

Impact of microbial activity on the performance of planted and unplanted wetland at laboratory scale

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Abstract

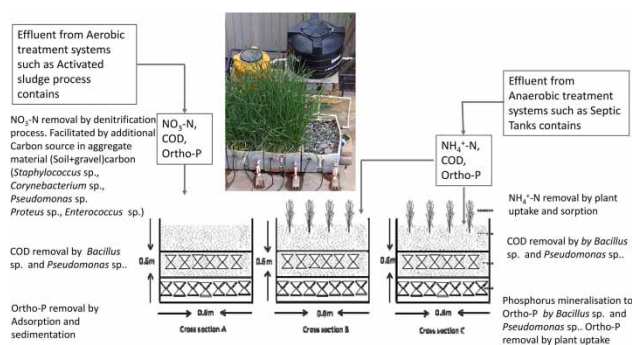
Three horizontal subsurface flow constructed wetland prototypes were set up to identify and understand the role of microflora in nutrient removal under diverse operating conditions. Out of three setups, one setup served as a control (without plants), and rest were planted with *Typha domingensis*. The setups were operated at two different hydraulic loading rates (5 cm/day and 16 cm/day) for two months each. Among 27 bacteria species isolated, 80% of nitrate-reducing bacteria were observed in control, and 50–77% of nitrate-reducing bacteria were observed in the plant setups. Presence of diverse denitrifying bacteria and soil organic carbon contributed to high Nitrate-N removal in control at both HLRs. Similar Ammonium-N (29%) and Ortho-P removal (30%) efficiency was observed at both HLRs in the control setup. Processes such as chemical sorption and adsorption dominated the Ammonium-N and Ortho-P removal in control setup. High average Ammonium-N removal efficiency of 89 and 52% was observed in plant setups at 5 cm/day and 16 cm/day HLR. At low HLR Ammonium-N removal in plant setups was dominated by nutrient uptake. In the plant setups, 35 and 15% Ortho-P removal efficiency was observed at low HLR (5 cm/day) and high HLR (16 cm/day) respectively. Hydraulic Retention Time (HRT) limited the uptake of Ortho-P thereby allowing mineralised phosphorus to escape the system without being absorbed by the plants.

Key words: HSSF-CW, hydraulic loading rates (HLR), microflora, *Typha domingensis*, wastewater treatment

Highlights

- At low HLR, Ammonium-N removal in CWs systems is dominated by plant uptake.
- Unplanted CW systems are efficient in removing Nitrate-N. Removal mechanism is dominated by the presence of diverse denitrifiers and organic carbon present in soil media.
- Ortho-P removal is dominated by physical processes in unplanted CW systems. In planted, Phosphorus mineralization leads to poor ortho-P removal at high HLR.

Graphical Abstract



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INTRODUCTION

Wastewater treatment is necessary to address contamination of surface water bodies affected by the release of the domestic and industrial effluents. Low cost and efficient wastewater treatment technology are crucial, especially in developing countries where resources to treat wastewater are limited (Iasur-Kruh *et al.* 2010).

The sewage generation estimated from class I and class II town in India is 38,524 Million litres per day (MLD), which accounts for 72% of the population. Out of the total wastewater generated, Centralised Wastewater Treatment (CWWT) plants have capacity to treat 30% and rest is discharged untreated into rivers lakes and open storm water drains (Sharma & Sharma 2018). In Bengaluru city, 30% of the total sewage generated receives treatment. Many a times, the effluents produced from CWWT systems fail to comply with the discharge standards set by Central Pollution Control Board (CPCB). CWWT system relies on expensive equipment and requires skilled personnel for operation and maintenance. Lack of underground drainage network (UDGs) for sewage collection, skilled personals to operate the treatment plants and frequent power cuts are listed as common reasons for the failure of CWWT systems to comply with the discharge standards (Jamwal *et al.* 2008, 2015). In addition, high operation and maintenance cost per year and high capital costs are main barriers towards effective scaling up of these systems (Massoud *et al.* 2009; Capodaglio 2017).

Unlike CWWT plants, Decentralized Wastewater Treatment Systems (DWWTS) such as constructed wetlands (CWs) do not rely on sophisticated operation and maintenance system and have reasonably manageable drainage network. These systems use less energy and produce a limited amount of sludge as compared to the CWWT systems (Singh *et al.* 2009). Effluent quality from CWs is less variable as the systems are resilient to shock loads and treated effluent can be reused for landscape irrigation, toilet flushing etc (Philip *et al.* 2012). In addition to CWs, Septic tanks and anaerobic digesters are some of the prevalent low cost technologies deployed for decentralized wastewater treatment (Capodaglio 2017). Constructed wetlands are proven technologies that are efficient in treating industrial, municipal and agricultural wastewater with minimal energy inputs (Almukhtar *et al.* 2018).

Nutrient uptake by wetland plants play a significant role in reducing nutrients in the wastewater (Wu *et al.* 2019). Plants improve wastewater quality by absorbing nutrients such as phosphorous, nitrogen and other elements during their growth cycle (Almukhtar *et al.* 2018). Depending on the species, inflow quality and nutrient loading rate; plants are capable of removing 3% to 47% of nitrogen and 3% to 60% of phosphorous from wastewater (Gottschall *et al.* 2007). *Typha* species are one of the emergent plants extensively used in the constructed wetlands. It has a dense root system, that provides oxygen to the rhizosphere region which helps in maintaining the contaminant removal efficiency of CWs (Shehzadi *et al.* 2014). Contrary to this, few studies reported microbial degradation process as a major contributor to nutrient removal in constructed wetlands (Iasur-Kruh *et al.* 2010; Gorgoglione & Torretta 2018; Saeed *et al.* 2019). Unplanted constructed wetlands has also shown comparable nutrient removal efficiencies. Biofilm growth on aggregate material and microbial degradations contributes to organic matter and nutrient removal in unplanted systems (Coleman *et al.* 2001). Microorganisms enhance the transformation and mineralization of the contaminants in and contribute to the nutrient removal in wastewater (Wang *et al.* 2016a). Also, bacteria helps in degradation and reduction of organic and inorganic compounds such as Ammonium-N and Nitrate-N by utilizing carbon and other inorganic species for its metabolic pathway (Llanos-Lizcano *et al.* 2019).

