Original Research Article

Enzymatic activities in the rhizosphere soil as influenced by filterbeds and hydrophytes in the vertically constructed wetland

ABSTRACT

An experiment with different filter beds and macrophytes was carried out to study their phytoremediation capacity on the efficiency of domestic wastewater treatment through constructed wetland (CW) from November to March 2017–18 at the University of Agricultural Sciences, Dharwad campus, Karnataka. Twenty treatment combinations involving five types of filter beds (FB-1: *gravel*, FB-2: *gravel-sand-gravel*, FB-3: *gavel-sand-brick-gravel*, FB-4: *gravel-sand-charcoal-gravel* and FB-5: *gravel-sand-(charcoal+brick)-gravel*) and four macrophytes (MP-1: *Typha latifolia*, MP-2: *Brachiariamutica*, MP-3: *Canna indica* and MP-4: *Phragmites sp.*) were evaluated for treating domestic waste water. After 120 days from the start, across treatment combinations, the plant in each column was uprooted, and the soil adhered to the root was collected in a polyethylene cover. The samples were brought to the laboratory, refrigerated, and analysed for dehydrogenase, phosphatase, and urease activities, as well as for biofilm formation. More biofilm growth was recorded on brick material compared to other filterbed materials.

Keywords: Sewage effluent; constructed wetland; filter bed-filter beds; macrophytes; Biofilm.

Introduction

Seventy percent of the freshwater used for irrigation worldwide is used for agriculture, making it the largest user of water worldwide. Currently, the agriculture sector in India, which is the foundation of the country's economy, uses almost 90% of all available water resources. However, the amount of freshwater available to agriculture is starting to decline due to growing competition from industry, domestic sectors, and agriculture itself. Furthermore, India is finding it challenging to supply enough freshwater for irrigation due to the rapid depletion of groundwater supplies and severe water pollution. In India, the problem has become more severe and challenging to handle due to the obvious lack of fresh water and the notable rise in the amount of urban wastewater produced by the expanding cities.

Worldwide scientific community support and experimentation with the innovative wastewater treatment method known as "constructed wetland" (Plate 1). Increased interest in using manmade wetlands for wastewater treatment and reuse has been sparked by the possibility of achieving improved water quality while creating vital habitat for wildlife. This structure, despite requiring a lot of land, provides attractive ways to integrate resource enhancement with wastewater treatment, frequently at a price that is comparable to established wastewater treatment options. A constructed wetland, which can be vertical or horizontal in shape, is a framework for treating wastewater that consists of one or more treatment cells arranged in an artificially regulated way. Several types of wastewater have been treated at varying degrees of treatment using constructed wetlands. In order to create a wetland that replicates the physical, chemical, and biological processes of natural wetland systems, common characteristics are linked to emergent hydrophyte stands. Different kinds of wastewaters have been successfully treated by hydrophytes, also known as macrophytes. With regard to the treatment procedures, the hydrophytes have a number of characteristics. The physical impacts of the plant tissues, which result in a filtering effect and offer surface area for attached microorganisms, are the most significant effects of the hydrophytes (Plate 3) on the wastewater treatment processes. The removal of contaminants from hydrophytes through plant uptake and oxygen release has varying effects on wastewater treatment procedures. Additionally, the hydrophytes act as habitat for wildlife.

The three main nutrient removal processes connected to artificial wetland systems are filtration, precipitation, and biodegradation. The selection of filterbed materials and their vertical placement in terms of thickness and depth should be done with the goal of reducing treatment costs and optimising the effectiveness of the aforementioned operations. In order to treat the domestic sewage effluent of the UAS, Dharwad campus, the current study (column study) was carried out using locally accessible materials such as gravel, sand, charcoal, and brick materials as filterbed (Plate 2).

Material and methods

Biological property

After 120 days, the plant in each column was uprooted and the soil adhered to root was collected in polyethylene cover. The samples were brought to the laboratory, refrigerated and analyzed for dehydrogenase, phosphatase, urease activities and biofilm formation.

