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Phytoremediation of Domestic Sewage in Constructed Wetland Integrated with Cultivation of *Chlorella* sp.: A Novel Technique for Remediation and Resource Recovery

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Abstract: Domestic sewage-based constructed wetland (CW) showed that the effluent from CW-system contain enough plant nutrients and enhanced the growth of microalgae. Hence, a pilot CW system employing *Typha latifolia* in domestic sewage, integrated with the cultivation wild type *Chlorella* sp. was investigated. Phytoremediation at 48 hours of detention time caused significant changes in its physicochemical properties and the generated effluent was notably attractive for the cultivation of microalgae. The microalga was grown in 6 treatments: treated-mixotrophic (T_1), treated-heterotrophic (T_2), treated-autotrophic (T_3), control-mixotrophic (T_4), control-heterotrophic (T_5) and control-autotrophic (T_6) conditions for 8 days inside an incubator. The results suggested that phytoremediation effluents integrated with mixotrophic cultivation of microalgae, utilising both light and carbon sources could be the most efficient, environmentally safe, sustainable and novel technique for synergistic resource generation and bioremediation.

Key words: CW-system, phytoremediated effluents, emergent macrophytes, wild-type *Chlorella*.

Introduction

Currently, one of the major percentages of the world's population which is steadily increasing, lives in the tropics and subtropics, thereby, producing enormous nutrient-loaded domestic sewage in residential areas. However, poor economic conditions in these regions inhibit the application of modern or even conventional wastewater treatment systems, as a result of which a huge quantity of untreated sewage has accumulated in the environment. Nevertheless, due to their regional conducive climate, the application of constructed wetlands (CWs) technology for the treatment of generated domestic sewage is highly rewarding (Kadlec and Knight 1996; Kivaisi, 2001). However, Juwarker et al. (1995) indicated that effluents from macrophytes-based CWs-system are rich in plant-available forms of nutrients, whereas others have claimed that the nutrient-rich domestic effluents could be a viable option for both productions of microalgae based biofuel and remediation of the environment (Gao et al., 2021; Wang et al., 2010). Studies employing wastewater industry-based feedstock for microalgae cultivation and lipid content were successful (Mitra et al., 2012; Pittman, 2011; Wang et al., 2010) since all the nutrients needed for algal growth are readily available in conventionally treated effluents (Greenwell et al., 2010). Cultivation strategies of microalgae (Cheirslip and Torpee, 2012)

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and CO_2 utilization method (Lin et al., 2012) in algae are reported to be crucial to enhance their biomass yield and oil content.

Evidently, on one hand, commercial-scale algal biomass demands wastewater-based feedstock for its viable production and on the other hand such feedstock in excess, particularly in domestic sewage has damaged the environment of the developing world. In this context, the generation of huge quantities of nutrient-rich domestic sewage, mostly in developing countries of tropical and subtropical regions offers great promises as inexpensive substrates for cost-effective microalgae cultivation as well as bioremediation of contaminated environments. Hence, a pilot experiment was conducted to study the phytoremediation of domestic sewage integrated with the cultivation of wild-type *Chlorella* sp. in autotrophic, mixotrophic and heterotrophic conditions for sewage-based resource recovery.

Material and Methods

Design of Pilot Wetland Treatment System

Round and identical pots, each (80 litres) made of concrete/cement, were designed to mimic emergent macrophytes with a vertical flow CWs-system. Pots were perforated from the bottom and fixed on stands with plastic pipes for the outflow of treated wastewater. For phytoremediation of wastewater, *Typha latifolia* with identical weight and height was planted on the bed (06 inches) of coarse sand/gravel in previously prepared pots and grown for two months in normal tap water. Two treatments (macrophytes and without macrophytes), each in triplicates were kept/grown in the open garden under identical environmental conditions (Table 1). For suitable effluent flow with 48 hours retention time in each CWs-system, two pots were connected/combined to a capacity of 160 litres. Then, circulations of normal tap water into all the pots were replaced with raw sewage from an overhead tank and continued for a stabilisation period of seven days, followed by a flow rate with 48 hours retention time. For continuous sewage flow, the tank was refilled at an interval of 12 hours for 18 days. Treated effluent was allowed to move vertically downward through the rhizospheric zone of CWs-system. Finally, phytoremediation effluent was discharged through a drain-out pipe at the bottom and collected/analysed (Table 2) before the cultivation of microalgae.

