6b).

Nile red staining of the pale green cultures of 10% TL further confirmed the sigificantly increased lipid content when compared with 50% TL cultures (Figures 4.6a,b; 4.7a,b,c,d). Almost all cells surviving in 10% TL had lipid storage in them, which absorbed nile red staining and readily responded to florescence microscopy. Since phosphorus addition and limitation had no significant effect on lipid content for higher dilutions (10-50% TL) in the 3 experimental sets, the only reason for increased lipid content in 10% TL can be attributed to nitrogen limitation (Figure 4.4, 4.5). Under N-limited conditions microalgal cells degrade the intracellular abundant proteins to recycle amino acids into proteins more suited for survival. Another quick nitrogen source utilised under stress conditions is chlorophyll and any changes in chlorophyll content are directly reflected in the nitrogen content of microalgal biomass (Procházková and Brányiková, 2014). In the present study the pale green cultures in 10% TL can be attributed to intracellular degradation of chlorophyll for survival of stressed microalgal cells.

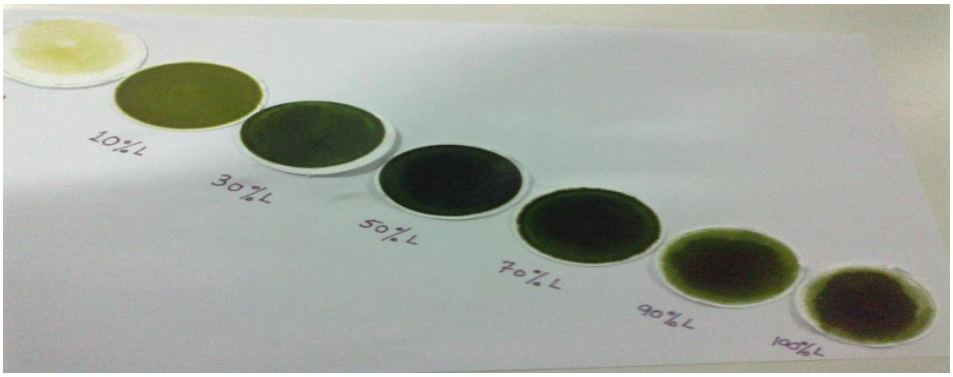


Figure 4.6a : Oven dried filtered dry weights of microalgal cells grown in different dilutions of TL.

In the present study dry biomass and lipid productivity was opposite to what Zhao *et al.* (Zhao et al., 2014) had observed in their study. They grew Chlorella *pyrenoidosa* in a mixture of leachate and municipal wastewater with no external phosphate addition and their evaluated microalgal biomass was 1.58 g L^{-1} and lipid production was 24.1 mg L^{-1} d^{-1} in 12 days. While in the present study lipid productivity in 10% TL (with highest total lipid content per cell) was very low (~ 4.42 mg L^{-1} day^{-1}) but dry biomass was higher 2.5 gL^{-1} (50% TL). The lipid productivity observed in the present study was in the lowest range according to literature (Lam and Lee, 2012b; Pittman et al., 2011). Leachate is a complex mixture of constantly varying compounds and it might have some possible inhibition or toxic effect on lipid production. Also the possible positive impact of waste addition (stressed condition) on lipid induction may some times not proceed as expected. As observed by Kim *et al.*, (Kim et al., 2007) where microalgal growth following the addition of fermented swine urine was increased by nearly 3-fold (197 mg L^{-1} dry biomass in treated cells over 76.5 mg L^{-1} in control cells) over a 31 day growth period, but the total fatty acid content was significantly reduced (9 mg g^{-1} dry biomass in treated cells compared to 46 mg g^{-1} in control cells).

Figure 4.6b : Different dilutions of TL at the end of batch cultures.

