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Growing Fresh Water Microalgae in High Ammonium Landfill Leachate

Zareen Taj Khanzada*, Süleyman Övez

Department of Environmental Engineering, Faculty of Civil Engineering, Istanbul Technical University, Istanbul, Turkey

Email address:
xaripink@yahoo.com (Z. T. Khanzada)

*Corresponding author

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Abstract: Municipal landfills are being employed for the disposal of communities solid waste. The compacted waste in landfills naturally generate leachate (liquid) which contain high concentrations of ammonium-nitrogen NH_4^+-N along with other toxic compounds. NH_4^+-N can be used as a cheap nitrogen source for microalgae biomass production, thereby facilitating tertiary treatment of landfill leachate. Two sets of studies, laboratory scale and pilot scale open raceway pond cultivation, were conducted to evaluate the potential of indigenous fresh water microalgal species to grow in ultra-membrane treated landfill leachate TL and simultaneously remove nutrients (NH_4^+-N-NO_3^- etc.). Microalgae growth was better in 50% diluted TL (1.5 gL^{-1} dry biomass) with 66.27% NH_4^+-N removal in the lab study. Onsite raceway pond cultivation had reduced biomass growth and nutrient removal. Nitrate-nitrogen NO_3-N removal was minimum from both the setups. Microalgal assimilation and nitrification was the main cause of NH_4^+-N removal from both the setups. When lab study duration was extended, NH_4^+-N was found to be released back into the leachate medium. Batch cultures (when prolonged) were observed to be not an effective nutrient removal strategy in terms of NH_4^+-N removal via microalgal system. Further research is needed to optimize microalgal growth and nutrient removal from landfill leachate.

Keywords: Leachate Tertiary Treatment, Prolonged Batch Culturing, NH_4^+-N and NO_3-N Removal

1. Introduction

Microalgae are aquatic relatives of plants and thrive in aerated, liquid cultures where they have sufficient access to sun light, carbon dioxide 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 production costs, making the price of microalgal large scale production cost prohibitive. Since atmospheric carbon dioxide 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 algal production [1, 2]. 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 [3, 4]. Microalgae cultivation requires N-fertilizer consumption in the range of 0.29 to 0.37 kg/kg oil, which is higher than that for Jatropha (0.24 kg/kg oil) and is nearly ten times higher than that for oil palm (0.048 kg/kg oil) [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 [2, 5]. 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 [6]. Optimizing cost effective and energy efficient technologies for one-step tertiary treatment of wastewaters remain a problem for industries and municipalities [2, 5, 7]. 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 [2, 5, 8]. Production of 1ton 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 1ton of algal biomass [9]. 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 [2, 10].

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, 2, 8, 11–14]. Open pond system resemble natural aquatic ecosystem which rely on symbiotic relation between algae and bacteria to degrade environmental pollutants [15–17]. Microalgae-bacterial consortia can break down various nitrogenous compounds from wastewater and show tremendous potential in treating wastewaters [3]. 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 [10, 14]. 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 [4]. 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 [18]. Dry biomass attained is usually low (0.25-1 gL^{-1}) when compared to photobioreactors (1.5-1.7gL^{-1}) [2]. 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 [19].

Landfill leachate LFL is a highly polluted waste stream generated in landfills (over time) by the dumbed solid wastes undergoing degradation (physical, chemical and biochemical reactions), rainwater percolation and inherent moisture content of waste. The leachate migrates down through spaces within the disposed of waste mass, and in modern containment landfills, drains down in the engineered drainage layer, collecting at the bottom in a storage reservoir. LFL consists of high levels of ammonia-nitrogen (3000–5000 mg/L NH_3^+-N), dissolved toxic xenobiotic organic compounds with low ratio of biochemical and chemical oxygen demand (BOD_5: COD → 0.2 or less) and heavy metals. LFL are considered high ionic strength wastewater owing to dissolved inorganic nutrients, which can lead to salinity with increased levels of chloride ions (~5g Cl^-/L), total dissolved salts (TDS) and conductivity [20–23].

