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Phosphorus removal from landfill leachate by microalgae

Zareen T. Khanzada[*]

Environmental Biotechnology Programme, Istanbul Technical University, Turkey

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ABSTRACT

Phosphorus is an essential constituent of all living organisms but it is non-renewable and its natural reserves are fast depleting. Phosphorus discharged in wastewater could be sustainably reused by microalgae. Knowledge about cellular phosphorus dynamics in microalgae has been rapidly advancing and luxury phosphorus (poly-P) uptake phenomenon by microalgae is becoming the focus point for many research studies. Ultra-membrane treated landfill leachate was used as a nutrient medium for the growth of indigenous microalgal species with simultaneous removal of phosphorus ($P\text{-}PO_4{}^{-3}$) and nitrogen ($N\text{-}NH_4{}^+$ and $N\text{-}NO_3$). Different concentrations of phosphorus (15–100 mg. L^{-1} $P\text{-}PO_4{}^{-3}$) was added to leachate. Highest nitrogen removal (69.03% $N\text{-}NH_4{}^+$) was observed for 100 mg. L^{-1} $P\text{-}PO_4{}^{-3}$ supplemented medium. P removal efficiency was 100% for all the tested $P\text{-}PO_4{}^{-3}$ concentrations. Intracellular poly-P was detected by florescence microscopy. Microalgae can be grown and utilized for the sustainable recovery of P and N from landfill leachate.

1. Introduction

Phosphorus (P) is an essential nutrient element for life of plants and animals. It serves an integral role in aspects of cellular metabolism ranging from energy storage (ATP and NADP), to cellular structure (phospho-lipids), to the very genetic material (DNA and RNA) that encodes all life on the planet [1]. However, global reserves of high-quality phosphate rock (PR) are limited, being a non-renewable natural resource, they are being consumed rapidly [2]. Most modern phosphate ($P\text{-}PO_4{}^{-3}$) fertilizers are made from PR. The input of $P\text{-}PO_4{}^{-3}$ based fertilizer is critical to the production of sufficient food, feed, fiber, and fuel to support a growing world population [3,4]. The appropriate and sound utilization of PRs as $P\text{-}PO_4{}^{-3}$ sources can contribute to sustainable agricultural intensification, particularly in developing countries endowed with PR resources.

Experts argue that a potential P crisis is coming and that it will leave the world's future food supply hanging in the balance [5]. P recovery and recycling are of considerable importance for sustaining profitable agricultural production in the long term since $P\text{-}PO_4{}^{-3}$ is mostly used in fertilizers. On the other hand, increased

discharge of inorganic phosphate (Pi) to lakes, bays, and other surface waters needs to be regulated [6].

Wastewater treatment facilities have enough nutrient rich water available with excess of nitrogen (N) and phosphorus (P) in discharged wastewaters. Overloading of such nutrients may negatively impact receiving natural ecosystem (rivers, lakes, ponds etc.) by creating nuisance algae growth (eutrophication), oxygen depletion and fish kills, undesirable pH shifts, and cyanotoxin production [7,8]. Considerable attention has been paid to an efficient means of Pi removal from wastewaters. Enhanced biological phosphorus removal (EBPR) through phosphorus accumulating organisms (PAOs) has become a well-established process and is currently applied in many full-scale wastewater treatment processes. Waste sludge (bacterial biomass) generated from an EBPR process contains large amounts (4–8% of dry biomass weight) of Pi and creates problems for safe disposal after use [6, 9].

P is also an essential macro-nutrient for microalgae growth. Although microalgae do not need large amounts of P, as it contains less than 1% of it, P is an important growth limiting factor, especially in natural environments where P is limited. Low P concentration is related to low cell densities [10]. Dual use of microalgae to remove nutrients from wastewater and produce biomass has been studied well since last decade or so. During research studies, it was found that some microalgae could assimilate more P (upto 3.3% dry biomass weight) than required for growth under nutritional conditions unfavorable for growth [9,11–13]. It was observed that microalgal biomass P content is dependant on species and cultivation condition. Cellular P content

* Corresponding author at: Environmental Biotechnology Programme, Department of Environmental Engineering, Istanbul Technical university İTÜ, Ayazağa Campus, Maslak, 34469, Istanbul. Turkey.

