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Studies on effects of soil enzymes for removal of nitrogen and phosphorus from sewage water treatment by constructed wetlands

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Abstract
Continuous application of sewage water to agricultural land can pose a serious threats to surface and ground water. Constructed wetlands (CWs) are becoming increasingly a major option worldwide for removing contaminants from domestic wastewater. One of the biological processes in CW is enzyme activity which plays a major role in releasing nutrients from organic substances. In this study, the enzyme activities and their relation to removal of nitrogen and phosphorus were investigated under three different vertical flow wetlands namely *Canna indica, Typha angustifolia* and *Xanthosoma sagitifolium* with hydraulic laoding rate of 0.00516cm/day. The activities of enzymes (Urease, Dehydrogenase and Phosphatase) were higher in planted system than unplanted system. Urease and dehydrogenase activity were significantly related with removal of Total nitrogen and phosphatase were significantly related with removal of Total phosphorus. This results suggests that increasing enzyme activity may increase nitrogen and phosphorous removal. Therefore this study concluded that the activity of urease in the root zones could be an important indicator for N purification in wastewaters.

Keywords: hydraulic retention time, constructed wetlands, enzyme activity, macrophytes

1. Introduction

The widespread of water quality degradation along with nutrient enrichment leads to eutrophication which poses a serious threat to water environment. Traditionally, there are variety of strategies and treatment methods had been taken and developed to minimize the water degradation. However these practices are not sufficient for wastewater treatment which has been used in agricultural purposes because, these may cause impact to surface water and ground water quality due to infiltration and percolation. Alternate methods of treating wastewater should be implemented to reduce contamination of surface and ground water and one of the methods to treat sewage water is the use of Contructed wetlands (CWs).

Constructed wetland have been used for pollution control by people for centuries (Mitsch and Jorgensen, 2004) [16]. CWs is one of the effective method at a reliable cost, energy efficient, natural means of treating sewage, agricultural and industrial wastes, at the same time offering the potential for multiple benefits (Wetzet, 2001) [20]. An artificial or constructed wetlands is a natural system designed and constructed to imitate hydrodynamic mechanisms as well as biogeochemical pollutant degradation processes that occur in natural wetlands, but with a greater degree of control over hydraulic regime of the systems (Langergraber, 2008) [13]. Constructed wetlands are commonly used for secondary treatment in small communities (Kadlec, 2009) [10]. Wetlands rely on natural microbial, biological, physical and chemical process to remove organic matter and nutrients (Cui, 2010) [3]. CWs can be divided into surface (horizontal flow constructed wetland, HFCW) and subsurface flow (horizontal subsurface flow constructed wetland, HSCW, and vertical flow constructed wetland, VFCW) systems. Horizontal flow constructed wetlands (HFCW) can provide a reliable treatment for Biochemical Oxygen Demand (BOD) and Total Suspended Solids (TSS), but frequently are less effective for nitrogen removal, unless a longer hydraulic retention time and enough oxygenation are provided.

Recently, research works are directed toward the vertical flow constructed wetland (VFCW) system since it is more effective not only for the mineralization of biodegradable organic matter but also for nitrification even at a low loading rate. The VFCW system possesses an unsaturated flow and it has greater oxygen transport ability than the horizontal system it also has the advantages of high hydraulic load rates and good removal of nutrients during any season and their small size, although pore clogging could be a problem. The wetland plant is one of the components used for wastewater treatment. Plants play both direct and indirect roles in wetlands. Even though the pollution removal efficiency varies with different methods of constructed wetland systems, the effect of micro-organisms plays a major role in pollutant adsorption and degradation (Savin and Amador, 1998) [17]. Plants play the main role in the wetlands by creating attachment sites for microorganisms and to release oxygen. The effectiveness of the desired treatment will be increased based on the selection of suitable plant species (Jethwa and Bajpai, 2016) [9].