Table 1: Variation of atmospheric temperature (°C) during outdoor purification of stock culture (seed) used in the pilot study for cultivation of wild-type *Chlorella* sp.

	Minimum (Jan-Feb)	*Maximum (Feb-March)*	*Mean*
Temperature (°C) range	14.5	32.5	–
Mean (±95% confidence limits, *n*=42)	–	–	22.8±1.25

Table 2: Mean (± SE, *n* = 3) physicochemical properties of fresh sewage, phytoremediation (CWs) effluent and untreated effluent (control)

Parameters	*Sewage (Fresh)*	*Treated effluent (CWs remediated)*	*Control (Untreated effluent)*
pH (0.38)#	8.9±0.06	7.2±0.17	8.8±0.12
BOD (15.9)	129.5±8.8	42.1±3.6	104.5±10.5
COD (67.8)	446.5± 22.6	134.8±16.6	408.2±25.8
TKN (30.2)	128.5± 11.5	56.2±5.9	112.5±11.2
TSS (43.2)	305.8± 18.5	38.2±4.6	182.3±15.0
TP (6.2)	28.5±2.4	8.2±0.69	25.2±2.3
NH_4^+ N (16.1)	84.2±5.2	46.8±5.6	78.5±4.8
NO_3^- N	ND	8.5±0.43	2.4±0.25
DO	ND	3.8±0.28	1.50±0.17

All properties (except pH) in mgL^{-1}; #: Values are Least-Significant-Difference($p < 0.05$); ND: Not detectable

Development and Purification of Wild-Type *Chlorella* sp.

Previously, employing high powered compound microscope, we have identified microalgae *Chlorella* sp. and many other algal species from the "Laxmital sewage pond" in Jhansi. For the present investigation, wild-type *Chlorella* sp. was isolated, subsequently, an axenic culture was developed following single cell isolation technique using an inverted microscope (400× magnifications) with serial dilutions method as described by Gour et al. (2014). About one litre of pure microalgae culture (in sterilised phytoremediation effluent media) with high density and viability was prepared using open sunlight (4000 lux) and nutrient media (NH_4Cl, H_2PO_4, $CaCl_2$, $MgCl_2$ and $CuCl_2$). Penicillin 100 mg/L, streptomycin 50 mg/L and chloramphenicol 10 mg/L were applied to restrict the growth of bacteria and cyanobacteria. Germanium dioxide 5 mg/L was also added to the control of diatoms. At an interval of 48

hours, $NaHCO_3$ 200 mg/L was added as the inorganic carbon source (Lin et al., 2012). Every alternate day, the pH of the culture medium was maintained at about 8.0 by the addition of concentrated H_3PO_4 or 12% NaOH. On the 21st day, the high concentration/vigor culture of wild-type *Chlorella* sp. was employed as inoculums for further cultivation under control conditions.

Cultivation of Microalgae in Phytoremediated-Effluent

Wild-type *Chlorella* sp. was cultivated by inoculating 5 ml inoculum of stock/working culture into a total/culture volume (250/100 ml) conical flask, each containing control (untreated effluent) or phytoremediation effluents in mixotrophic, heterotrophic and autotrophic medium inside an incubator (30°C). Then, an interval of every 2 days, 2.0 g/L glucose (heterotrophic nutrition) and 400 mg/L $NaHCO_3$ (photosynthetic need of carbon) were supplemented on a rotary shaker (at 120 rpm, 15 minutes) for mixotrophic/heterotrophic and autotrophic/mixotrophic cultures, respectively. Autotrophic and mixotrophic cultures (conical flasks in inverted condition) were exposed to fluorescent light intensity (3000 lux) with 16:8 (light:dark) cycle, whereas heterotrophic cultures were kept in total darkness (sealed black polyethylene packet) within the same incubator to ensure heterotrophic growth. After a period of 8 days of cultivation, microalgae biomass in each flask was estimated separately by centrifuging (3900 rpm for 20 minutes at 30°C) and drying the pellets at 40°C to constant weight. Three independent operations were carried out for the estimation of the final microalgae biomass. The supernatant was decanted and analysed to study the effects of microalgae growth in effluents.