Knowledge regarding the type of microflora and their contribution to contaminant removal helps with designing efficient constructed wetland systems (Rajan *et al.* 2018). In addition, understanding the role of microorganisms and plants in constructed wetland and their response in diverse operational condition is crucial to optimize the biological wastewater treatment (Lee *et al.* 2009). Here, we conducted a laboratory experiments to identify the microflora and understand its contribution

to contaminant removal under diverse operational conditions in planted and unplanted constructed wetland systems.

METHODOLOGY

Constructed wetland setup

The laboratory-scale horizontal subsurface flow constructed wetland (HSSF-CW) prototypes were setup at Ashoka Trust for Research in Ecology and the Environment (ATREE), Bangalore. Each setup was constructed using three plastic rectangular open tanks of dimension 1 m × 0.6 m × 0.6 m (length × width × height). Out of three containers, one served as control setup (without plants) and the other two served as experimental setups (Planted). Each setup was filled with three layers of aggregate material (0.2 m height). The bottom layer comprised of terracotta pieces, second layer comprised of the mixture of terracotta and soil and third (topmost) layer comprised of soil, respectively (Figure 1). Finally, to finish the look gravel pieces were added above the inflow pipes of the three systems (Figure 2). The gravel aggregate material remained dry throughout the test period.

Terracotta is a lightweight porous material that provides high organic removal efficiency when used as a substrate in a constructed wetland for wastewater treatment (Jamwal *et al.* 2019). The soil used in

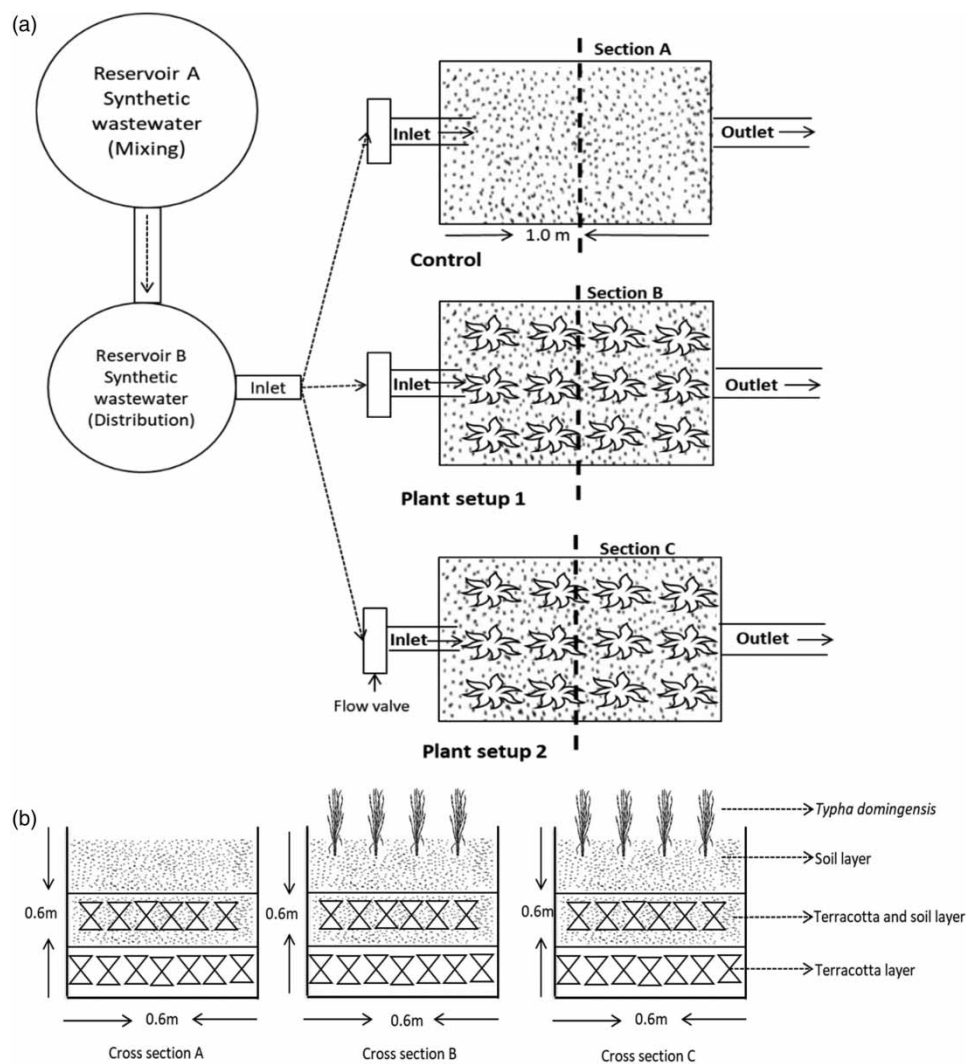


Figure 1 | (a) Flow diagram of the Lab setups, (b) cross-section view of the setups.



Figure 2 | View of constructed wetland setups at ATREE.

CW setups was obtained from the Jakkur Lake, Bangalore, which is sandy clay loam soil with medium infiltration rate (Suma & Srinivasa 2015). The seeds of emergent plant *Typha domingensis* were washed thoroughly after soaking for 24 hours. The seeds were added at 3 cm depth of wet soil of the two plant setups. The plants density was maintained at 34 plants/m² (Mcmullen 2018).