From bioassay studies using different test organisms, LFL toxicity have been monitored, and it can be inferred that of all the toxic compounds that remain in stabilized LFL, Ammoniacal nitrogen (NH_4^+-N-NH_3) has been identified as one of the major toxicants to living organisms [20, 23, 24]. Organic compounds in dumped waste produce NH_3-N due to hydrolysis and fermentation of the nitrogenous fractions of biodegradable substrates [23]. Ammoniacal-nitrogen (NH_4^+-N-NH_3) can take any one form, ammonium ion (NH_4^+) or un-ionised ammonia (NH_3) in aqueous solution, with relative concentrations being pH and temperature dependent [25]. Ammoniacal-nitrogen (NH_4^+-N-NH_3) concentration increases over time and may constitute as one of the major long-term pollutants in LFL. One of the main issues regarding management of closed landfills is the disposal of leachate which still continues to be produced (with high concentration of NH_4^+-N) for a long time even after the closure of landfills [23].

Researchers around the world have evaluated LFL as a resource for growing microalgae coupled with nutrient removal (N, P), heavy metals and toxic organics etc. [11, 26–29]. Due to the variability in composition (nutrient load and toxicity etc.) and characteristics (age and structure) of dumped waste in different regions [22], which alters produced leachate accordingly, there is a wide margin for researchers to exploit this problematic waste stream further and sustainably reuse its nutrients [20, 30]. For this purpose ultra-membrane filtrated municipal landfill leachate TL from Istanbul municipal landfill site (Odayeri Istaç- Istanbul Buyukşehir Belediyesi) was collected 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-NO_3^- removal as a means of sustainable tertiary treatment.

2. Materials and Experiments

Fresh water indigenous microalgal cultures of Chlorella v*ulgaris* 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.

2.1. 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 -4^0C 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 1).

Table 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 Cl^{-1}	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

2.2. Analytical Methods

Dry weight was determined after oven drying the centrifuged microalgal paste at 60^oC 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 [31]. TOC and IC were measured by Shimadzu TOC analyser-5000A and Carbon oxygen demand COD by open reflux method ISO 6060 [31]. 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. Results and Discussion

3.1. Microalgal Growth and Nitrogen (NH_4^+-N and NO_3-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 [4]. 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 [3], [32]. 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 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 1; Table 1). Zhao et al. [33] 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 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 1; Table 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 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 60th) 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 2.

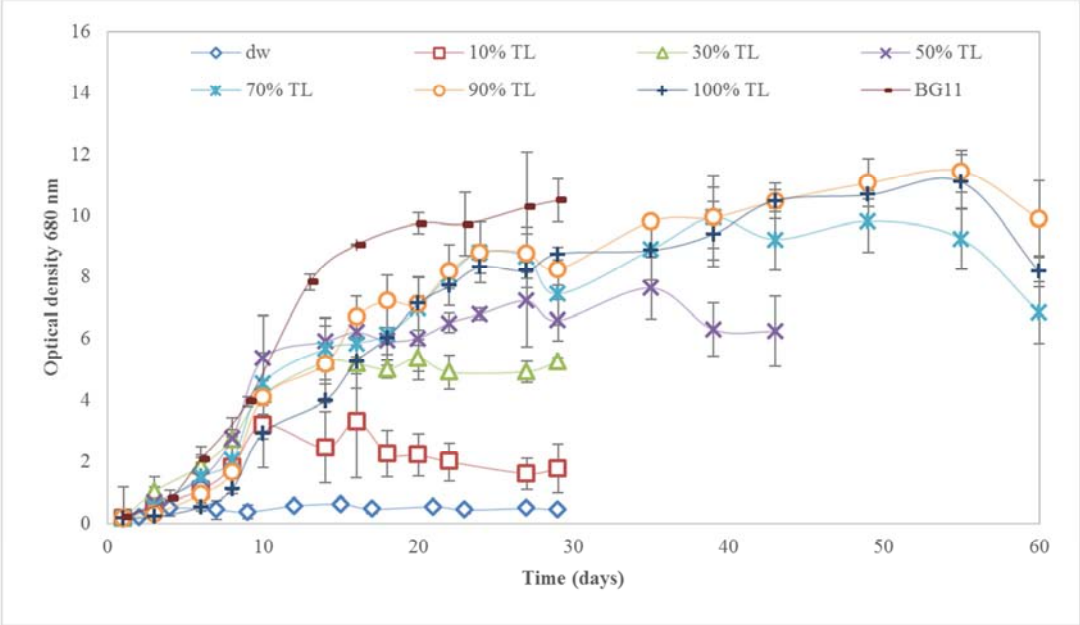

Figure 1. *Microalgal growth curve in the lab study (data shown is the mean ± SD, n=2).*