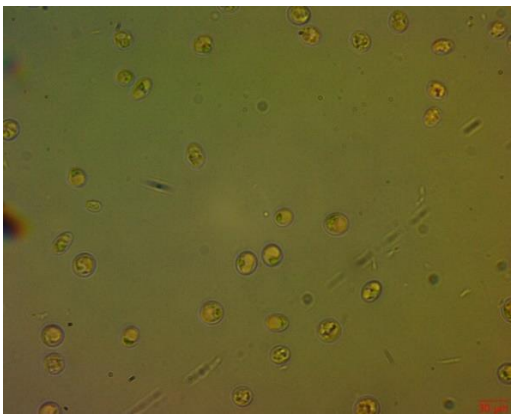
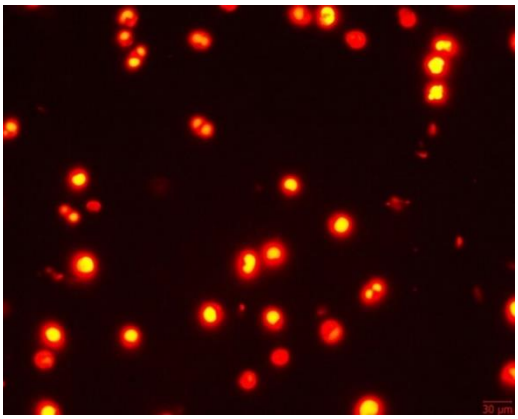
 a)  b)

Figure 4.7a, b : Nile red staining of dried microalgal cells grown in 10% TL. a). Shows the dried cells under 100x magnification using normal microscope. b). Shows same cells with their stored neutral lipids (yellow region) stained with nile red as observed under florescence microscope (green excitation filter at 604 nm).

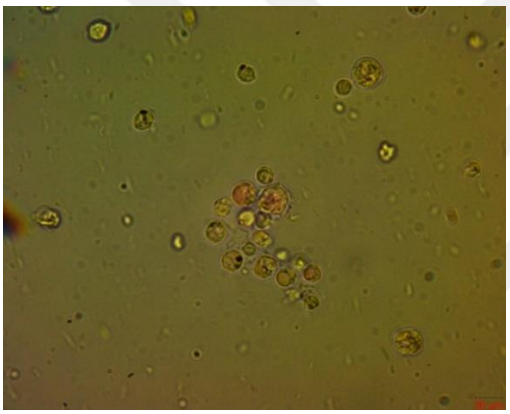
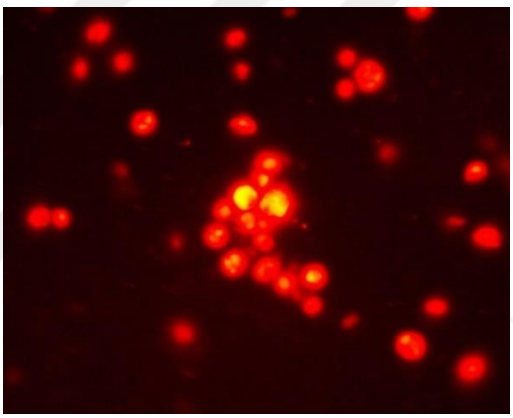
 c)  d)

Figure 4.7c, d : Nile red staining of dried microalgal cells grown in 50% TL. c). Shows the dried cells under 100x magnification using normal microscope. d). Shows same cells with their stored neutral lipids (yellow region) stained with nile red as observed under florescence microscope (green excitation filter at 604 nm).

4.3 Conclusion

In the present study priliminary results suggested that irrespective of high N-NH_4^+ and other stresses in leachate media, fresh water microalgae were able to grow biomass in all the experimental sets and produced lipids. Altering pH of the medium had no significant effect on biomass and lipid content in the 3 experimental sets but addition of phospate significantly increased the biomass in higher dilutions (10-50% TL) and lipid content in lower dilutions (70-100% TL) of microalgal cultures. Total lipid content and productivity of produced dry biomass was on the lowest range according to literature survey.