Synthetic wastewater was fed to the setups through two overhead tanks. Reservoir A (300 L) was used for mixing and storing of synthetic wastewater. Reservoir B (200 L) was used for distribution of synthetic wastewater to three setups. The synthetic wastewater was prepared in the laboratory using urea, regular granular sugar, NH₄Cl, K₂HPO₄, fertilizer (N, P, K, S, Mg, Cu, Fe and Mn) and partially treated sludge (Weerakoon *et al.* 2013). The partially treated sludge was obtained from the Sewage Treatment Plant in Jakkur, Bangalore, India.

Constructed wetlands/green infrastructure are generally used as polishing/tertiary treatment system to remove nutrients and organic matter before the effluent is discharged into the surface water bodies. The composition of synthetic wastewater mimics the secondary treated wastewater with COD levels <200 mg/l (theoretical) and Nitrate-N (mg/L) 40.4 ± 9.7, Ammonium-N (mg/L) 10.4 ± 3.1, Total nitrogen (mg/L) 55.1 ± 11.9, Ortho-P (mg/L) 7.6 ± 0.4. The well mixed synthetic wastewater was daily added to Reservoir A. The volume of the wastewater added depends on the hydraulic loading rate (HLR). Table 1 presents the quantity of the compounds used for the preparation of synthetic

Table 1 | Composition of synthetic wastewater

Compounds	Quantity/100 L
Urea	2.4 g
Sugar	8.0 g
NH ₄ Cl	0.4 g
K ₂ HPO ₄	0.004 g
Fertilizer	10.025 g
Partially treated sludge	260 ml

wastewater. The theoretical COD, Total Nitrogen and Total Phosphorous in synthetic wastewater were 158.5 mg of Oxygen/L, 25.6 mg/L and 19.0 mg/L respectively.

Operation and maintenance of setups

The nutrient removal efficiency of the synthetic wastewater was investigated at a high flow rate (100 L/day) corresponding to 16 cm/day hydraulic loading rate (HLR) and low flow rate (30 L/day) corresponding to 5 cm/day HLR. The system was operated for two months at each HLR. The HLR designed were based on the previous lab based studies focused on understanding the pollutant removal efficiencies of constructed wetland systems (with/without plants) operated at different HLR. The HLR ranged from 2.5 cm/day to 30 cm/day (Weerakoon *et al.* 2013). This study is an extension to published work to assess the impact of aggregate material on pollutant removal efficiency at different HLRs (Jamwal Phillips & Karlsrud 2019; Jamwal Biswas & Phillips 2020). Each set of experiment (at constant HLR) was run for two months, after which the plants were harvested and new set of experiment was launched at different HLR. The experiments were started with 5 cm/day of HLR. The plants matured at two months after which the plants were harvested and system was set to operate at 16 cm/day HLR. To keep the analysis simple data from four months of operation is presented.

Synthetic wastewater was added to the reservoir A, which is connected to reservoir B with a check valve installed on the feeder pipe (Figure 1). The check valve ensured delivery of constant and approximately equal flows into three setups. The inflow was adjusted using a control valve mounted on the inlet pipe. Hydraulic retention time (HRT) for each setup was calculated considering the pore volume. The average porosity estimated for control, plant setup 1 and plant setup 2 were 12, 10 and 15% respectively. The pore volume for each setup was determined at the beginning of the study by pouring a known amount of water into the setup filled with aggregate material.

Table 2 presents the mass loading rates at two different flow rates for control, plant setup 1 and plant setup 2.

Table 2 | Mass loading rate, HLR and HRT of control and plant setups

Setup units	Desired inflow rate (L/day)	Actual average inflow rate (L/day)	HLR (cm/day)	HRT (Hrs)	Average mass loading rate (g/m ² /day)				Overhead tank refilling frequency
					NO ₃ -N	NH ₄ -N	PO ₄ -P	Total COD	
Control	30	33	5	27	2.20	0.57	0.42	4.83	Once in two days
Setup 1		34	6	41	2.30	0.59	0.43	5.05	
Setup 2		31	5	30	2.08	0.54	0.39	4.56	
Control	100	95	16	8	4.38	3.22	1.33	9.56	Once in a day
Setup 1		98	16	6	4.50	3.31	1.36	9.83	
Setup 2		98	16	10	4.48	3.30	1.36	9.78	

Soil sample collection and microflora analysis

For plant setups, soil samples (20 g) were collected from the rhizosphere region of the soil layer (3.8 cm deep) and for control setup, samples were collected from the soil layer. The samples were collected in a sterile bag from four corners and centre of each setup. The samples from each setup were mixed thoroughly to create a composite soil sample. Serial dilution and plating on Nutrient agar (NA) and Reasoner's 2A agar (R₂A) was carried out for each composite soil sample (a mixture of 5 soil samples from each setup). NA is a general-purpose nutrient medium used for cultivation and growth of a broad range of non-fastidious bacteria. Nutrient agar has been successfully used to grow nitrifier bacteria such as *Nitrosomonas sp.* (Kramer 2016). Whereas R₂A media is used for isolation of slow growing aerobic and facultative heterotrophic bacteria. The low nutrient in R₂A helps

the growth of slow growing and stressed bacteria. Among the common conventional nutrient medias, R₂A is best for cultivation of diverse strains in the soil sample including potentially new species (Hu *et al.* 2007; Pham & Kim 2016). The methodology used here is a general method deployed to find all the microorganisms present in soil.