E-mail address: xaripink@yahoo.com (Z.T. Khanzada).

varies greatly between species and even for each species it varies among different cultivation conditions [14,15].

Further investigation on the phenomenon of luxury P uptake by microalgae using 31PNMR analysis demonstrated that assimilated P was in the form of poly-phosphate (Poly-P) [16]. Poly-P is a biological polymer composed of Pi residues linked by high energy phosphohydride bonds [13]. Poly-P plays a significant role in enhancing cell resistance under unfavorable environmental conditions. Dyhrman [1] reviewed that cellular poly-P has been variously attributed to a stationary phase adaptation, an energy storage compound, a metal chelator, a factor in DNA competency, an osmotic regulator, a buffer against alkali conditions and in phosphorus homeostasis (as a phosphate storage molecule) among other potential functions. More studies have been undertaken focusing on Poly-P metabolism in algae in order to enhance P removal from wastewater, and these studies found that environmental factors such as light, osmotic shocks, nutrient availability all affected intracellular P accumulation. The microalgal species studied include *Chlamydomonas*, *Skeletonema*, *Thalassiosira*, *Synechocystis*, *Nostoc*, *Calothrix*, *Synechococcus* and *Trichodesmium* among others [1,16]. Microalgae based treatment approaches could be particularly researched for non profit agricultural run off waters (or landfill leachate) where EBPR process cannot be easily implemented. Once P is accumulated and concentrated in the micoalgal biomass, it can be recovered and utilized as bio-solids (fertilizer product) [13]. Landfill leachate LFL is a highly polluted waste stream naturally 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. One of the main issues regarding management of closed landfills is the disposal of leachate which continue to be produced (with high concentration of ammonium nitrogen $N-NH_4^+$) for a long time even after closure of the landfills [17–19].

Landfill leachate has been used as a growth medium of microalgae for the removal of nutrients, heavy metals and organics etc. [22–24]. Since landfill leachate characteristics (age and structure) and composition (nutrient load and toxicity) differ in regions according to their respective climate and dumped solid waste, data on P removal dynamics particularly luxury P uptake using leachate collected from Istanbul municipal landfill (Odayeri Istaç- Istanbul Buyukşehir Belediyesi) was lacking. In an attempt to sustainably reuse and utilize landfill leachate nutrients, microalgae was grown in ultra-membrane filtered (0.2 μm pore size) leachate (TL) in the authors previous study (khanzada 2017). Tertiary treatment (polishing) of TL was carried out simultaneously by growing microalgae, but the biomass production was significantly reduced in the authors previous work. One of the reasons was suggested to be P limitation (5 mg. L^{-1} P-PO_4^{-3}), since TL had enough N ($\square$500 mg. L^{-1} $N-NH_4^+$) to support microalgal growth. The concentration of N and P (N:P ratio) is considered to be a fundamental factor and has a direct effect on microalgal growth kinetics, which relates to wastewater nutrient removal and biomass production [16,25,26]. N to P ratio for an effective nutrient removal is often assumed to match the Redfield ratio of 16:1 (for phytoplankton) but is actually species specific and most of the microalgae adjust their internal stoichiometric N:P biomass ratio according to the aquatic media in which they are growing [26,27]. Xin et al. [25] observed N:P ratios of 5:1 - 12:1 to be optimum for growth, nutrient uptake and lipid production by microalga *Scenedesmus sp.*

Based on these research findings, present study was conducted with five different Pi concentrations (15, 25, 50, 75 and 100 mg. L^{-1} P-PO_4^{-3}) added to TL, inorder to understand which combination of N:P in TL medium promoted better biomass growth and nutrient removal (particularly $N-NH_4^+$, $N-NO_3$ and P-PO_4^{-3}). Intracellular luxury P uptake was also observed by the tested indigenous microalgal strains and cross checked by flourescence microscopy.