The biochemical reaction processes such as degradation of organic compounds, hydrolysis and transformation of plants and nutrients, microbial reisudes are controlled by activities of microbial enzymes. Plants can influence enzyme activity directly by excreting exogenous enzymes and also affect microbial species composition by releasing exudates and oxygen into the rhizosphere that in turn affects enzyme activity indirectly. Some recent studies have reported that enzymes in wetland systems play very important roles in transformation of organic matter and nutrients. Enzymes like dehydrogense describes the general condition of soil microbial population (Margesin *et al.*, 2000) [15]. The objective of this study was to compare the enzyme activities of planted (*Canna indica*, *Xanthosoma sagitifolium* and *Tyha angustifolia*) and unplanted vertical flow contructed wetlands in reducing nitrogen and phosphorous from sewage water.

2. Material and Methods

2.1 Characterization of wastewater

The raw sewage effluent was collected from ukkadam sewage treatment plant, Coimbatore. The samples analysed for physical and chemical properties were collected in polycarboxyl container from the treatment plant, Ukkadam and samples for Dissolved Oxygen (DO) were collected and fixed by adding with one ml of manganous sulphate and one ml of alkaline potassium iodide solution. The collected effluent samples were stored at 4^{o}C under refrigerated condition and used for analysis.

2.2 Plant material

Three macrophytes species, *Canna indica*, *Typha angustifolia* and *Xanthosoma sagitifolium* were collected from the Muthanakulam Lake, Pusaripalayam Lake and nearby wetland area of Coimbatore, located in Tamil Nadu. The collected plants were authenticated and certified by botanical survey of India, Coimbatore. Then, plants were grown in the nursery for further research studies.

2.3 Experimental set up

The experiment was conducted in the Department of Environmental Sciences, Tamil Nadu Agricultural University. A lab scale constructed wetland model (Reactors) was designed with vertical flow system with uniform size of 45×21.5×30 cm (L×B×H) column made up of glass with an provision of inlet at the top and outlet at the bottom for all total seven reactors. From bottom to top, the wetland filled with gravels, river sand and garden soils in sandwich manner. The size of gravel is 20 mm and the porosity of sand and soil was 50% and 32% respectively for each reactor. Inlet from main tank was arranged manually and several types of PVC (Polyvinyl chloride) pipes were used to distribute the wastewater flow into wetland model system (fig 1). The wastewater was pumped into wetlands by peristaltic pump, with drip irrigation pipes and valves to optimize the average flow rate passing through constructed wetland system. Outlet for the treated effluent collection was arranged at bottom of the reactor with silica tubes.

2.4 Constructed wetland operation and sample analysis

Each CW system was operated for 15 days with hydraulic loading rate of 0.00516 cm/day with three different plants such as *Canna indica, Typha angustifolia* and *Xanthosoma sagitifolium*. A total of seven reactors were used for this study with two replication (Planted) along with one control (Unplanted) as Fig 1. The collected aquatic plants were taken in uniform size and weighed before transplanting and left undisturbed one week for establishment in reactors. After establishment, the wastewater was pumped to CWs and monitored regularly with an hydraulic loading rate of 0.00516 cm/day. In this study, the treated water from the outlet was collected at different-hydraulic retention time besides the soil samples collection near rhizosphere zone of plants upto 15 days. The activities of urease, phosphotase and dehydrogenase were measured with nesslerization and colorimetry. The water samples were analyzed according to the procedures described in the standard-methods.