Analytical Methods

All physicochemical analyses were performed under strict laboratory conditions. Analytical reagent-grade chemicals and double distilled water were used for all chemical analyses. Borosilicate glass apparatus was used for all analytical works. Fresh sewage and effluents were carried to the laboratory and kept in ice kits and analysed for pH, BOD, COD, DO, total Kjeldahl nitrogen (TKN), total suspended solids (TSS) and total phosphorus (TP) following standard procedures (APHA, 2005). The steam distillation method (Allen, 1998) employing MgO with and without Devarda's alloy was used to determine NO_3^- + NO_2^- -N and NH_4^+ -N in fresh sewage and effluents, respectively.

Statistical Analysis

Statistical analyses were conducted using the software Microsoft Excel 2010. All parameters were carried out in triplicates. Data generated in the experiment were analysed using one way analysis of variance (ANOVA) as per the standard statistical procedure (Gomez and Gomez, 1984). To perform an analysis of significance, *F* values were obtained; the least significant differences (LSD, at $p < 0.05$) value was used to test the significance between treatment means.

Results and Discussion

Phytoremediation of Domestic Sewage

Table 2 summarises the initial physicochemical properties of fresh sewage, phytoremediation-effluent and control. The mean pH values in different wastewater ranged between 7.2 and 8.9. The pH value as observed in the phytoremediation effluent was significantly ($p < 0.01$) decreased than fresh sewage, probably due to the uptake of NH^+_4N and concomitant release of a proton by macrophytes. The BOD was reduced from 129.5 mgL^{-1} to 42.1 mgL^{-1}, while COD came down from 446.5 mgL^{-1} to 134.8 mgL^{-1} during phytoremediation. TKN was reduced from 128.5 mgL^{-1} to 56.2 mgL^{-1} during phytoremediation, representing more than a 56% reduction. TSS in phytoremediation effluents was 38.2 mgL^{-1} compared to 305.8 mgL^{-1} in the fresh sewage, corresponding to a reduction of 87%. During phytoremediation, TP content decreased from 28.5 mgL^{-1} to 8.2 mgL^{-1}. The reductions in all these values are attributed to the symbiotic association between bacteria and the root of *Typha latifolia*, and causing degradation of organic matter. Our observation revealed that the settling of suspended particulate matters stimulated microalgae growth in control as well as in phytoremediation effluents. A little increase in DO in case of control might be due to sluggish microalgae photosynthesis. However, a substantial increase of DO in case of phytoremediated effluents may be referred to both the growth of microalgae and rhizospheric diffusion of O_2 from plant tissues as well. The results showed that the fresh sewage and control sewage were suboxic and the condition became aerobic after 2 days of phytoremediation. Statistical analysis of pH, COD, NH_4^+, TKN and TP values in phytoremediation effluents were significantly ($p < 0.05$) decreased in comparison to fresh sewage. It is therefore, evident that the physical and chemical quality of sewage was modified to a significant extent when subjected to phytoremediation (Giri et al., 2012; Kivaisi, 2001).

Growth and Productivity of Wild Type *Chlorella*

The biomass yield and productivity of microalgae have been summarised in the Table 3. It is evident from Figure 1, that the microalgae markedly increased biomass in all phytoremediation cultures than their control counterparts. The mean values for the algal biomass yield revealed that growth occurred in all treatments, while less growth was observed in cultures containing either phytoremediated-autotrophic (T_3) or control-autotrophic (T_6) culture. A comparison of microalgal yield between phytoremediation and control cultures indicated that the biomass increased in both treatments, however, the microalgae cultivated under phytoremediation-mixotrophic culture (T_1) had the highest biomass concentration (2.62 gL^{-1}), which was significantly (p<0.01) higher than control-mixotrophic (T_4) concentration (0.42 gL^{-1}). The photoautotrophic microalgae production registered a biomass concentration of 1.22 gL^{-1} and a productivity rate of 153mg/L/d in culture T_3, which is significantly (p<0.01) higher than its control counterpart T_6. The high volumetric biomass yield and productivity in phytoremediation-photoautotrophic culture T_3 could be related to the efficient fixation of inorganic carbon from $NaHCO_3$ by the process of photosynthesis at optimal pH (7.2-8.8). Furthermore, our results on biomass yield and productivity in phytoremediated-phoautotrophic culture tend to suggest that the microalgae could be grown photoautotropically using $NaHCO_3$ as a source of inorganic carbon. A similar suggestion was also given by previous workers (Lin et al., 2012).