2. Methodology

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, 0.2 μm pore size) was kindly provided by Istanbul municipal landfill management (Odayeri Istaç- Istanbul Buyukşehir Belediyesi) and stored at 4 °C in 20 L air tight plastic containers in dark until use. Physico-chemical parameters of TL is presented in Table 1. 1 L glass bottles with 500 ml working volume was used for the experiment. Continuous air bubbling was supplied at a rate of 3 L. min^{-1} (flow rate in each 500 mL culture was ∼0.31 mL. min^{-1}), continuous artificial irradience of 55 μmol photon $m^{-2}s^{-1}$ through white fluorescent lamps (measured by a digital quantum meter- Model MQ-200 Apogee, USA) was provided with room temperature of 24–25 °C. pH was maintained between 6.5–7.5 manually on alternate days with 0.5 N H_2SO_4/ NaOH. For lab study, five different concentrations of elemental P (15, 25, 50, 75, 100 mg. L^{-1} P-PO_4^{-3}) was made from P stock solution (0.67 mM K_2HPO_4) and added to autoclaved TL (20 min at 121 °C). BG11 nutrient media was set as positive (+ive) control and TL (without any P-PO_4^{-3} addition) as negative (-ive) control. Experiment was run in batch cultures (after priliminary trials with same conditions) for 30 days in dublicate.

2.1. Analytical methods

Biomass dry weight was determined after oven drying (at 60 °C) the centrifuged microalgal paste until constant weight was reached (∼ 3 days). Nitrogen- ammonium ($N-NH_4^+$) and nitrogen-nitrate ($N-NO_3^-$) were measured using an ion-selective electrode (Orion 95– 12) with an Orion IonAnalyser 701A meter (Orion Research Inc., Boston, MA). P-PO_4^{-3} concentration was determined following standard methods SM 4500- PBD [28]. The data was statistically analysed by ANOVA (single factor) followed by Student's *t*-test comparing with controls at $p \leq 0.05$ using microsoft excel. Averaged values are presented here.

Table 1
Physico-chemical characteristics of TL.

| Parameter	(mg. L^{-1})
BOD_5	211
BOD_5/COD	0.26
COD	800
P-PO_4^{-3}	5.42
$N-NO_3$	150
$N-NH_4^+$	1100
TDS (g. L^{-1})	11.84
Conductivity (mS/cm)	23.5
Salinity (‰)	14.37
Alkalinity	1700
pH	7
Chloride (Cl)	10000
Sodium (Na)	1540
Potassium (K)	2710
Mercury (Hg)	5.9
Lead (Pb)	5.15
Cadmium (Cd)	4.66
Zinc (Zn)	4.88
Nickel (Ni)	4.42
Copper (Cu)	4.44
Cromium (Cr)	3.78
Iron (Fe)	11.09
Sulphur (S)	17.54
Calcium (Ca)	1.5
Magnesium (Mg)	3.26

2.2. Fluorescence microscopic analysis

The samples (collected every week) were centrifuged and stored at $-20\,^{\circ}$C for the detection of poly-P granules using fluorescence microscopic analysis. 10 ul frozen pellets were incubated in solutions of 40,6-diamidino-2-phenylindole (DAPI; Sigma Inc., 2 ug ml^{-1}) dye over night and mounted on slides to be observed by fluorescence microscope with attached CCD camera (Zeiss Imager M2, DAPI Filter excitation/emission 338–378 nm/441-481 nm).