Estimation ofDehydrogenase activity

In the dehydrogenase enzyme assay, 2,3,5-triphenylformazone (TPF), which is created when soil microbes reduce 2,3,5-triphenyltetrazolium chloride, is measured colorimetrically. Five grammes of soil, 0.1 gof $CaCO_3$, one millilitre of TTC's 3% aqueous solution, and three millilitres of distilled water were added to each test tube, which was then incubated for twenty-four hours at 37˚C in the incubator. Following that, each tube's 2,3,5-triphenylformazone was extracted and placed individually into a 50 ml volumetric flask by pouring the soil through a funnel that had been plugged with non-absorbent cotton. Small amounts of methanol were used to wash the soil multiple times until the filtrate was colourless. Then, using methanol as a blank, the filtrate's volume was increased to 50 ml, and the red color's intensity was measured in a spectrophotometer at 485 nm. According to Casida *et al.* (1964), the amount of TPF that was generated was determined and reported as µg TPF g-1 day^{-1}.

Estimation of phosphatase activity

The phosphatase activity of soil samples was measured using the method described by Evazi and Tabatabai (1979). The reaction mixture consisted of 1 g of soil, 0.2 ml toluene, 4 ml of modified universal buffer (pH 7.5), and 1 ml of para-nitrophenol phosphate solution. The mixture was mixed and incubated at 37˚C for an hour. After that, 1 ml of 0.5 M $CaCl_2$ and 4 ml of 0.5 M NaOH were added, swirledand filtered. The intensity of yellow colour was measured at 420 nm against the reagent blank. The concentration of para-nitrophenol phosphate formed in soil samples was calculated using a standard curve made using graded concentration of para-nitrophenol phosphate. The phosphatase activity was expressed as µg P-NP formed g^{-1} soil hr^{-1}.

Estimation of urease activity

Urease activity of soil samples was determined by following the procedure of Tabatabai and Bremner (1972). Ten-gram soil sample was treated with 1 ml toluene and 10 ml phosphate buffer and incubated at 30˚C for 24 hr. Following the incubation period, 15 ml of 1N KCl were added, and the mixture was filtered using Whatman No. 42 filter paper. Distilled water was added to the filtrate volume until it reached 100 ml.To one ml of the extractant, 2 ml of 10 per cent sodium tartarate and 0.5 ml Nessler's reagent were added and incubated for 30 minutes. The volume was increased to 25 ml with distilled water after 30 minutes. Using a spectrophotometer, the amount of yellow colour that had developed was measured at 610 nm in comparison to a blank sample that had no urea solution. A standard curve created using graded concentrations of ammoniacal nitrogen was used to calculate the concentration of ammoniacal nitrogen generated in soil samples. The urease activity was expressed as µg NH_4^+-N formed g^{-1} soil day^{-1}.

Quantification of biofilm formation

The biofilm formation ability of different filterbed materials was assessed in column study. We used a 2-inch-diameter, 30-centimeter-long PVC pipe. A small hole was left for drainage and an end cap was used to seal the bottom of the column. Different filterbeds, such as sand, gravel, brick, and charcoal, were filled in these columns in triplicate without any plantings, and untreated home sewage effluent was regularly watered on them.The study was conducted for a period of 120 days. The samples were drawn from different depths (0-5 cm, 5-15 cm and 15-30 cm) and analyzed for quantification of biofilm formation.

The amount of bio-film that developed on various filterbed materials was measured gravimetrically (Besciak and Surmacz, 2011). Known samples were combined with 15 ml of a solvent mixture (acetone, petroleum ether, 3:1 v/v) for 10 seconds, and the mixture was centrifuged for 10 min at 20,000 rpm. The biofilm samples were then filtered through filter paper that had been pre-weighed, and the filter paper was dried to constant weight in a vacuum oven at 70º C. Based on weight differences, the weight of biofilm was computed and expressed as mg g-1 for every filterbed material.