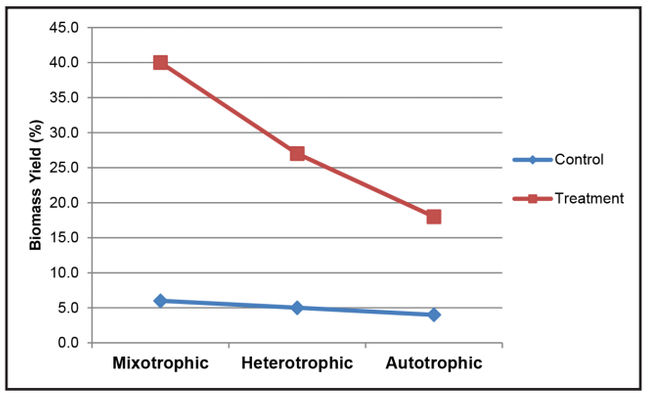

Figure 1: Variation in biomass yield of wild-type *Chlorella* sp. in different treatments.

The statistical examination of inoculums weight did not reveal any significant difference among different treated cultures, whereas the biomass production rate (mg/L/d) and production per unit inoculums (mg/L/ml) were highly significant among different treatments of microalgae cultivation at the end. The microalgae yielded maximum biomass under mixotrophic culture in phytoremediation effluent and lowest under autotrophic-control culture. Furthermore, biomass production per day (Figure 2) and biomass production per unit inoculums (Figure 3**)** also recorded consistently a similar trend. Nutrient-rich culture medium at nearly

Table 3: Mean (± SE, *n* = 3) biomass concentrations and mean biomass production rates of wild-type *Chlorella* sp. in treated (phytoremediated) effluent and control (untreated effluent) during 8 days cultivation in controlled condition

No.	*Treatment type/media*	*Cultivation volume (ml)*	*Inoculums weight (g)*	*Inoculums concentration (g/L)*	*Biomass rate (mg/L/d)*	*Production inoculums (mg/ml)*	*Production per unit*
Treated-(phytoremediated)-effluent							
T_1	Mixotrophic	5.0	4.54±0.01	2.62±0.15	328±14.3	524±18.2	
T_2	Heterotrophic	5.0	4.55±0.02	1.81±0.12	226±13.4	362±15.6	
T_3	Autotrophic	5.0	4.55±0.02	1.22±0.13	153±12.2	244±18.2	
Untreated-(control)-sewage							
T_4	Mixotrophic	5.0	4.56±0.03	0.42±0.04	53±1.1	84±6.9	
T_5	Heterotrophic	5.0	4.54±0.01	0.34±0.03	43±0.81	68±6.5	
T_6	Autotrophic	5.0	4.55±0.05	0.23±0.02	29±0.52	46±5.9	
ANOVA							
CV(%)	-----	---	0.39	15.4	13.8	14.56	
$LSD_{(p< 0.05)}$	-----	---	0.032	0.30(0.37)	14.1 (18.4)	17.9	
F value	-----	---	0.54	95.2	105.4	89.8	

neutral pH with added glucose might have enhanced biomass yield and productivity in T_1. In heterotrophic cultivation, we observed that even in absence of light the microalgae yielded significantly higher biomass than concomitant autotrophic-cultivation, which could be due to the inducible hexose transport system in wild-type *Chlorella* sp. that allows it to utilise glucose. With respect to the biomass production trend in microalgae, the biomass yield and productivity was significantly higher, when they were cultivated under mixotrophic condition. Our hypothesis is that the additional biomass yield and productivity in mixotrophic cultivation could be related to the photosynthetic growth of microalgae, while the presence of glucose confirmed and equalized the concomitant and self-sufficient heterotrophic growth in both heterotrophic and mixotrophic conditions (Mitra et al., 2012).