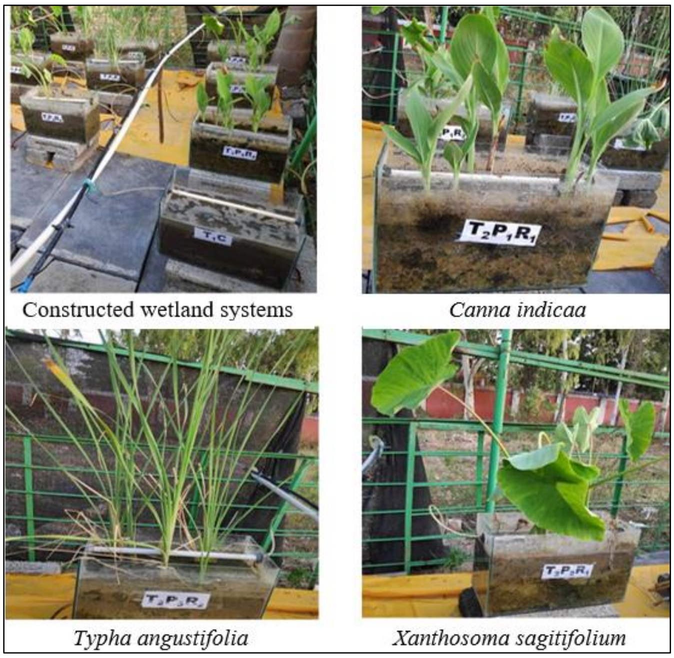

Fig 1: Constructed wetland in experimental set up

2.5 Soil enzymatic activity assay

2.5.1 Dehydrogenase-triphenyl tetrazolium choride (TTC) method

Five gram of samples was taken in a 50mm boiling tube. To the sample, 5 ml of 2, 3, and 5- triphenyl tetrazolium chloride solution was added and incubated at 3˚C for 24 hours. After incubation, 40 ml acetone was added and again incubated for 2 hours in dark. Using Whatman No.1 filter paper, the contents were filtered. The red colour developed was read at 485 nm in SHIMADZU UV- 1800 spectrophotometer, against a control of Tris buffer without TTC. The concentration of dehydrogenase in the sample was obtained from the standard graph using triphenyl farmazan. The results were expressed in µg of TPF g^{-1} soil (Casida *et al.*, 1964) [2].

2.5.2 Urease
Five gram of soil was taken in a 100ml volumetric flask. To the sample, 2.5ml of 0.08M urea solution was added, mixed well and incubated for 2 hours at 37˚C. then 50 ml of 1N potassium chloride (KCl) solution was added and kept for shaking in the mechanical shaker for 30 minutes. Whatman No. 1 filter paper was used to filter the contents and 1ml of filtrate was pipette out into 50 ml volumetric flask.
To this filtrate 9ml of double distilled water, 5ml Na salicylate (Prepared by mixing 100ml 0.12% Na nitroprusside, 100ml 17% Na salicylate and 100 ml distilled water) and 2ml of 0.1% ml Na dichlorisocyanurate were added, mixed well and allowed to stand for 30 minutes. The bluish green color developed was read at 690 nm in SHIMADZU UV-1800 spectrophotometer. Simultaneously, a blank was also prepared as above with 2.5ml distilled water, the urea being added at the end of the incubation and immediately before KCL addition and the results were expressed as µg $NH^{4+}N$ g^{-1} soil h^{-1} (Klose and tabatabai, 2000) [11].

2.5.3 Phosphatase
One gram of soil was placed in a 50ml conical flask and 4ml of modified universal buffer (MUB), 0.25 ml of toluene and 1ml of p-nitro phenyl phosphate (PNP) solution were added, mixed well and incubated at 37C for 1 hour. After that, the stoppers were removed and 1 ml of 0.5M calcium chloride and 4 ml of 0.5M sodium hydroxide were added. Then the soil suspension was filtered through what man No.1 filter paper and color intensity was read at 420nm in SHIMADZU UV- 1800 spectrophotometer. Simultaneously a blank was also prepared as above, the PNP solution being added at the end of the incubation, after the addition of calcium chloride ($CaCl_2$) and sodium hydroxide (NaOH) reagents. The concentration of phosphatase was obtained from a standard graph and the results expressed as µg nitro phenyl g^{-1} soil h^{-1}tabatabai and bremner (1969) [18] and Eivazi and tabatabai (1977) [5].

3. Results and Discussions
The present study investigates about soil enzymes activity, removal of total nitrogen and total phosphorous related to the retention time which had significant difference. The characteristics of the paper and board mill effluent (raw) used for the study is tabulated in the Table 1.