10 g composite soil samples from each setup were dispensed into the buffer solution (0.85% NaCl solution). The diluted sample was kept on a shaker for 20–30 minutes to obtain a uniform suspension. Fivefold serial dilution was prepared for each soil sample. The samples were plated by pour plate in NA and spread plate in R₂A. The Petri plates were incubated at 28 °C for 24–48 hours. After the incubation, the colony counter was used for the enumeration of the bacteria. The dry weight was measured by weighing soil after drying it in a hot air oven overnight at 105 °C. The colony-forming units (CFU) were calculated using Equation (1) (Aneja 2007).

$$\text{CFU/g dry weight} = \frac{\text{Plate count} \times \text{Dilution factor}}{\text{Dry weight of soil} \times \text{Volume of sample plated}} \quad (1)$$

The distinct colonies from the agar plates were subcultured in NA and R₂A agar. Ten colonies from the control and eight colonies from plant setup 1 and nine colonies from plant setup 2 were subcultured. Morphological test like gram staining was done to identify the morphology of the bacteria. Biochemical tests were carried out for the identification of isolated bacteria from the soil in the genus level. This includes catalase test, oxidase test, indole test, methyl red test, voges-proskauer test, citrate utilization test, Hydrogen sulfide production test, carbohydrate fermentation test, gelatin hydrolysis test, casein hydrolysis test, litmus test and nitrate reduction test (Aneja 2007).

Water sample collection and analysis

Water samples were collected at the inlet (I) and the outlets of the plant setup 1 (S1), plant setup 2 (S2) and control (C). The samples were collected in a sterile borosilicate glass and plastic bottles (LDPE, medical-grade USP Class VI autoclavable) and were tested for physical parameters, i.e. temperature and conductivity (once in two days); chemical parameters, i.e. pH (once in two days), dissolved oxygen (DO) (fortnightly once), organic matter (total COD) (two times a week), Ortho-Phosphates (Ortho-P) (once in two days), Nitrate-N (once in two days), Total Nitrogen (TN) (weekly once) and Ammonium-N (once in two days); biological parameter, i.e. *E. coli* (Fortnightly once). All the parameters were tested following the methods described in APHA Standard methods for the examination of water and wastewater (Federation and Association 2005). Conductivity, pH and temperature were measured using YSI Pro 1030 sensor (YSI, Yellow springs the USA) and Nitrate-N was measured using Hach Nitrate Pocket Colorimeter (Hach Company, Loveland, USA). Ammonium-N, TN, Ortho-P was analyzed photometrically (Merck KGaA, Darmstadt, Germany). Samples were tested for total Chemical Oxygen Demand (COD) by following the methods described in APHA standard methods. *E. coli* was detected and enumerated by Colilert 18 Quanti tray/2000 method (IDEXX Laboratories, Westbrook, USA).

The contaminant removal efficiency and Mass Loading Rate (MLR) of Nitrate-N, Ortho-P, Ammonium-N and total COD was estimated using Equations (2) and (3) respectively. Considering shorter HRTs, inflow was assumed to be equal to outflow hence the MRR removal efficiency (Equation (5)) was similar to contaminant removal efficiency (Equation (2)).

$$\text{Removal efficiency (\%)} = \frac{C_i - C_o}{C_i} \times 100 \quad (2)$$

$$\text{Mass Loading Rate (g/m}^2\text{/day)} = C \times \text{HLR} \quad (3)$$

$$\text{Mass Removal Rate (MRR) (g/m}^2\text{/day)} = (MLR_i - MLR_o) \quad (4)$$

$$\text{Mass Removal Rate (\%)} = \frac{(MLR_i - MLR_o)}{MLR_i} \times 100 \quad (5)$$

where C_i is the concentration of contaminant in the influent and C_o is the concentration of contaminant in the effluent. MLR_i ($g/m^2/day$) is the mass loading rate at the inlet and MLR_o ($g/m^2/day$) is the mass loading rate at the outlet.

HLR is the hydraulic loading rate (cm/day).

Considering that, the mass loading rate is a function of flow rate and pore volume, different values of MLRs were obtained at 5 cm/day and 16 cm/day HLR. Also at similar HLR, slight variations in MLR was observed which could be attributed to difference between the inflows and pore volume of three setups (Table 1).

Statistical analysis

Descriptive statistics of effluent quality was run through IBM SPSS Statistics 23.0 (Armonk, NY: IBM Corp.). An independent sample t-test was used to test the significance of the difference between the (a) quality of influent and effluent of the control, plant setup 1 and plant setup 2, (b) removal efficiency between control and plant setups and (c) contaminant load reduction at 5 cm/day and 16 cm/day HLR.

RESULTS AND DISCUSSION

Microflora identification

Similar number of colonies (5 log CFU/g) were observed in R_2A agar for both control and plant setup, whereas in NA, more number of colonies (5 log CFU/g) were observed in control compared to plant setups (4 log CFU/g). This could be attributed to the presence of anaerobic bacteria in control setup that thrived and grew inside the NA (pour plate method). In addition to aerobic and facultative bacteria, NA pour plates is known to support the growth of anaerobic bacteria. Few studies have reported significant increase in anaerobic bacterial community in unplanted system as compared to constructed wetland planted with *Typha angustifolia* (Gaballah *et al.* 2020). In the case of plants setups, radial oxygen leakage (ROL) leads to oxygenated conditions resulting in an environment dominated by aerobic and facultative bacteria. *Typha* species are known for the release of oxygen to the soil through the deep root system, thereby inhibiting the development of anaerobic bacteria (Samsó & García 2013).

Three *Staphylococcus* spp. and three *Pseudomonas* spp., one *Bacillus* sp., one *Corynebacterium* sp., one *Enterococcus* sp., and one *Proteus* sp., were isolated from the control. Three *Pseudomonas* spp., two *Bacillus* spp., one *Streptococcus* sp., one *Lactobacillus* sp., and one *Corynebacterium* sp. were isolated from setup 1. Four *Bacillus* spp., two *Staphylococcus* spp., one *Pseudomonas* sp., one *Lactobacillus* sp., and one *E.coli* were isolated from setup 2 (Annexure 1). These bacterial species are known to degrade organic matter and nutrients present in soil and groundwater. They tend to thrive in variable environmental conditions and helps with nutrient cycling. Study conducted by (Vaish *et al.* 2018), reported presence of *Staphylococcus* spp. in high pH condition (up to 9.5) in garden soil, sewage, groundwater and human gut. *Staphylococcus* spp. present in soil carries the NarG gene that is responsible for the conversion of Nitrate-N to nitrogen gas (Philippot *et al.* 2002). *Pseudomonas* spp. are abundantly found in the rhizosphere region and are known to carry out solubilization and mineralization of insoluble phosphorous (Rodríguez & Fraga 1999). Also due to their diverse metabolic activity, they are capable of carrying out bioremediation of organic pollutants (Kahlon 2016). *Bacillus* spp. play a significant role in degrading organic compounds in the constructed wetland (He *et al.* 2016). They promote release of ortho phosphorous in soil by producing organic acid and acid phosphatases (Saeid *et al.* 2018). *Corynebacterium* spp. are facultative anaerobic bacteria which are widespread in soil and water (Betts 2006). *Corynebacterium* spp. also contributes to the reduction of Nitrate-N to nitrogen gas in water and soil under anoxic conditions.