Table 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	Biomass dry wt.	Growth rate per day
(20th day for 10-30%, 30th day for 50-100% TL)	(gL^{-1})	(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 1). Tam and wong [34] 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 [5]. Osada et al. [24] 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. [28] 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 [5] 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 2) is similar to Edmundson and Wilkie [35] (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. [29] 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. [33] 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 [27] 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 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 2). NH_4^+-N removal was below 70% in all the dilutions with 50% TL showing maximum removal 66.27% (Figure 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 2), which could be due to phosphorus P limitation, which is recently considered as rate limiting factor for NH_4^+-N removal from leachate [26]. Tam and wong [34] 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. [7] also observed the same pattern with high strength wastewater led to poor nutrient removal.

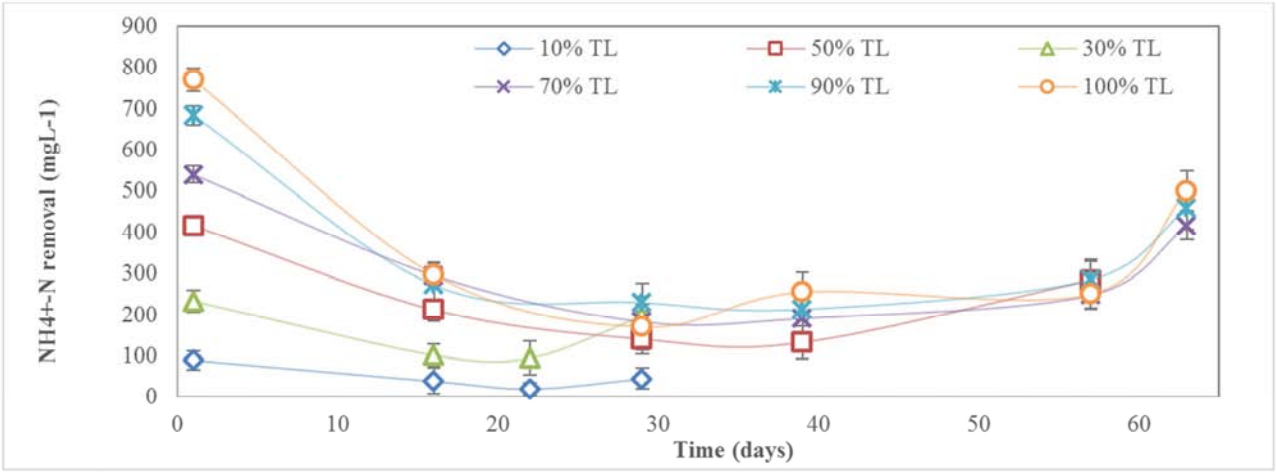

Figure 2. *NH_4^+-N removal in the lab study (data shown is the mean ± SD, n=2).*

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 20th onwards in 10-30%TL and day 30th onwards in 50-100% TL) (Figure 2). Zhou et al 2013 [36] 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. [37] 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 [17, 38]. Wang et al. [16] observed that microalgae produced more EPS containing proteins (in large proportions) when grown in high N wastewater.

3.2. 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 [5] 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). Scherholz and Curtis [32] 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 [3, 14].