Table: 1 Initial characteristics of raw sewage effluent

S. No	Parameters	Sewage effluent
1.	pH	7.80
2.	Electrical conductivity (dS m^{-1})	2.84
5.	Biological oxygen demand(mg L^{-1})	540
6.	Chemical oxygen demand (mg L^{-1})	1560
7.	Total nitrogen (%)	0.34
8.	Total phosphorous (%)	0.31

3.1 PH
The pH values found to be ranged from 7.8 to 6.6 in the constructed wetland system. The pH values did not differ among the three plants and however there was significant difference, where highest value in control and lowest in canna indica at 15th day with hydraulic loading rate of 0.00516cm/day (Fig 3) was observed.

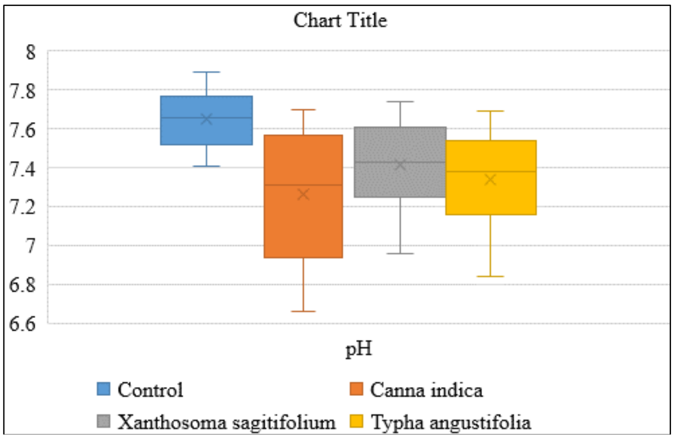

Fig 2: Represents the pH values in contructed wetlands

3.2 Removal of nitrogen and phosphorus
The total nitrogen content in treated effluent ranged from 0.07 to 0.27 % with retention time of 1st day to 15th day. Among the plants, *Canna indica* recorded higher total nitrogen reduction with a mean of 0.17 at retention time of 15th day whereas *Typha angustifolia* and *Xanthosoma sagitifolium* recoreded total nitrogen with a mean of 0.19 respective to retention time of 15th day.
The effect of plants on total phosphorus of the treated sewage effluent for all the three plants was furnished in Fig 3. The total phosphorus content showed the decreasing trend with respect to days of sampling (1st day to 15th day). Among the plants, *Canna indica* showed the finest performance in reduction of total phosphorus followed by *Typha angustifolia and Xanthosoma sagittifolium.*

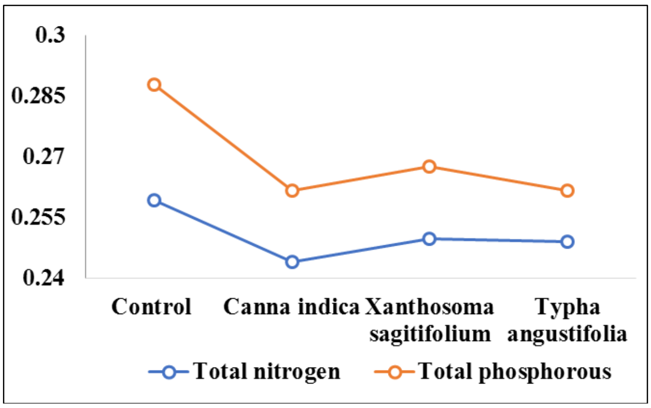

Fig 3: Represents values of total nitrogen and total phosphorus

3.3 Soil enzymes activity
In this study, wastewater quality was monitored and the enzymes activites were obtained at different hydraulic retention time