3. Results and discussion

3.1. Effect of different $P-PO_4^{-3}$ concentrations on microalgal growth

When using wastewater streams, one or more elements can be added to reach proper nutrient molar ratio (C, N, P), required for microalgae growth [7]. The leachate used in this study was P deficient (5 mg. L^{-1} P-PO$_4^{-3}$) as compared to N (<1000 mg. L^{-1} N-NH$_4^+$). Chu et al. [16] observed that P-PO$_4^{-3}$ concentrations significantly affected biomass production. In the present study P-PO$_4^{-3}$ addition significantly enhanced microalgal growth curves as compared to TL (-ive control) (except for 15 mg. L^{-1} P-PO$_4^{-3}$) but still significantly lower than control group BG11 in all the tested concentrations of P (15, 25, 50, 75, 100 mg. L^{-1} P-PO$_4^{-3}$) (Fig. 1). Growth curve showed no proper onset of stationary phase and cultures continued to grow slowly until the end of 30 day experiment (Fig. 1). Microalgae growth showed a lag phase of atleast 2–3 days in all the P-PO$_4^{-3}$ concentrations tested, even after the cultures were pre-acclimated to leachate medium for 3–4 weeks prior to starting the experiment. High salinity ($\sim$14 g. L^{-1}), brown color and imbalance nutrient composition of leachate seemed to have delayed the growth of cultures and increased lag phase in all the treatments (Fig. 1; Table 1). Zhao et al. [29] also observed a lag phase of 6 days for 20% leachate (338 mg. L^{-1} NH$_4^+$-N) in their study.

In the present study, N:P ratio (of 25 mg. L^{-1} P-PO$_4^{-3}$ treatment) in the leachate media was close to BG11 media (+ive control) as 40:1, but growth was still significantly lower (Figs. 1,2). It could be due to high concentration of N-NH$_4^+$ (<1000 mg. L^{-1}) which can be toxic to microalgae [30]. Biomass growth in 15 mg. L^{-1} P-PO$_4^{-3}$ treatment was as low as TL (-ive control). This is consistant with Markou et al. [31] who provided 10–50 mg. L^{-1} P and observed that growth was significantly reduced than TAP media (control group) and poly-P accumulation was enhanced in their 10 mg. L^{-1} P treatment. *Chlorella* sp. showed significant growth in 50 mg. L^{-1} P where P was mainly used for proliferation of cells. Dry biomass weight, growth rate and volumetric biomass productivities from
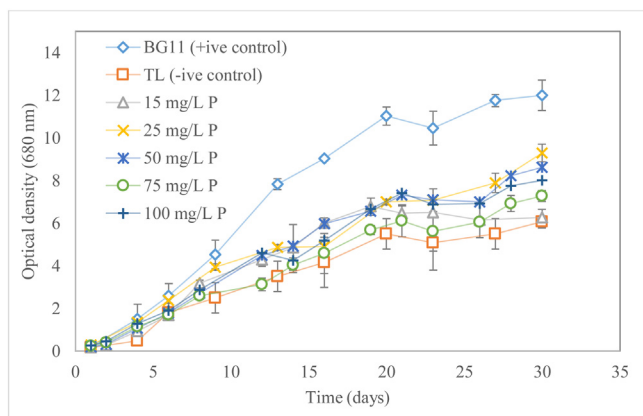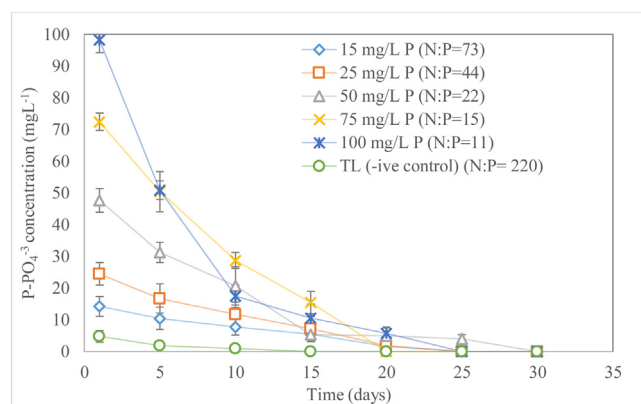
Fig. 2. P-PO$_4^{-3}$ removal from the leachate media along with N:P ratios (in legends) after addition of different P-PO$_4^{-3}$ concentrations (data shown is the mean $\pm$ SD).

different P-PO$_4^{-3}$ treatments and control groups are presented in Table 2.