Proteus spp. are proteolytic bacteria and considered as a fecal indicator in wastewater. They have high metabolic activities and contributes to organic matter reduction in water and soil (Drzewiecka 2016). *Streptococcus* spp. are fecal indicators bacteria that persist longer in the environment and are found in soil, water, food and dairy products (Gerba 2015). *Lactobacillus* spp. (Lactic Acid Bacteria) are found in soil and helps with removing odor in the sewage treatment process and also helps with composting of organic wastes (Higa & Kinjo 1989). *Escherichia coli* thrives under extreme conditions and persists for an extended period in tropical, subtropical and temperate condition. The naturally occurring *Escherichia coli* strain promotes plant growth and nutrient uptake in soil (Nautiyal *et al.* 2010).

Table 4 presents the bacterial species and percent diversity that contributed to the transformation of organic carbon, Ammonium-N, Nitrate-N and Ortho-P in experimental setups. As compare to planted setups, control setup exhibited highest diversity of Nitrate-N reducing bacterial species (80%) and lowest diversity of organic carbon oxidising species (40%). This could be attributed to anaerobic conditions responsible for inhibiting the growth of aerobic bacteria in control setup. None of the bacterial species identified contributed to removal/transformation of Ammonium-N in control setup. Ammonium-N removal in plant setups is mainly dominated by plant uptake (Wu *et al.* 2019). Maximum percentage of Ortho-P transforming bacterial species were observed in plant setup 2 followed by plant setup 1 and control setup. The lowest percentage of Ortho-P transforming bacterial species could be attributed to anaerobic conditions that inhibited the growth of *Bacillus* spp. and *Pseudomonas* spp. in control setup.

Performance evaluation

The contaminant removal efficiency of three setups was estimated using Equation (2). Table 5 presents the contaminant levels at the inlet and outlets and removal efficiency of control and plant setups. The theoretical COD estimated is greater than the actual COD measured in the influents. Given that synthetic wastewater stayed in the reservoirs, degradation of organic matter and nutrients within the storage tanks might have led to this difference (Table 2). Similar differences were reported in the study conducted by other researchers (Weerakoon *et al.* 2013; Jamwal *et al.* 2019). For example, one of the study reported BOD₅ levels in the inflows as 25 mg/l compared to the theoretical BOD₅ levels of 44.7 mg/l (Weerakoon *et al.* 2013).

Nutrient reduction and transformation

At both HLRs (5 cm/day and 16 cm/day), a significant reduction in the Ammonium-N levels was observed at the outlet of control and the plant setups ($p < 0.05$). Physical process such as volatilisation and sorption to the filter media are reported to contribute to Ammonium-N removal. Ammonium-N volatilisation occurs at high pH (>8). This might not have contributed as lower pH levels were observed in the all the setups (Table 5). Sorption to the filter media and soil could be an important factor contributing to the removal of Ammonium-N. In control setup, similar removal efficiency was observed at both HLRs indicating dependence of Ammonium-N removal on the availability of sorption sites in filter media rather than the HRT. Lab experiments conducted by various researchers have attributed 40%–60% Ammonium-N reduction to sorption on gravel media (Riley *et al.* 2005; Hedström 2006).

High average Ammonium-N removal efficiency of 89 and 52% was observed in plant setup as compared to control at 5 cm/day and 16 cm/day respectively (Figure 3). Both physical and biological process contributed to Ammonium-N removal in plant setup. At low HLR additional 60% Ammonium-N removal could be attributed to plant uptake. Whereas at high HLR the contribution of plants to Ammonium-N removal dropped to 20%, suggesting that retention time limits the

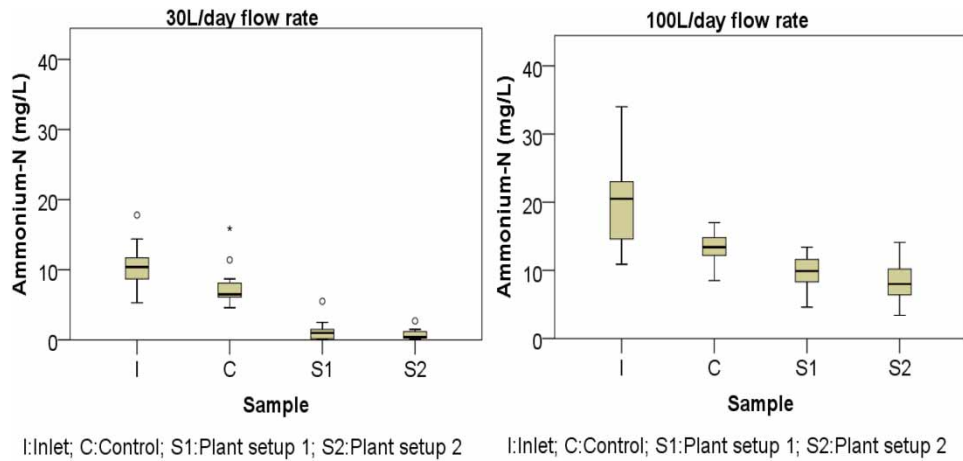


Figure 3 | Ammonium-N levels at the inlet and outlet of control and plant setups.

uptake of Ammonium-N by plants. Previous studies suggest that both nutrient uptake by plants and transformation by nitrifying bacterial species contributes to Ammonium-N removal in planted CW systems. Plants require less energy for absorption and uptake Ammonium-N than Nitrate-N and thus have been reported to contribute significantly to Ammonium-N removal (Wu *et al.* 2019). In a similar study, 83% Ammonium-N removal efficiency was observed in constructed wetland planted with *Thalia geniculata* as compared to an unplanted constructed wetland where 59% reduction was observed (Llanos-Lizcano *et al.* 2019).