Results and discussion

Enzymatic activities in the rhizosphere soil as influenced by filterbeds and hydrophytes in the constructed wetland

Dehydrogenase activity

The result related to soil dehydrogenase activity (µg TPF g^{-1} day^{-1}) at 120 DAS as influenced by different filterbeds and hydrophytes is presented in Table 1.

The dehydrogenase activity of soil at 120 DAS was significantly influenced by both hydrophytes and filterbeds. Interestingly, the highest soil dehydrogenase activity was found in the hydrophyte phragmites (26.92 µg TPF g^{-1} day^{-1}) and the filterbed*"gravel"* (35.30 µg TPF

g^{-1} day^{-1}). The dehydrogenase activity of hydrophytes and filterbeds ranged from 17.04 to 26.92 µg TPF g^{-1} day^{-1}, respectively. The interaction between the hydrophytes and filterbeds was significant. The combination of "*gravel-sand-(charcoal+brick)-gravel*" and typha recorded significantly higher dehydrogenase activity in the rhizosphere (63.27 µg TPF g^{-1} day^{-1}).

Soil dehydrogenase is an enzyme from the oxido-reductase class that catalyses the oxidation of organic molecules. Soil dehydrogenase enzyme is a key component of enzymatic activity that participates in and ensures the right sequence of all biochemical pathways in soil biogeochemical cycles. Dehydrogenase activity is an indicator of microbiological redox systems and may be considered a good measure of microbial oxidative activities in soil, as well as the overall microbial load in the rhizosphere. Enzymes play an important part in the degradation of organic contaminants, and dehydrogenase enzyme is a constitutive enzyme that determines the overall microbial population.

The present study clearly showed that gravel had more porosity and aeration which might have increased dehydrogenase activity.A study conducted by Smith (2020) investigated the impact of different filterbed materials on dehydrogenase activity in a pilot-scale constructed wetland treating domestic wastewater. The researchers found that filterbeds containing organic-rich materials, such as peat, exhibited higher dehydrogenase activity compared to those composed of inert materials like sand. This suggests that the presence of organic matter in filterbeds can enhance microbial activity and promote the degradation of organic compounds.The highest dehydrogenase activity was recorded in rhizosphere of phragmites. It indicated that microbial dependency and rhizosphere competence are high for this species compared to other hydrophytes. Activity of dehydrogenase was significantly high in root zone of phragmites. This finding is similar in line with the findings of Kong *et al*.(2009).Plant species root morphology and development seem to be a key factors in influencing microbial–plant interaction (Gagnon *et al*., 2007). In a study by Johnson (2019), the researchers investigated the effect of different hydrophyte species on dehydrogenase activity in a laboratory-scale constructed wetland. They found that wetlands planted with specific hydrophyte species, such as *Typha latifolia* and *Phragmites australis*, exhibited higher dehydrogenase activity compared to unplanted control systems. This suggests that hydrophytes can enhance microbial activity and create favorable conditions for organic matter degradation.

Phosphatase

The result related to soil phosphatase activity (µg P-NP g^{-1} hr^{-1}) at 120 DAS as influenced by different filterbeds and hydrophytes is presented in Table 1.

Both filterbeds and hydrophytes had significant influence on soil phosphatase activity at 120 DAS. Significantly, the highest soil phosphatase activity was recorded in filterbed*'gravel-sand-gravel'* (93.43 µg P-NP g^{-1} hr^{-1}) and hydrophyte canna (74.56 µg P-NP g^{-1} hr^{-1}). The phosphatase activity of filterbeds and hydrophytes ranged between 29.24 to 93.43 and 52.45 to 74.56 µg P-NP g^{-1} hr^{-1}, respectively. The interaction between the filterbeds and hydrophytes was significant. The combination of '*gravel-sand-gravel'* and canna recorded significantly higher phosphatase activity in rhizosphere (196.44 µg P-NP g^{-1} hr^{-1}). The high phosphatase activity in canna rhizosphere showed positive relationship with phosphorus concentration in plant.