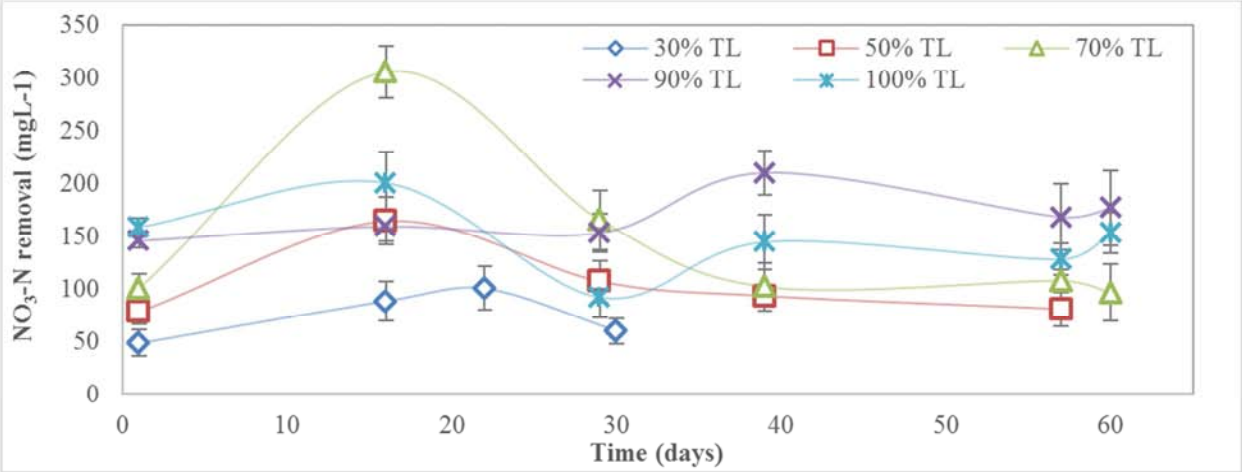

Figure 3. *NO_3^--N removal in lab study (data shown is the mean ± SD, n=2).*

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 [15, 38].

3.3. 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 4; Table 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. [39] 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. [13] 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. [12] 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. [39] observed appearance of pennate diatom towards the end of semi-continuous growth of mixed species, which was not in batch mode. Nwoba et al. [13] also observed an increase in cyanobacteria contamination in semi-continuous mode. Godos et al. [40] observed a fluctuating dominance of algal species while treating high organic carbon wastewater (~2000-7000 mgL^{-1}) in continuous mode HRAP.

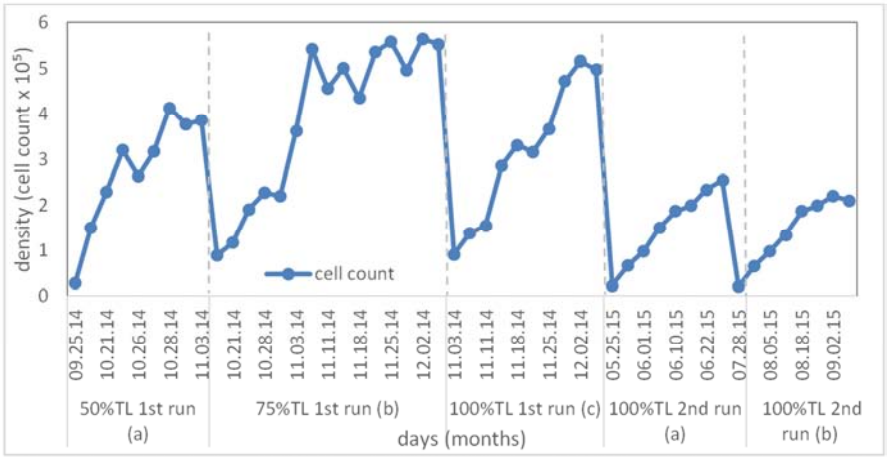

Figure 4. *Microalgal growth in raceway pond during five batches from Sept, 2014 to Sept. 2015.*

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 [38]. EPS are negatively influenced by nutrient availability and microalgae growing in P depleted, high N media release EPS (containing polysaccharides) [16, 17, 38]. Excess oganic matter accumulation (such as in continuous mode) can also induce autoinhibition of monocultures [12]. 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 [12].

In the present study NH_4^+-N concentration in the open raceway cultivation kept on fluctuating in all the five batches (Figure 5) which was not observed in the lab study, where after reaching certain minimum value NH_4^+-N concentration successively increased (Figure 2). Fluctuation of NO_3^--N was not as pronounced as NH_4^+-N (Fig. 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. [11] 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. [41] 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. [37] 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. [14] 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. [39] 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. [41] 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. [40] 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 ^{0}C in all the batch runs and pH was constantly decreasing below 7 (Figure 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).