The nitrifying bacterial species are chemotrophic and grow when the organic matter in the wastewater drops significantly (Gajewska *et al.* 2020). The growth of nitrifying bacteria such as *Nitrospirae* is inhibited by (a) root exudates (organic carbon) released by the plants (b) low concentration of Ammonium-N in the rhizosphere region and (c) low oxygen levels within the treatment zone of constructed wetland (Table 5). Given the presence of either of the three conditions, the growth of nitrifying bacteria was limited in control as well as plant setups. Therefore, physical process and plant uptake dominated the Ammonium-N removal in control and plant setups respectively.

At both HLRs (5 cm/day and 16 cm/day), significant reduction in Nitrate-N was observed at the outlet of control and the plant setups ($p < 0.05$) (Figure 4). Unlike Ammonium-N removal efficiencies, no significant difference was observed in the Nitrate-N removal efficiency between control and plant setups ($p > 0.05$) (see Table 5). At 5 cm/day HLR, the Nitrate-N removal efficiency of control, plant

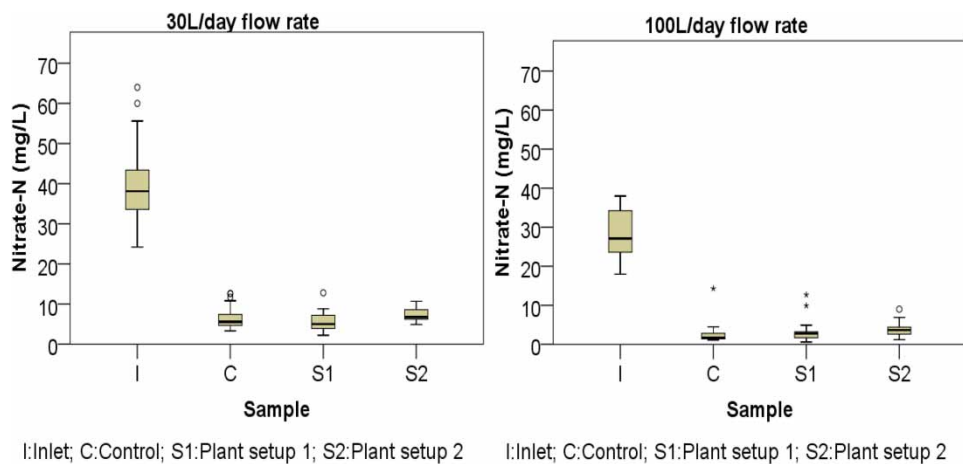


Figure 4 | Nitrate-N levels at the inlet and outlet of control and plant setups.

setup 1 and plant setup 2 were 82, 85 and 81% respectively. At 16 cm/day HLR, the Nitrate-N removal efficiency for control, plant setup 1 and plant setup 2 were 90, 87 and 85% respectively.

Presence of conditions favourable for the growth of denitrifying bacteria contributed to Nitrate-N removal in control setup. Whereas radial oxygen leakage (ROL) from the roots of plants inhibited the growth of denitrifying bacteria and contributed to low bacterial diversity and denitrification rates in plant setups (Table 3). In control setup, 80% of the bacteria showed positive results for Nitrate-N reduction test as compared to 50 and 77% in plant setup 1 and plant setup 2 (see Table 4). Previous studies reported effectiveness of denitrifying bacteria in removing nitrates in marsh ponds and unplanted wetlands (Dong & Reddy 2010; García *et al.* 2010). Absence of O₂, redox potential, temperature, pH, moisture content are some of the environmental factors that influence denitrification rate (García *et al.* 2010).

Table 3 | Colony-forming units in control and plant setups under two media types

Sample	Media	CFU/g of soil
Control	NA	2.08×10^5
Setup 1	NA	1.90×10^4
Setup 2	NA	5.39×10^4
Control	R ₂ A	1.18×10^5
Setup 1	R ₂ A	1.97×10^5
Setup 2	R ₂ A	3.73×10^5

Table 4 | Bacterial species diversity and their role in removing contaminants from the wastewater

Setup	Bacterial species putatively responsible for the removal of/transformation of			
	Organic Carbon (% diversity)	Oxidation of Ammonium-N (% diversity)	Nitrate-N (% diversity)	Ortho-P (% diversity)
Control	<ul style="list-style-type: none"> • <i>Bacillus sp.</i> • <i>Pseudomonas sp.</i> (40%) 	Limited Microbial influence. Removal of Ammonium-N dominated by plant uptake	<ul style="list-style-type: none"> • <i>Staphylococcus sp.</i> • <i>Corynebacterium sp.</i> • <i>Pseudomonas sp.</i> • <i>Proteus sp.</i> • <i>Enterococcus sp.</i> (80%) 	<ul style="list-style-type: none"> • <i>Bacillus sp.</i> • <i>Pseudomonas sp.</i> (40%)
Plant setup-1	<ul style="list-style-type: none"> • <i>Bacillus sp.</i> • <i>Pseudomonas sp.</i> (62%) 		<ul style="list-style-type: none"> • <i>Bacillus sp.</i> • <i>Pseudomonas sp.</i> • <i>Corynebacterium sp.</i> (50%) 	<ul style="list-style-type: none"> • <i>Bacillus sp.</i> • <i>Pseudomonas sp.</i> (62%)
Plant setup-2	<ul style="list-style-type: none"> • <i>Bacillus sp.</i> • <i>Pseudomonas sp.</i> (66%) 		<ul style="list-style-type: none"> • <i>Bacillus sp.</i> • <i>E. coli</i> • <i>Staphylococcus sp.</i> • <i>Pseudomonas sp.</i> (77%) 	<ul style="list-style-type: none"> • <i>Bacillus sp.</i> • <i>Pseudomonas sp.</i> (66%)