Effects of Microalgae Growth on pH and Conductivity

The effects of biomass yield of microalgae on phytoremediated effluent at different culture were examined (Table 4) to test its potential for further remediation. The results on pH values indicate a consistently increasing trend, from sluggish control-mixotrophic to sharp rise in treated-autotrophic culture in comparison to their respective initial values. In contrast, the pH was found to decrease significantly in treated-heterotrophic to sinking slightly in control heterotrophic during the period of cultivation. The consistent pH rise found under autotrophic and mixotrophic conditions could be attributed to the growth of microalgae and the resultant uptake of dissolved carbon species such as CO_2 from dissolved bicarbonate, leaving OH^- species with less efficiently under ammonium, and more efficiently under nitrate growth conditions (Garcia et al., 2010). Further, the regular declining pH under heterotrophic cultivation could be explained by the fact that ammonium consumption during dark phase growth of microalgae released protons and decreased the pH (Grobbelaar, 2004). Our observations also demonstrate that microalgae cultivation could be used within the wastewater treatment process for bioremediation and sterilisation of domestic sewage, and support the claims by Park et al. (2011).

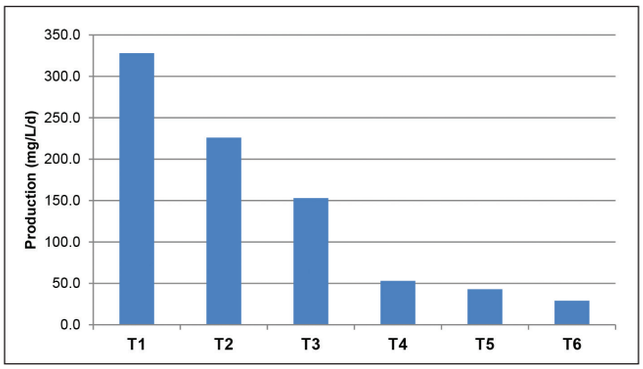

Figure 2: Variation in biomass production of wild-type *Chlorella* sp. in different treatments.

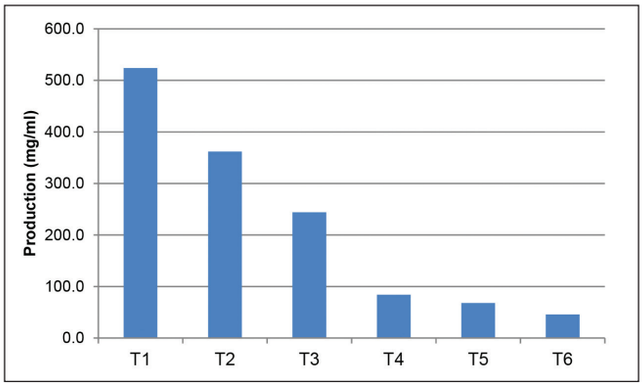

Figure 3: Biomass production in per unit of inoculum of wild-type Chlorella sp. in different treatments.

The conductivity in the final effluent shows that the growth of microalgae causes the lowest values under mixotrophic conditions in comparison to heterotrophic as well as autotrophic conditions. Among phytoremediation cultures, the mean minimum conductivity was recorded in treated-mixotrophic (1.28 mS/cm), whereas, the maximum in treated-heterotrophic culture (1.77 mS/cm). Likewise, among the control, the mean conductivity was in control-mixotrophic (2.45 mS/cm) and maximum in control-heterotrophic (2.82 mS/cm). It is suggested that the final value of conductivity in these cultures depended on the extent of uptake by microalgae biomass. The conductivity value as observed in heterotrophic cultivations was significantly ($p < 0.01$) increased than all other cultures, which is due to the high concentration of NH^+_4-N (Table 4).