3.2. $P-PO_4^{-3}$ removal from leachate media

In natural environment and wastewater, P is present in various forms, such as ortho-phosphate (containing one phosphate unit), poly-phosphate, pyro-phosphate, meta-phosphate and their organic complexes. The major form in which microalgae acquire P is inorganic phosphate P-PO$_4^{-3}$ (ortho-phosphate). The transport of P-PO$_4^{-3}$ into the microalgal cell is an energy dependent process and its uptake rate is slower in dark than in light environments [32]. Moreover, the uptake of phosphate is influenced by pH; uptake rates decrease in acid and relatively alkaline environments [33]. In the present study continuous light was provided and pH was kept close to neutral, which seemed to facilitate P-PO$_4^{-3}$ removal from TL media in all the concentrations of P-PO$_4^{-3}$ tested (Fig. 2).

In the present study, P-PO$_4^{-3}$ concentration in the medium reached to minimum on day 20$^{\text{th}}$ in all the treatments (Fig. 2). The P-PO$_4^{-3}$ was observed to reach to minimum quicker than N-NH4$^+$, which is similar to Rasoul-Amini et al. [34], who observed a faster removal efficiency for P-PO$_4^{-3}$ (94.77%) than nitrogen (51.41%) within first 4 days using urban wastewater. Qu et al. [35] also evaluated that P uptake was a relatively rapid process. Su et al. [36] also observed a faster 100 mg. L^{-1} P-PO$_4^{-3}$ removal than N-NH$_4^+$. P-PO$_4^{-3}$ was completely consumed (99%) in their experiment within 6 days.

This could imply that microalgal cells accumulated P from the media and utilized this intracellular (stored) P later for N-NH$_4^+$ consumption and other cellular functions [10]. Microalgal cells absorb N and P from wastewater and use these nutrients to produce biomass and the removal of one nutrient depends on the availability of the other. At high N supply, the concentration of P in the microalgal biomass was a function of the supply of P [27]. In the present study, N was in excess, which also might have favoured the removal of all (tested concentrations of) P from leachate medium.

In the present study, apart from cultivation conditions (continuous light and neutral pH) and excess N availability, rapid P uptake could be due to genetics of the tested microalgal strains, as *Chlorella vulgaris* and *Chlamydomonas reinhardtii* are known poly-P accumulator organism (PAOs) [12,16,35,37]. The luxury uptake (by the PAOs) could likely drive the accumulation of poly-P in aquatic systems or areas where P-PO$_4^{-3}$ is in excess, like the coastal zone. Poly-P concentrations of $\sim$7 % in coastal diatoms, *Skeletonema* sp. was observed under nutrient replete conditions and hypothesized a luxury uptake response. Ota et al. [13] observed

Fig. 1. Growth curve showing microalgal cell density in leachate media against different concentrations of P-PO$_4^{-3}$ (data shown is the mean $\pm$ SD).

Table 2
Dry biomass weight, growth rate and volumetric biomass productivities of different P-PO$_4^{-3}$ treatments and control groups in the experiment (data shown is the mean $\pm$ SD).