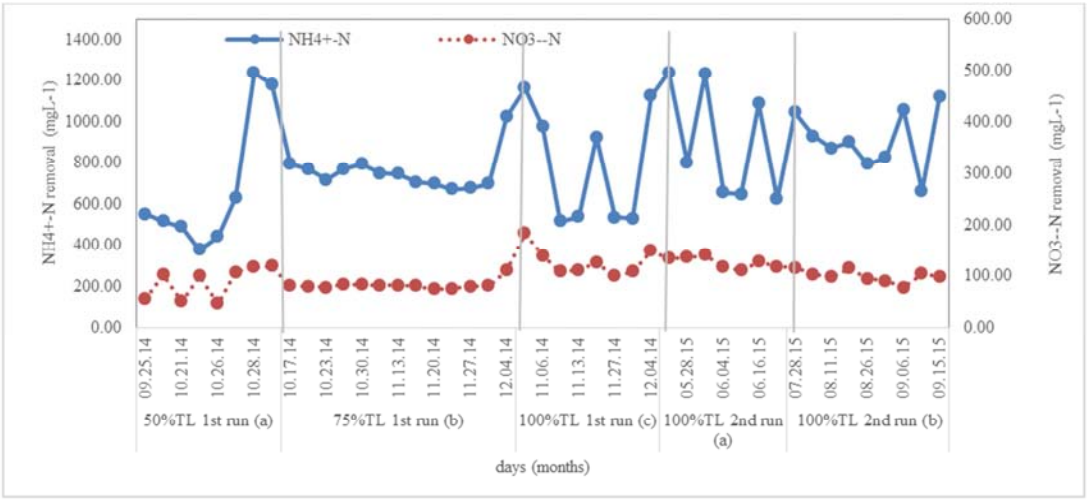

Figure 5. *NH_4^+-N and NO_3^--N removal dynamics in raceway pond cultivation.*

3.4. 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 6). Organic carbon in TL was mostly inert and recalcitrant based on BOD_5/COD ratio (Table 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 6). Microbial EPS can reach over 40 gL^{-1} under biotic and abiotic stresses while adapting to extreme environments [42]. Wang et al. [16] 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. [11] 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. [41] 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. [40] 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 (↑30^0C) [43] 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 6). The only inorganic C source for microalgae in the present study was CO_2 (from air) and inherent alkalinity of TL used (Table 1). Nwoba et al. [13] did not observe any increase in biomass after the addition of inorganic carbon ($NaHCO_3$) in open raceway pond when compared with biocoil reactor.

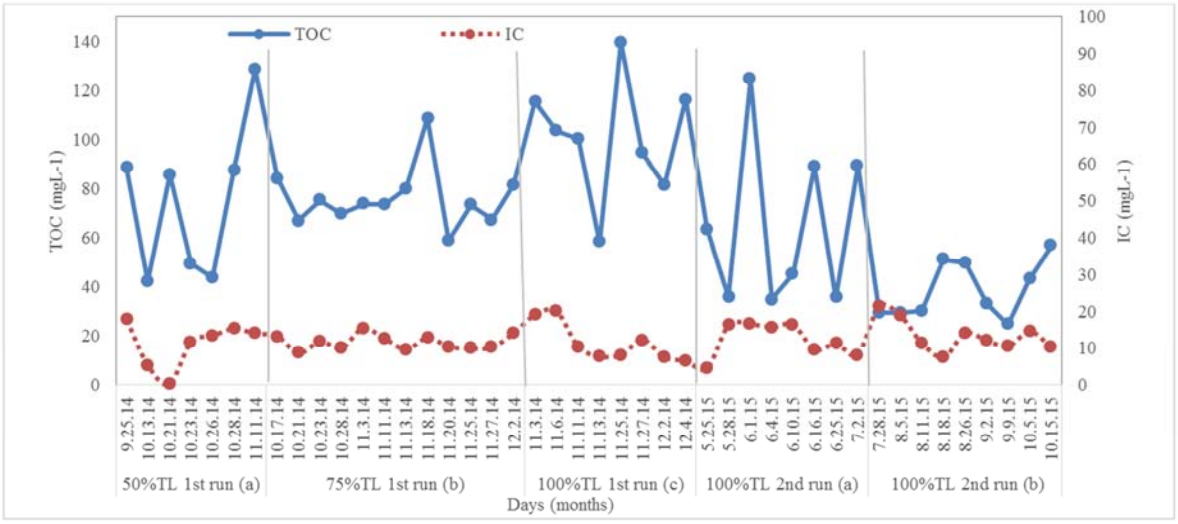

Figure 6. *TOC and IC removal dynamics during raceway cultivation.*

3.5. 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 [6, 14]. 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 [14]. 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 7.