Effects of Microalgae Growth on NH^+_4-N and NO_3^--N Concentration

Table 4 summarized the effects of microalgae growth on NH^+_4-N and NO_3^--N concentrations of final effluents. The examination of effluents (Figure 4) clearly demonstrates that the growth of microalgae causes a sharp reduction of NH_4^+-N with a minimum of 56.1% (T_5) to a maximum of 99.1% (T_1) with respect to their initial values. Our observation also exhibits a remarkable reduction of NH_4^+-N in phytoremediation cultures, suggesting the hypothesis that regardless of culture

Table 4: The effects of wild-type *Chlorella* sp. growth on mean(±SE, *n* =3) conductivity, pH, NH_4^+-N, NO_3^-N, total-N and total-P status in final effluents of different treatments over 8 days of cultivation

Treatment No.	*Cultivation type/media*	*pH*	*Conductivity (mS/cm)*	*NH_4^+-N content-(mg/L)*	*NO_3^-N (mg/L)*	*Total N(TKN) (mg/L)*	*Total P content (mg/L)*
Treated-(phytoremediated)-effluents							
T_1	Mixotrophic	8.2±0.06	1.28±0.04	0.4±0.13	1.2±0.11	10.5±0.36	1.0±0.15
T_2	Heterotrophic	6.4±0.05	1.77±0.10	4.8±0.14	0.4±0.05	12.2±0.34	2.2±0.26
T_3	Autotrophic	8.8±0.10	1.45±0.10	0.5±0.05	0.8±0.06	18.2±0.38	3.5±0.21
Control-(Untreated)-effluents							
T_4	Mixotrophic	9.2±0.06	2.45±0.08	24.8±2.2	1.0±0.11	69.5±0.51	12.8±0.46
T_5	Heterotrophic	8.4±0.06	2.82±0.06	34.5±3.2	0.6±0.05	73.2±0.80	14.2±0.41
T_6	Autotrophic	9.5±0.05	2.58±0.09	28.5±2.9	2.2±0.10	78.4±0.53	18.5±0.51
ANOVA							
CV(%)	-----	2.4	6.9	11.4	15.8	2.5	7.2
$LSD_{(p< 0.05)}$	-----	0.20(0.24)	0.25(0.31)	2.7(3.3)	0.29(0.35)	1.6(1.9)	1.12(1.35)
F value	-----	311.1	61.6	254.9	45.8	4139.5	414.4

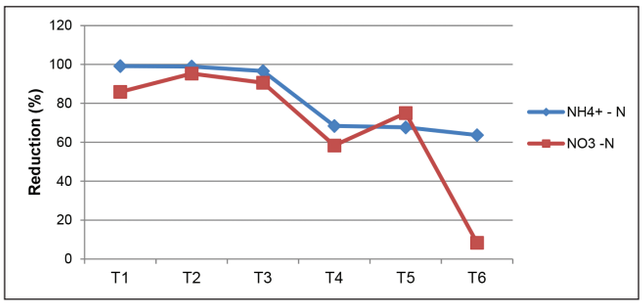

Figure 4: Reduction of NH_4^+-N and NO_3-N in the final effluents of different treatments.

conditions of phytoremediation effluents, NH_4^+-N is fairly and effectively taken up by microalgae for biomass production. Furthermore, the highest reduction of NH_4^+-N, both in absolute values and relative terms, advocates the hypothesis that the phytoremediation effluents under mixotrophic cultivation of wild-type *Chlorella* sp. are excellent for use in wastewater treatment. Unlike ammonification, nitrification was very limited as indicated by a fairly low concentration of effluent NO_3^- -N. The investigation of NO_3^--N values reveals the highest reduction in heterotrophic-cultivation than in comparison to autotrophic and mixotrophic cultivation. It is feasible that the added amount of glucose reached a high level of anaerobic condition resulting in loss of nitrogen by denitrification under heterotrophic cultivation.

Effect of Microalgae on TKN and TP

Table 4 records the TKN and TP contents of different treatments in final effluents. Cultivation of microalgae demonstrated a sharp reduction in the TKN and TP contents in all treatments at the end with respect to their initial values (Figure **5**). The percentile reduction in TKN and TP contents in the final effluent was in the order: $T_1>T_2>T_3>T_4>T_5>T_6$. In view of the treatment of domestic sewage, microalgae cultivation in phytoremediation effluents removed 68-81% of TKN and 57-88% of TP. The statistical examination revealed significant differences in TKN reduction (F = 203.5; p<0.01) and TP removal (F = 118.4; p<0.01) among different culture conditions. It is worth mentioning that reduction of TKN and TP in treatment T_1 was highly significant (p>0.05) than in treatment T_2 which clearly suggests the hypothesis that ideal biochemical and environmental conditions have prevailed in the treatment T_1 medium to maximise these nutrients uptake by microalgae in phytoremediation effluents.