Treatments ↓	Optical density OD 750 nm	Dry weight (g/L) $R^2 = 0.921$	Growth rate per day $\mu = (lnOD_t - lnOD_0)/t$	Volumetric productivity mg/L/day
BG11 (+ive control)	11.06 ± 0.365	2.72	0.29	69.74
TL (-ive control)	6.02 ± 0.51	1.47	0.15	36.79
15 mg. L^{-1} P-PO$_4$-	6.29 ± 1.13	1.49	0.16	38.20
25 mg. L^{-1} P-PO$_4$-	7.58 ± 0.83	2.00	0.24	57.40
50 mg. L^{-1} P-PO$_4$-	7.67 ± 0.164	1.92	0.19	49.20
75 mg. L^{-1} P-PO$_4$-	6.8 ± 0.164	1.84	0.17	47.10
100 mg. L^{-1} P-PO$_4$-	7.12 ± 1.10	1.87	0.18	47.90

that 43% of P from TAP media was transformed into poly-P as a storage molecule by the end of early stage (3 day culture), i.e., total P and poly-P was accelerated during the early stage of sulphur-deficient growth conditions for microalgae. Markou and Geogakakis [33] reviewed that P deficient cells take up P-PO$_4^{-3}$ at higher rates than P sufficient cells do. In the present study, the inoculum was growing in P deficient TL media and this might have triggered the microalgae to accumulate P faster once P sufficient experimental conditions were aquired. All of the tested concentrations of P-PO$_4^{-3}$ (15–100 mg. L^{-1}) were removed from the leachate media, which is consistant with the results abtained from Ota et al. [13] who observed □82 mg. L^{-1} P removed from TAP media to be found as intracellular poly-P. Li et al. [38] observed 79% P removal using autoclaved centrate with high P concentrations of 215 mg. L^{-1}.

3.3. Intracellular poly-P storage

Fluorescence microscopy using DAPI dye staining of cultured microalgal cells was also carried out to check the presence of poly-P inside cells, apart from conventional assay of P-PO$_4^{-3}$ residual measurement in TL. Fluorescence images also confirmed the presence of poly-P in the tested cultures against –ive control (TL), which was without any added PO$_4^{-3}$, i.e., Pi deficient cells (Figs. 3,4). DAPI is a useful staining tool in the fluorometric analysis of DNA and PolyP [39]. DAPI-poly-P complex can be seen as yellowish green color (of intracellular poly-P) against blue DAPI-DNA stained cells (Figs. 3,4). These findings suggested that illumination and autotrophic growth might significantly increase the metabolic requirement for P and enhance P assimilation by *Chlorella* and *Chlamydomonas* species. This also suggested that, when extracellular P-PO$_4^{-3}$ is plentiful, Microalgae (*Chlorella* and *Chlamydomonas* species) continue to assimilate and store poly-P in their cells.