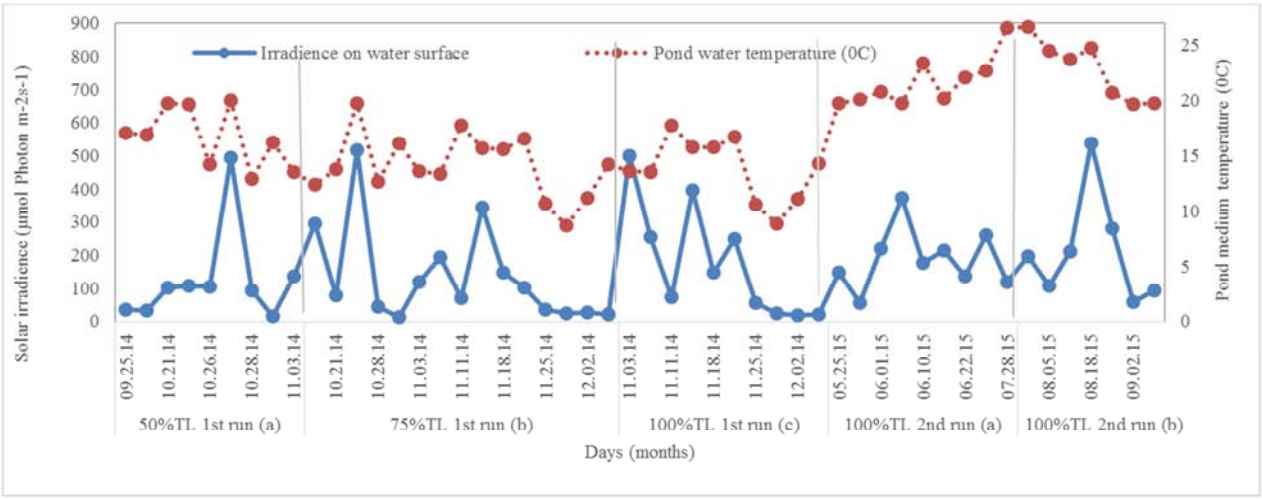

Figure 7. *Solar irradiance and pond water temperature.*

Optimum pH range is between 7-9 but pH tolerances vary among different species [3]. 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 [6]. 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. [11] in their raceway cultivation with high loading rate of NH_4^+-N (4%) using leachate. Liang et al. [44] 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. [13] 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 [45].