Denitrification is the process by which Nitrate-N is converted to nitrogen gas. The facultative anaerobic bacteria carry out denitrification. It uses Nitrate-N (and nitrite) as an electron acceptor under anoxic condition, utilises organic carbon, and converts it to nitrogen gas. Studies reported that COD/TN ratio ~5 is optimal for effective removal of TN via nitrification and denitrification process. Few studies reported 40–50% TN removal efficiency at low COD/TN ratio of 2 (Collison & Grismer 2013; Zhu

et al. 2014; Wang *et al.* 2016b). An average COD/TN ratio observed in inflows was approximately two, which was further enhanced by the presence of organic carbon in soil layer. This might have contributed to high Nitrate-N removal efficiency at 16 cm/day HLRs in all three setups.

Ortho-P reduction

Figure 5 presents the Ortho-P levels at the inlet and outlet of control and plant setups. At both HLRs, significant reductions ($p < 0.05$) in the Ortho-P levels were observed at the outlet of three setups. Similar removal efficiency (30%) was observed in control at both HLRs, whereas lower removal efficiencies were observed at high HLR in plant setups. In plant setups, higher Ortho-P removal efficiency was observed (35%) compared to control (30%) at low HLR (5 cm/day) ($p < 0.05$). Ortho-P removal in unplanted CW is dominated by chemical adsorption of soluble reactive phosphorous (SRP) to the soil/aggregate material (Yang *et al.* 2001). Whereas in plant setups, in addition to chemical adsorption, higher retention times (5 cm/day) allowed for uptake of the reactive Ortho-P by plants, therefore, contributing to high removal efficiencies in plant setups. Higher HRT allows the phosphorus to interact/react with the microorganisms, substrate and plants in a constructed wetland. Studies have reported a linear relationship between phosphorous removal efficiency and HRT in the planted CW (Quan *et al.* 2016).

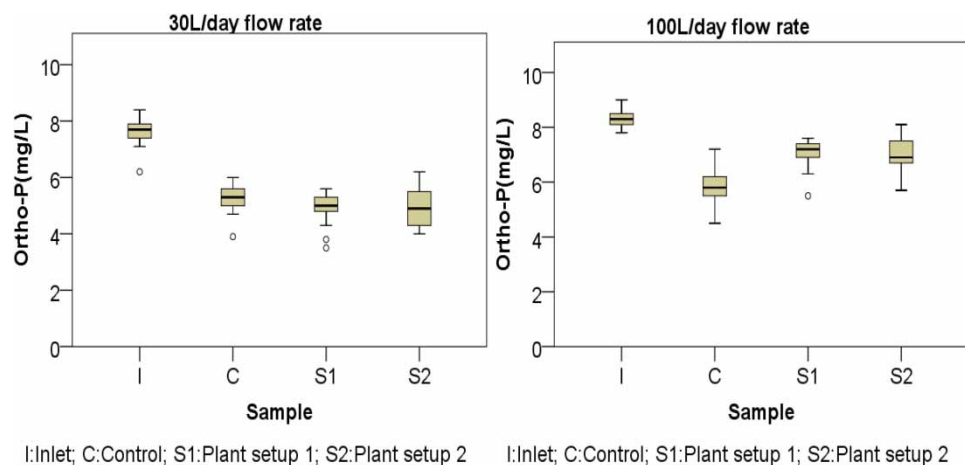


Figure 5 | Ortho-P levels at the inlet and outlet of control and plant setups.

At higher HLR (16 cm/day) greater Ortho-P removal efficiency was observed in control unit (30%) as compared to plant setup 1 (15%) and plant setup 2 (16%) ($p < 0.05$). Chemical adsorption to aggregate material and soil particle contributed to the removal of Ortho-P in control (Ghosh & Gopal 2010). Also low percentage of phosphate solubilizing bacteria (40%) prevented phosphorus mineralisation thereby controlling Ortho-P levels in the outlet of control setup. Whereas in plant setups, inorganic P-solubilizing bacteria (IPSB) like *Bacillus* spp. and *Pseudomonas* spp. solubilized inorganic form of phosphorous to Ortho-P and made it available for plant uptake. Organic P-mineralizing bacteria (OPMB) such as *Bacillus cereus* and *Bacillus megaterium* are also known for making Ortho-P available to plants in agricultural soil. Both IPSB and OPMB encourage conversion of inorganic and organic phosphorous to Ortho-P (Cao *et al.* 2018). The inorganic phosphate compounds such as dicalcium phosphate, tricalcium phosphate, hydroxyapatite and rock phosphate present in the rhizosphere region of the soil is converted into Ortho-P (Rodríguez & Fraga 1999). Whilst high microbial diversity have contributed to Ortho-P levels, high flow rates and low HRT (16 cm/day) allowed Ortho-P to escape without getting absorbed by the plants thereby contributing to lower Ortho-P removal in plant setups.

Organic matter reduction

Figure 6 presents the total COD levels at the inlet and outlet of control and plant setups. At both flow rates, a significant reduction in total COD levels was observed at outlets of control setup and plant setups ($p < 0.05$). Control setup provided 20% COD removal efficiency at both HLRs, whereas an average of 34% (5 cm/day) and 27% (16 cm/day) total COD removal efficiencies was observed for plant setups (Table 5). No significant difference was observed in the total COD removal efficiencies between plant setups and control setup ($p > 0.05$). Considering that COD is removed during the secondary treatment process, this study was designed to assess nutrient removal efficiencies at HRTs lower than the HRTs deployed for removal of organic carbon in CWs (4 days) (Table 2). Hence, low HRTs contributed to low COD removal efficiency in both control and plant setups.