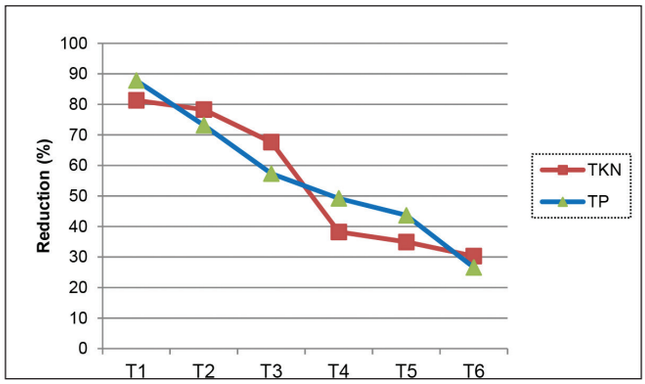

Figure 5: Variation in reduction of TKN and TP in the final effluents of different treatments.

This hypothesis is also supported by consistency in the highest biomass yield and productivity in treatment T_1 over the period of cultivation. The highest biomass yield and productivity with a subsequent maximum reduction of TKN and TP content in treatment T_1 credibly explain the fact that these nutrients were efficiently absorbed by microalgae during the period of growth and biomass production under mixotrophic-phytoremediation conditions, and were far superior to other treatments.

As shown in Figure 6, irrespective of culture conditions, phytoremediation effluents indicated a higher percentage of reduction of TKN than the cultures under control treatments. A consistently similar trend was also observed in the case of reduction of TP (Figure 7), which could be due to detoxification of domestic sewage following phytoremediation by *Typha latifolia*, consequently, encouraging higher growth and productivity of microalgae and higher uptake of these nutrients by microalgae biomass. The general comparison of the various cultivation regimes in our study showed that the reduction of TKN and TP in wastewater marked the highest under mixotrophic cultivation, followed by heterotrophic and lowest in autotrophic conditions. The higher percentage of reduction of these nutrients occurs in mixotrophic and heterotrophic conditions, because more ATP and NAD(P)H for the metabolic process is available, which is unrelated to autotrophic carbon assimilation (Yang et al., 2000).

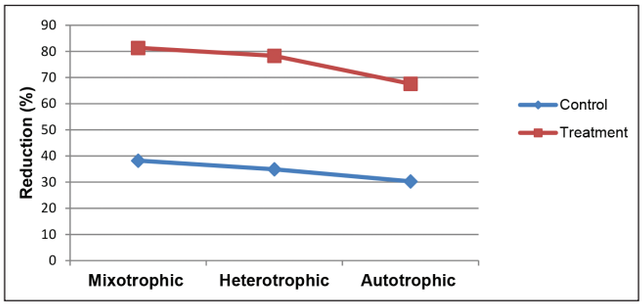

Figure 6: Reduction in TKN in the final effluents of different treatments.

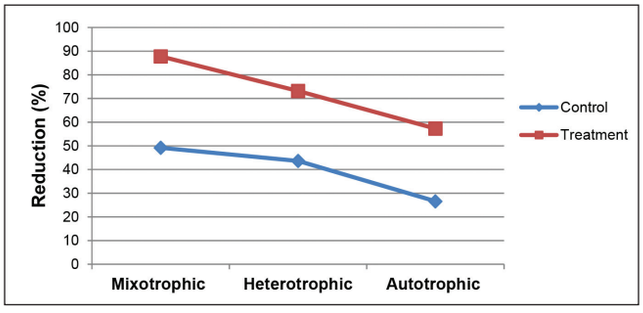

Figure 7: Reduction in TP in the final effluents of different treatments.

Conclusion

The results of the present investigation tend to conclude that *Typha latifolia* has significant effects on the phytoremediation of domestic sewage and resultant effluent is attractive for the cultivation of wild-type *Chlorella* sp. The examination of microalgae biomass suggests that phytoremediation of domestic sewage in CWs integrated with mixotrophic cultivation of microalgae utilizing both light and carbon sources could be the most efficient and unique strategy to recover sewage-based nutrients. The work also demonstrates that the phytoremediation of domestic sewage is very much efficient, environmentally safe and sustainable for synergistic resource generation and bioremediation.

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