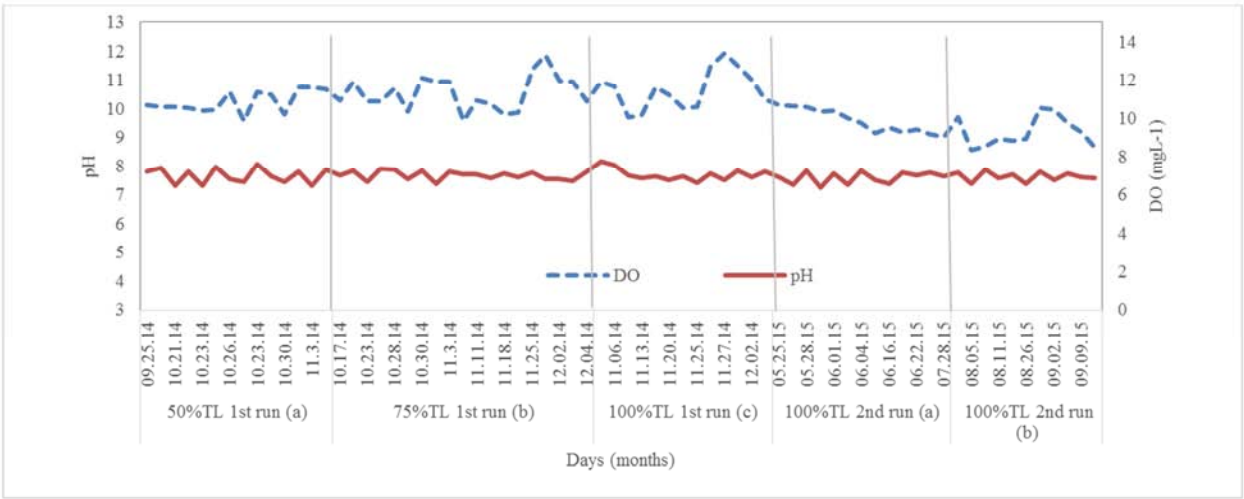

Figure 8. *pH and DO concentration during raceway pond cultivation.*

Production of algal biomass results in more oxygen (photosynthetic product) being produced [3]. Dissolved oxygen DO favours nitrification as a means of NH_4^+-N removal [14]. Nitrifiers (bacteria) require 4.57 g O_2 to oxidise per gram of NH_4^+ to NO_3^- [3]. 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 (a, b, c) DO was reaching to highest concentration, i.e., 12 mgL^{-1} (Figure 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 (a, b), DO was reaching to its lowest value, i.e., 8.5 mgL^{-1} (Figure 8). Godos et al. [40] 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. [11] 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. [13] 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. [12] 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^oC).

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. [46] observed a reduction of 30% in EC while treating leachate with bacto-algal system in lab for 10 days.

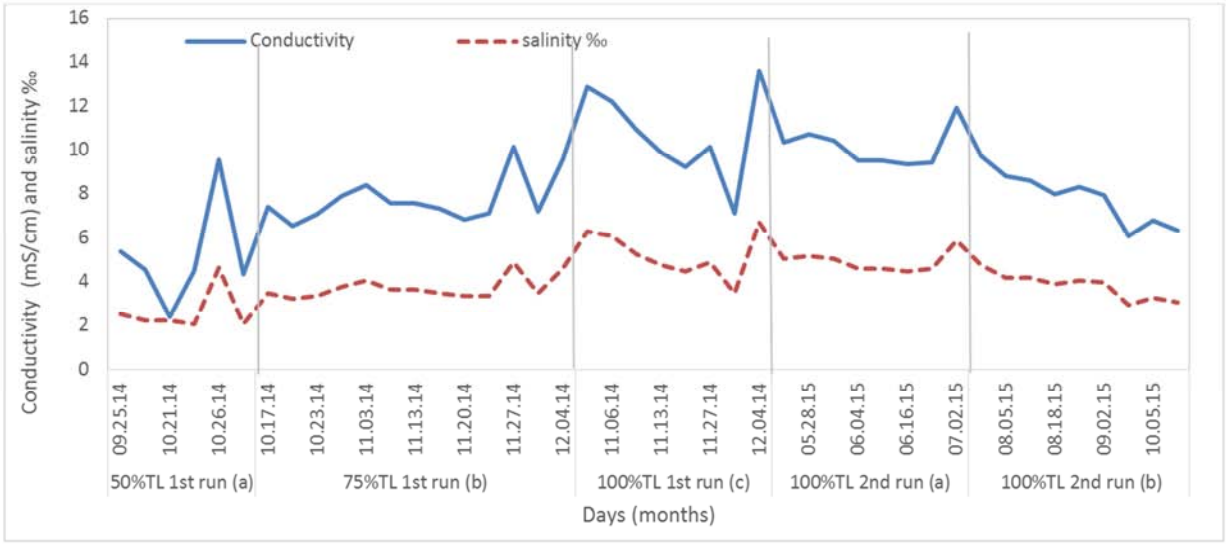

Figure 9. *Electrical conductivity EC and salinity data for raceway cultivation.*

4. Conclusion

Indigenous fresh water microalgal species were able to grow in high NH_4^+-N (~760 mgL^{-1}) and saline (10,000 mgL^{-1} chlorides), 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.

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