Urease activity (UA) in plants showed an increasing trend in all three plants according to the Hydraulic Retention Time (HRT = 15 days). The least enzyme activity were observed at unplanted system (Control) than planted system. The activity of urease enzyme was highest in *Canna indica* as 34.80 mg kg^{-1} at 15 days intervals whereas lowest in *Xanthosoma sagitifolium* (21.07 mg kg^{-1}) as in table 4. This study relates

that if urease activity increases, the total nitrogen values decrease along with increase in retention time (Fig. 4).
Huang *et al.*, (2012) [8] also reported that urease activity was significantly positively correlated with TN-N. Li *et al* (2011) [14] stated that there were close relationships between UR activity and TN removal efficiency in a subsurface wastewater. Urease activity depends on the availability of the wastewater, temperature and hydraulic retention time. Qiaolong *et al.*, (2016) reported that reason might be that during the operation period, the rhizosphere effects promoted the growth of micro-organism and aeration of the constructed wetland by radial oxygen loss where more the roots grows enzyme activity will increase. It is proved that the activity of urease in the root zone from CWs can be used as one of the major indicators for estimating the removal of nitrogen containing pollutants in wastewater (Cui *et al.*, 2013) [3]. Therefore, it is concluded that nitrogen removal was mainly through biological degradation by urease.

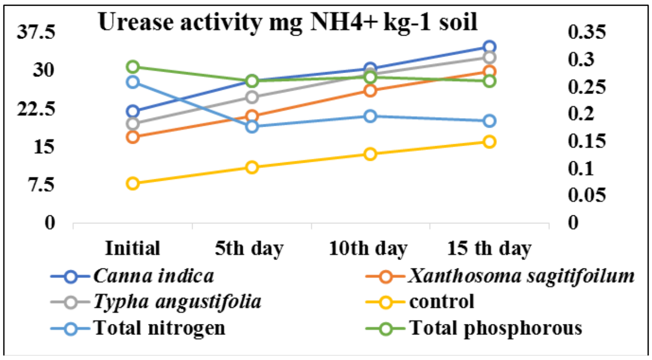

Fig 4: Correlation between urease enzyme activity and total nitrogen

Table 2: Urease activity at different retention time in constructed wetlands

Plants	Urease activity mg NH^{4+} kg^{-1} soil			
	Initial	5th day	10th day	15th day
Canna indica	22	28	30.5	34.80
Xanthosoma sagittifolium	17	21.07	26.2	29.8
Typha angustifolia	19.5	24.8	29.3	32.65
control	7.8	11	13.5	16

Dehydrogenase (DA) serves as an indicator of the microbiological redox-systems and could be considered a good and adequate measure of microbial oxidative activities in soil. the present investigation reveals that there was significant difference in the dehydrogenase enzyme activity among the three plants with the highest activity in the *Typha angustifolia* (23.87 mg kg^{-1}) and lowest in *Xanthosoma sagitifolium* (10.07 mg kg^{-1}) as Fig 5.
Factors such as oxygen availability, organic carbon availability, pH and soil nutrients could influence the soil enzymes activities in wetlands (Baddam *et al.*, 2016) [1]. Dehydrogenase is a kind of soil oxido-reductase that are widely distributed in soil microenvironments (ye *et al.*, 2010) [21]. DA was also significantly and positively correlated with the removal of TN-N and also had similar functions with Urease in CWs (huang *et al.*, 2012) [8]. The main reason was that the nitrogen pollutants in the CWs were decomposed and transformed by enzymes and microbes (Erler *et al.*, 2011) [6].

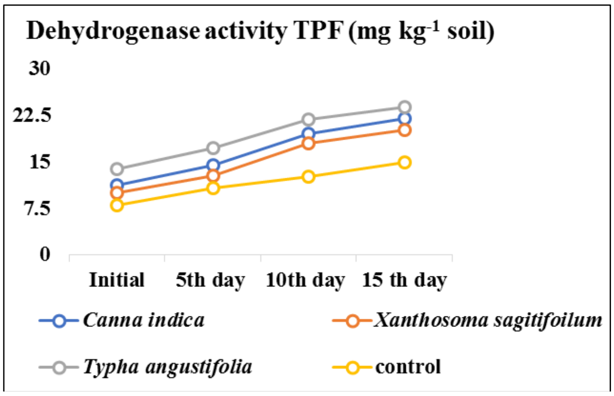

Fig 5: Dehydrogenase enzyme activity

Table 3: Dehydrogenase activity at different retention time in constructed wetlands