Urease

The result related to soilureaseactivity at 120 DAS as influenced by different filterbeds and hydrophytes is presented in Table 1.

Both filterbeds and hydrophytes had significant influence on soil urease activity at 120 DAS. Significantly, the highest soil urease activity was recorded in filterbed '*gravel-sand-*

charcoal-gravel' (0.75µg NH_4^+-N $g^{-1}day^{-1}$) and hydrophytes phragmites and typha (0.50µg NH_4^+-N $g^{-1}day^{-1}$). The soil urease activity of filterbeds and hydrophytes ranged between 0.25 to 0.75 and 0.45 to 0.50µg NH_4^+-N $g^{-1}day^{-1}$, respectively. The interaction between the filterbeds and hydrophytes was significant. The combination of '*gravel-sand-charcoal-gravel'* and canna recorded significantly higher urease activity in rhizosphere (0.99 µg NH_4^+-N $g^{-1}day^{-1}$). This indicated that urease activity dependency is high for these two plant types compared to paragrasss and canna. Among filterbeds, '*gravel-sand-charcoal-gravel'* (FB4) recorded the highest urease activity.

Urease is an enzyme that catalyzes the hydrolysis of urea into ammonia and carbon dioxide. This enzymatic reaction is essential in the nitrogen cycle as it converts urea, a common nitrogenous compound in wastewater, into ammonia, a form of nitrogen readily used by plants.Urease activity is crucial in constructed wetlands as it influences the overall nitrogen transformation process. Ammonia produced through urease activity can be subsequently nitrified and denitrified, converting it into harmless nitrogen gas and reducing its impact on the environment. Understanding the factors that influence urease activity is key to optimizing nitrogen removal in constructed wetlands.

Biofilm developmenton different filterbed materials after 120 DAS (in sole column without hydrophytes)

The data pertaining to biofilm development is presented in Table 2. Brick (1.18 and 0.05 mg g^{-1}) had the maximum biofilm formation, followed by sand (0.68 and 0.04 mg g^{-1}), charcoal (0.67 and 0.02 mg g^{-1}), and gravel (0.31 and 0.01 mg g^{-1}) at depths ranging from 0 to 5 and 5 to 15 cm. The growth of biofilms diminished as depth increased. No biofilm formation was found between 15 and 30 cm.

The bacteria present in the environment often grow in the form of biofilm, called also the biological membranes. This type of structure is very useful and advantageous for bacterial cells, because it allows them to better adapt to changing environmental conditions. Biofilms are communities of single or multiple populations, which are embedded on some type of surface. Bacterial cells included in this structure produce extracellular polymeric substances (EPS) thatsurround them outside and protect against harmful external factors. The composition of EPS may also include various organic or inorganic ingredients, such as sand or plant remains. Biofilms are found in every type of environment, both natural and anthropogenic origin. Their development is conditioned by the presence of water, nutrients and oxygen (for aerobic bacteria).

The highest biofilm growth was observed on brick followed by sand, charcoal and gravel. Biofilm formation decreased with increasing depth. Our results clearly shows that high biofilm growth on surface rather than subsurface. No biofilm growth was noticed below 15 cm depth. Brick can be used in top layer which might increases the efficiency of constructed wetland in terms of nutrient removal and increasing the microbial population and our results clearly indicated that biofilm growth requires good aeration and sunlight with nutrients. The results are in confirmation with Zhang *et al.* (2023)

Plant growth parameters as influenced by filterbeds and hydrophytes in constructed wetland

The diversity of the root system might result in linkages between specific root features and their functions. To acquire a complete image of the root system, several factors will be required. In this context, plant characteristics such as root length, root volume, root biomass, and shoot biomass of hydrophytes were measured.

Root length

Filterbeds and hydrophytes have a substantial influence on root length. The longest root length was measured in a gravel bed with no sand component. This could be due to the increased pore space formed between the gravel material in the *'gravel'* filterbed (Table 3).