3.4. Effect of P supplementation on nitrogen (N-NH$_4^+$ and N-NO$_3$) removal from TL

The presence of P-PO$_4^{-3}$ is of great importance in the process of N utilization in the microalgal cell and N uptake is considered to be a function of P-PO$_4^{-3}$ availability. Landfill leachate is normally P limited and P is considered as growth limiting factor when it comes to N-NH$_4^+$ assimilation by microalgae. Pasku-liakova et al. [40] achieved 90.7% N-NH$_4^+$ removal when 10% diluted raw leachate (100 mg. L^{-1} N-NH$_4^+$) was supplemented with phosphate (P-PO$_4^{-3}$) to make N:P molecular ratio 16:1 in 24 days. Growth rate was observed to be 0.14 day^{-1}, without P-PO$_4^{-3}$ addition N-NH$_4^+$ removal was 51%. Presence of residual nitrogen and phosphate in the system by the end of 24 days, was suggested to be due to the limitation of microalgal cells by the exhaustion of another micronutrient. In the present study, N-NH$_4^+$ removal was significantly increased in all the tested P-PO$_4^{-3}$ treatments against TL (-ive control) (Fig. 5). N:P ratio of
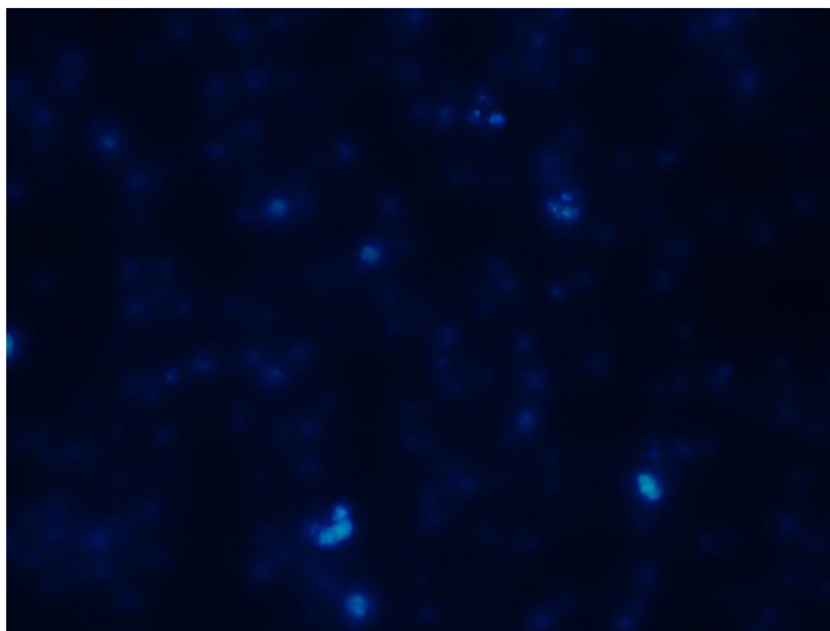
Fig. 3. Fluorescence microscopic image of Pi defcient cells in TL (-ive control) with no visual Poly-P presence.
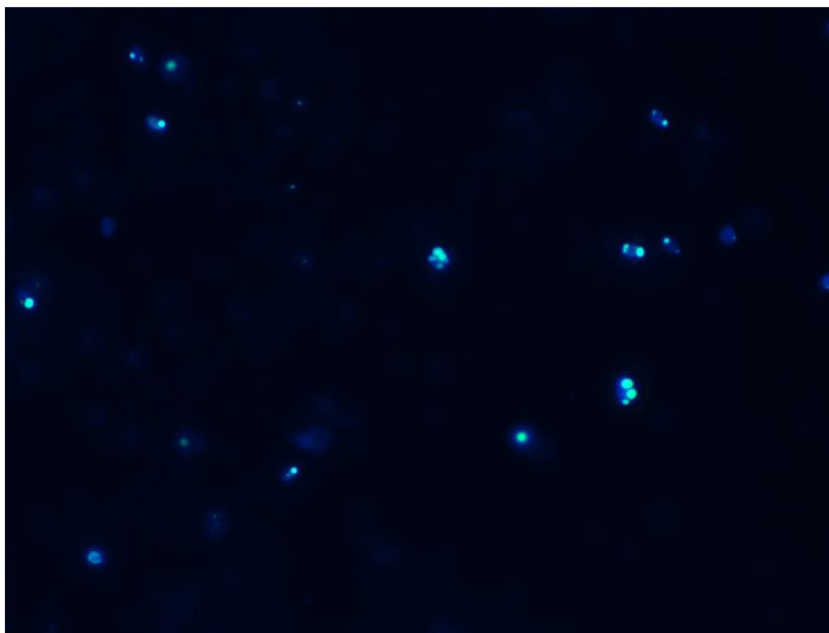
Fig. 4. Fluorescence microscopic image of poly-P accumulated in microalgal cells. Poly-P is stained yellowish green against the blue DAPI dyed microalgal cells (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
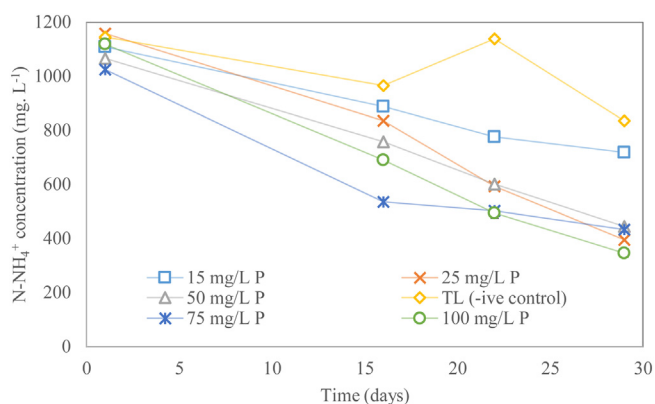
Fig. 5. N-NH$_4^+$ removal from TL media supplemented with different P-PO$_4^{-3}$ concentrations.