5. CONCLUSION AND RECOMMENDATIONS

Cultivation of microalgae in leachate offset both water and fertilizer consumption, reducing environmental footprint and increasing the potential sustainability of newly emerging micralgal based technology. Optimized biomass production is central to economic biodiesel production and this in turn requires careful optimization of microalgal cultivation systems. Results presented in the thesis showed that microalgal cultures were resilient and ammoniacal tolerant even at high concentrations of NH_4^+-N (750 mgL^{-1}). Highest biomass (2.5 gL^{-1}) was obtained in 50% TL (~242 mgL^{-1}), after that growth was slow in batch mode with continuous light for ~30 days. In terms of NH_4^+-N removal, 50 mgL^{-1} containing TL dilution showed 100% removal from the system. Removal efficiency was decreasing with increasing leachate concentration, and presence of residual NH_4^+-N was more obvious in lower dilutions (50-100% TL). I terms of oil production, again 50 mgL^{-1} NH_4^+-N (10% TL) proved better in inducing accumulation of oil in microalgal cells. Open raceway pond cultivation had significantly reduced biomass growth and nutrient removal (including NH_4^+-N). NO_3-N was not utilized by the cultures in lab or pilot study. Altering pH within a narrow range had no effect on biomass growth or NH_4^+-N. PO_4-P addition to TL significantly enhanced growth and oil content (in lower diutions 50-100% TL). Preliminary results showed that lipid content and productivity was low and considered as not feasible (or economic) for biodiesel energy generation. Further screening is required to optimize the microalgae-to-bioenergy system using landfill leachate to produce more lipid for future biodiesel production. Overall microalgal system could be employed after ultra-membrane treament of leachate at Oda yeri-municipal landfill of Istanbul (after further optimization), for a cost effective nutrient removal (when compared to nano-filtration).

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APPENDICES

APPENDIX A: Odayeri- Istanbul municipal landfill, (a) Area specification. (b) Entrance. (c) Waste management. (d) Biological treatment (nitrification pond). (e) Ultra-membrane filteration system. (f) TL used in the experimental studies (blue star).

(a)

(b)

(c)

(d)

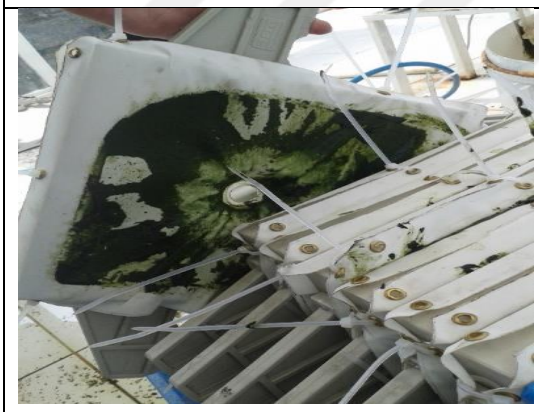
(e)

(f)

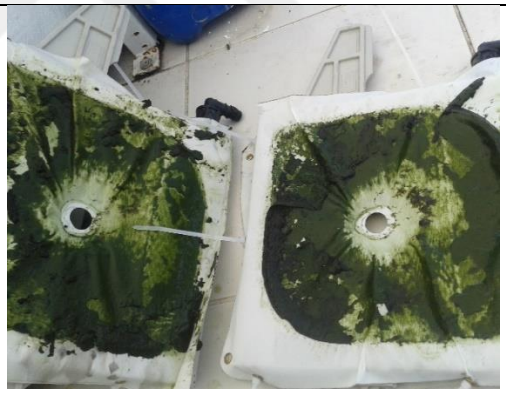
APPENDIX B : Onsite raceway pond cultivation, (a) Raceway ponds. (b) Filter press system (blue machine). (c) Stacked filters in the press system. (d) Microalgal biomass on filters. (e) TL used in the study (f) Crude microalgal oil**.**

(a)

(b)



(c)



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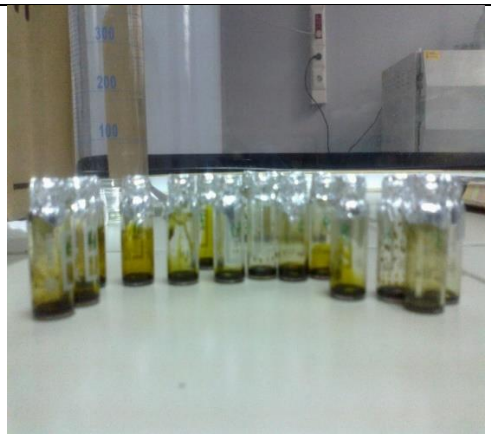
(e)



(d)

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- **Zareen T. Khanzada** and Süleyman Ovez. 2017. Microalgae as a sustainable biological system for improving leachate quality. Energy. 140: 757-765.

- **Zareen T. Khanzada** and Süleyman Ovez. 2017. Leachate for producing 3rd generation microalgal oils. *Modern Environmental Science and Engineering.* 3(9): 614-624.

CONFEENCE PARTICIPATION ON THE THESIS:

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- Great Water Cities Summit 2017. USA.