Allfourhydrophytes were perennial monocots, however the root systems varied between species. Paragrass had much longer effective root lengths than phragmites (Plate 4). As a result, these two grasses eliminated the majority of the physicochemical elements of sewage effluent more effectively than the other two species.

Root volume

The volume of a plant's rootzone governs its vegetative and reproductive development (Table 3). There was no substantial difference between filterbed treatments. Paragrassrecorded the largest root volume. The root volume of canna was mostly owing to a rhizome-based root system rather than effective roots. Typha had a limited root volume due to its short root length. Phragmites had a lower root volume than canna. However, phragmites' root systems were longer and more fibrous than rhizome-based root systems (Plate 4).

Root biomass and shoot biomass

Both filterbeds and hydrophytes had a considerable impact on root and shoot biomass production by hydrophytes. The highest root and shoot biomass was found in filterbed*'gravel-sand-brick-gravel'*. It could be attributed to increased nitrogen intake, which results in increased removal from effluent. Paragrass showed the highest shoot and root biomass, followed by canna and phragmites. Canna's increased root mass was primarily related to rhizome weight, making it less effective in boosting the quality of use. Whereas paragrass and phragmites had increased root biomass due to their fibrous root systems, which led to their better efficiency in increasing USE quality (Table 3).

Conclusion:

Paragrass had the highest recorded values for root length, root volume, root biomass, and shoot biomass, whereas phragmites and typha had the lowest. The *'gravel'*filterbed showed higher dehydrogenase activity, phosphatase in *'gravel-sand-gravel'*, and urease in *'gravel-sand-charcoal-gravel'*; on the other hand, phragmites showed higher urease and dehydrogenease activities. At a depth of 0–5 cm, brick material exhibited a significantly greater rate of biofilm growth among the filterbed materials. Beyond 15 cm of depth, no biofilm formation was seen, and biofilm growth decreased with depth.

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Table 1. Enzymatic activities in the rhizosphere soil as influenced by filterbeds and hydrophytes in the constructed wetland at 120 DAS

Treatments	**Dehydrogenase (µg TPF g^{-1}day^{-1})**					**Phosphatase (µg P-NP g^{-1} hr^{-1})**					**Urease (µg NH$_4^+$-N g^{-1} day^{-1})**				
Hydrophytes Filterbeds	**Typha**	**Paragrass**	**Canna**	**Phragmites**	**Mean**	**Typha**	**Paragrass**	**Canna**	**Phragmites**	**Mean**	**Typha**	**Paragrass**	**Canna**	**Phragmites**	**Mean**
Gravel	4.31	24.63	30.29	81.98	**35.30^{a}**	34.72	48.43	67.61	53.91	**51.17^{c}**	0.25	0.25	0.25	0.25	**0.25^{d}**
Gravel -Sand-Gravel	21.54	14.00	33.92	7.27	**19.18^{c}**	46.60	58.48	196.44	72.18	**93.43^{a}**	0.50	0.50	0.50	0.50	**0.50^{b}**
Gavel-Sand-Brick-Gravel	7.67	5.92	30.56	9.96	**13.53^{d}**	39.29	47.51	51.17	63.05	**50.25^{c}**	0.50	0.25	0.25	0.75	**0.44^{c}**
Gravel-Sand-Charcoal-Gravel	4.04	16.29	13.60	17.63	**12.89^{d}**	95.94	94.11	38.38	46.60	**68.76^{b}**	0.75	0.75	0.99	0.50	**0.75^{a}**
Gravel-Sand-(Charcoal+Brick)-Gravel	63.27	24.37	3.63	17.77	**27.26^{b}**	49.34	21.93	19.19	26.50	**29.24^{d}**	0.50	0.50	0.25	0.50	**0.44^{c}**
Mean	**20.17^{c}**	**17.04^{d}**	**22.40^{b}**	**26.92^{a}**		**53.18^{b}**	**54.09^{b}**	**74.56^{a}**	**52.45^{b}**		**0.50^{a}**	**0.45^{b}**	**0.45^{b}**	**0.50^{a}**	