Fig. 6. N-NO$_3^-$ removal from TL media supplemented with different P-PO$_4^{-3}$ concentrations (data shown is the mean ± SD).

50, 75, 100 mg. L^{-1} P-PO$_4^{-3}$ treatments (11:1 – 22:1) was close to redfield ratio of 16:1 and showed better N-NH$_4^+$ removal than TL (–ive control) and 15 mg. L^{-1} P-PO$_4^{-3}$) (Fig. 5).

Since wastewater are usually complex medium and nutrient removal are not as direct as can be observed using regular nutrient media, Procházková et al. [32] observed that extent of P limitation can provoke different responses in algae. Also bacteria can effect nitrogen (NO$_3$-N-NH$_4^+$) removal dynamics in microalgal cultures. In the present study, N-NH$_4^+$ was not fully removed from the system in all the tested P concentrations (Fig. 5). Addition of 100 mg. L^{-1} P-PO$_4^{-3}$ showed highest N-NH$_4^+$ consumption 69.03% as compared to TL (–ive control) (Fig. 5). But growth in the same 100 mg. L^{-1} P-PO$_4^{-3}$ showed a similar growth curve to the rest of P concentrations tested (Fig. 1). N-NO$_3$- removal was significant between 50–75 mg. L^{-1} P-PO$_4^{-3}$ treatments and TL (–ive control) but less than 50% was removed from the system in 30 days experiment in all the treatments (Fig. 6).
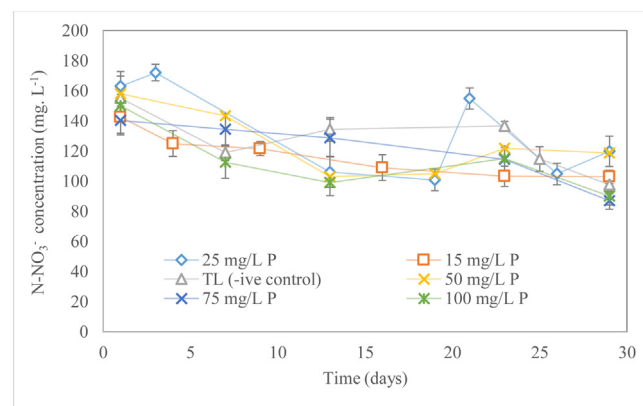
4. Summary

In the present study, irrespective of landfill leachate's high N-NH$_4^+$ concentration, salinity and dark color microalgal cultures (*Chlorella vulgaris* and *Chlamydomonas reinhardtii*) were able to grow sustainably and remove N and P from the system. N-NH$_4^+$ removal was 69.03% from 100 mg. L^{-1} P-PO$_4^{-3}$ added TL, but N-NO$_3$- was not significantly removed among the tested different P-PO$_4^{-3}$ concentrations. At the end of 30 day experiment residuel N was still present in the system. P-PO$_4^{-3}$ removal was rapid and efficient with 100% removal efficiency. P-PO$_4^{-3}$ removal from leachate medium via microalgae was suggested to be a luxury uptake. Presence of intracellular poly-P granules (stored P-PO$_4^{-3}$) was confirmed by fluorescence microscopy. This intracellular stored P can be extracted from microalgal biomass or the produced microalgal biomass can be sustainably utilized for a number of agricultural uses.

Declaration of Competing Interest

None.

Acknowledgements

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2020. e00419.

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