Plants	Dehydrogenase activity TPF (mg kg^{-1} soil)			
	Initial	5th day	10th day	15th day
Canna indica	11.21	14.5	19.5	22
Xanthosoma sagittifolium	10.07	12.76	18.04	20.17
Typha angustifolia	13.86	17.21	21.87	23.87
Control	8.09	10.78	12.65	14.9

Phosphotase activity (PP)
The higher ativity was shown by *Typha angustifolia* (33 mg kg^{-1}) at 15th day and lowest value in *Canna indica* (30.2 mg kg^{-1}). There was significantly difference between planted and unplanted system in constructed wetlands. In similar trend of all other two enzymes studied, if retention time increases the enzymes activity also increases. Here the phosphatase activity as significantly related with removal of total phosphorus. Where PP increases, TP decreases along with increase in retention time of 15 days as Fig 6.

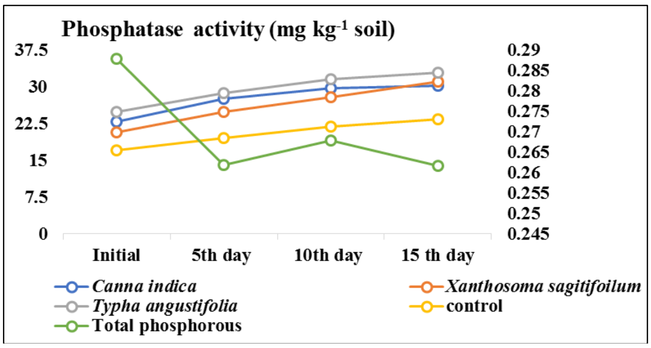

Fig 6: Correlation between Phosphate enzyme activity and total phosphorus

Table 4: Phosphatase activity at different retention time in constructed wetlands

Plants	Phosphatase activity (mg kg^{-1} soil)			
	Initial	5th day	10th day	15th day
Canna indica	23	27.6	29.76	30.2
Xanthosoma sagittifolium	20.8	24.98	27.86	31.05
Typha angustifolia	25	28.76	31.6	33
Control	17	19.6	22	23.4

Results showed that activity of phosphatase were related to concentration of total phosphorous in wastewater, whereas activity of urease related to concentration of total nitrogen and also the phosphorus removal and phosphatase activity were related. Similarly studies have reported that there will be positive correlation between PP activity and phosphorus removal which was revealed in most of the wetlands (Kong *et al.,* 2009) [12]. In some cases, inverse relationship between them was also found by (Wu *et al.*, 2013) [20] because there will be negative correlation, which indicated that phosphorous removal may be accomplished by CW matrix. When wastewater passes through CWs system, some physical and chemical process such as sorption, filtration, ion exchange and complexation occurred which removed phosphorous from wastewater.

3.4 Impact of HRT

Hydraulic retention time is a measure of the average length of time that a soluble compound remains in a constructed wetland. HRT plays an important role in the removal of nutrients by determining the time interval for interactions between the nutrients and roots as well as by allowing time for bacteria to flourish and transform the N. Lower the hydraulic loading rate and longer HRT resulted in a higher removal of TP (Cui *et al.*, 2010) [4]

$$\mathrm{HRT} = \frac{n * L * W * D}{\text{average flow (Q) passing though CWs}}$$

Where *n* is the media porosity, *L*, *W* and Dare the length, width and depth respectively of the CW

4. Discussions and Conclusions

This study concludes that more nitrogen and phosphorous concentration were removed from the influent with the presence of *Canna indica* when compared to other plants and unplanted system. Overall, lower hydraulic loading rate and higher retention time resulted in a higher removal of total nitrogen and total phosphorous. Relationship between the removal of TN-N and soil biological indicators were significantly positive in the planted system than unplanted system. Therefore this study revealed that the activity of urease in the root zones could be an important indicator for N purification from wastewaters and whereas removal of phosphorous and phosphatase activity were significant in planted CWs than other CWs. Further, soil enzyme activities had more significant relationship with removal rate of nutrients.

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