	S. Em. ±	**C. D. (P=0.05)**	**S. Em. ±**	**C. D. (P=0.05)**	**S. Em. ±**	**C. D. (P=0.05)**
Filterbeds	0.40	1.15	0.49	1.41	0.01	0.03
Hydrophytes	0.36	1.02	0.44	1.26	0.01	0.03
Filterbeds × Hydrophytes	0.80	2.29	0.98	2.83	0.02	0.06

Table 2. Biofilm development on different filterbed materials after 120 days (in sole columns without hydrophytes)

Filterbed materials	**Biofilm development (mg g^{-1}) at differentcolumn depths (cm)**		
	0-5	**5-15**	**15-30**
Brick	1.18	0.05	*
Sand	0.68	0.04	*
Charcoal	0.67	0.02	*
Gravel	0.31	0.01	*

* Biofilm formation was not observed at 15- 30 cm depth

Table 3. Plant growth parameters as influenced by different filterbeds and hydrophytes in the constructed wetland

Treatments	**Root length (cm)**					**Root volume (cm^{3})**				
Hydrophytes Filterbeds	**Typha**	**Paragrass**	**Canna**	**Phragmites**	**Mean**	**Typha**	**Paragrass**	**Canna**	**Phragmites**	**Mean**
Gravel	28.80	71.67	29.00	62.33	**47.95**^{a}	331	622	414	350	**429**
Gravel -Sand-Gravel	18.63	42.00	28.93	45.00	**33.64**^{d}	305	384	527	369	**396**
Gavel-Sand-Brick-Gravel	19.33	89.00	30.33	49.00	**46.92**^{ab}	193	840	429	293	**439**
Gravel-Sand-Charcoal-Gravel	26.67	70.83	31.17	47.67	**44.08**^{bc}	177	444	418	294	**333**
Gravel-Sand-(Charcoal+Brick)-Gravel	26.20	94.33	33.67	28.33	**45.64**^{b}	177	606	373	309	**366**
Mean	**23.93**^{d}	**73.57**^{a}	**30.62**^{c}	**46.47**^{b}		**236**^{c}	**579**^{a}	**432**^{ab}	**323**^{bc}	
	S. Em. ±		**C. D. (P=0.05)**			**S. Em. ±**		**C. D. (P=0.05)**		
Filterbeds	0.69		1.98			42.95		NS		
Hydrophytes	0.62		1.77			110.2		38.41		
Filterbeds × Hydrophytes	1.38		3.96			85.90		NS		
	Root biomass (g)					**Shoot biomass (g)**				
Gravel	8.47	20.47	7.10	8.23	**11.07**^{b}	27.29	98.96	45.87	48.79	**55.23**^{b}
Gravel -Sand-Gravel	8.20	9.83	7.57	8.43	**8.51**^{b}	40.08	141.74	60.29	65.69	**76.95**^{ab}
Gavel-Sand-Brick-Gravel	12.47	33.43	26.63	9.27	**20.45**^{a}	26.75	146.87	78.83	84.73	**84.30**^{a}
Gravel-Sand-Charcoal-Gravel	6.47	17.80	11.23	9.10	**11.15**^{b}	24.35	146.49	68.29	65.43	**76.14**^{ab}
Gravel-Sand-(Charcoal+Brick)-Gravel	3.63	33.97	8.20	6.73	**13.13**^{b}	39.23	119.47	61.75	53.43	**68.47**^{ab}
Mean	**7.85**^{b}	**23.10**^{a}	**12.15**^{b}	**8.35**^{b}		**31.54**^{c}	**130.71**^{a}	**63.01**^{b}	**63.61**^{b}	
	S. Em. ±		**C. D. (P=0.05)**			**S. Em. ±**		**C. D. (P=0.05)**		
Filterbeds	1.42		4.09			6.24		17.90		
Hydrophytes	1.27		3.66			5.58		16.01		
Filterbeds × Hydrophytes	2.85		8.18			12.49		NS		

NS-Non significant

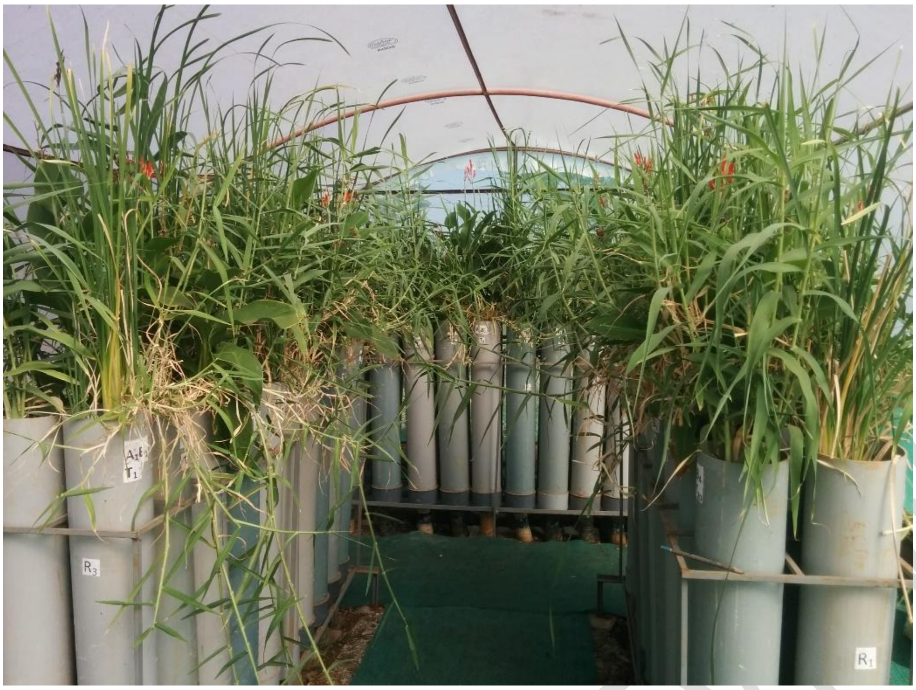


Plate 1. General view of experimental set-up

a) Gravel

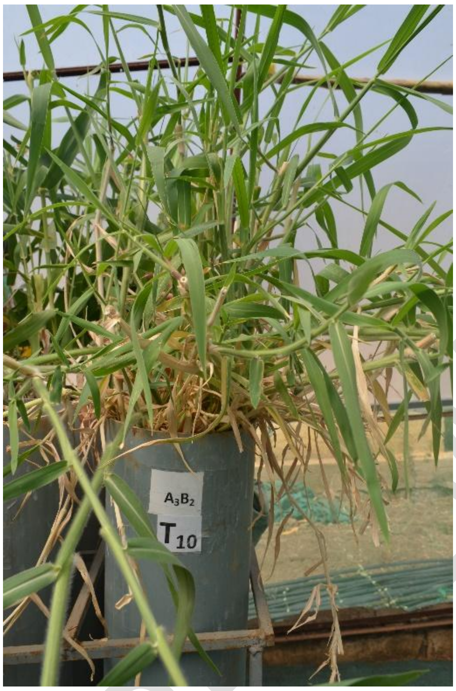
b) Sand

c) Brick

d) Charcoal

Plate 2. Filterbed materials used in constructed wetland system

a) *Typha latifolia* (Typha)



b) *Brachiariamutica* (Paragrass)

c) *Canna indica* (Canna)

d) *Phragmites sps.* (Phragmites)

Plate 3. Hydrophytes used in constructed wetland system

a) Phragmites **b) Typha** **c) Canna** **d) Paragrass**

Plate 4. Root growth of hydrophytes in the constructed wetland system