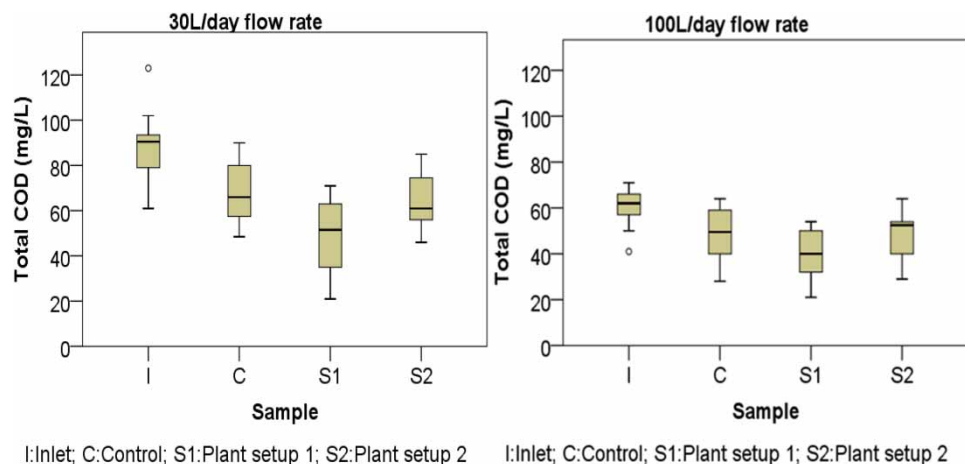


Figure 6 | Total COD levels at the inlet and outlet of control and plant setups.

We observed *Bacillus* spp. and *Pseudomonas* spp. in control setup (40%), plant setup 1 (62%) and plant setup 2 (66%). Relatively higher bacterial density contributed to higher but not statistically significant COD removal efficiency in plant setups. Both *Bacillus* spp. and *Pseudomonas* spp. are known to remove COD brewery effluent efficiently. The study reported 82% COD removal by *Bacillus* spp. and up to 79% COD removal by *Pseudomonas* spp. (Oljira *et al.* 2018). Another study reported an overall COD reduction from 108 mg/l to 4 mg/L and 206 mg/L to 16 mg/L at 5 days HRT by *Pseudomonas* spp. and *Bacillus* spp. respectively (Adebayo *et al.* 2013). Mixed cultures of *Pseudomonas aeruginosa*, *Bacillus* spp. have also been used to remove COD in lipid-rich and pharmaceutical wastewater (Oljira *et al.* 2018).

Impact of hydraulic loading rate on the contaminant load reduction

We estimated contaminant load reduction efficiency using Equations (4) and (5). Figure 7 compares the contaminant load reduction at 5 cm/day and 16 cm/day of control and plant setups.

In case of control setup, either higher or similar contaminant removal efficiencies were observed at different HLRs. No significant difference was observed in Ammonium-N, Ortho-P and COD load reduction at both 5 cm/day and 16 cm/day indicating that physical processes such as volatilisation, sorption and chemical adsorption to soil particles dominated contaminant removal and same was not affected by HLR (Riley *et al.* 2005; Hedström 2006). Significantly higher i.e. 90% Nitrate-N load reduction was observed at 16 cm/day as compared to 82% observed at 5 cm/day.

In case of planted systems except for Nitrate-N, contaminant removal efficiencies decreased at high HLR. Significantly higher Ammonium-N and Ortho-P load reduction were observed in plant setups at

Table 5 | Concentrations and removal efficiencies of control and plant setups in different HLR

Parameter	Inlet		Control		Control		Plant setup 1		Plant setup 1		Plant setup 2		Plant setup 2	
	HLR-5 cm/day Mean ± SD	HLR-16 cm/day Mean ± SD	HLR-5 cm/day Mean ± SD	HLR-16 cm/day Mean ± SD	HLR-5 cm/day % Removal	HLR-16 cm/day % Removal	HLR-5 cm/day Mean ± SD	HLR-16 cm/day Mean ± SD	HLR-5 cm/day % Removal	HLR-16 cm/day % Removal	HLR-5 cm/day Mean ± SD	HLR-16 cm/day Mean ± SD	HLR-5 cm/day % Removal	HLR-16 cm/day % Removal
pH (<i>n</i> = 27)	7.4 ± 0.2	7.31 ± 0.8	7.5 ± 0.2	7.4 ± 0.5	–	–	7.0 ± 0.2	7.4 ± 0.4	–	–	6.9 ± 0.1	6.9 ± 0.1	–	–
Conductivity (μ S/cm) (<i>n</i> = 27)	1,517 ± 65	1,358 ± 35	1,500 ± 82	1,333 ± 34	–	–	1,549 ± 136	1,369 ± 56	–	–	1,552 ± 107	1,361 ± 53	–	–
Temperature (°C) (<i>n</i> = 27)	24.7 ± 1.8	28.8 ± 1.9	23.6 ± 1.5	28.0 ± 1.9	–	–	24.5 ± 1.9	28.2 ± 2.2	–	–	23.9 ± 1.8	28.2 ± 2.0	–	–
Dissolved Oxygen (mg/L) (<i>n</i> = 4)	0.2 ± 0.2	0.7 ± 0.9	0.1 ± 0.1	0.1 ± 0.1	–	–	0.01 ± 0.1	0.05 ± 0.05	–	–	0.09 ± 0.14	0.06 ± 0.06	–	–
Nitrate-N (mg/L) (<i>n</i> = 26)	40.4 ± 9.7	27.5 ± 6.2	6.6 ± 2.8	2.7 ± 3.0	82	90	5.7 ± 2.5	3.3 ± 3.1	85	87	7.2 ± 1.6	3.9 ± 1.8	81	85
Ammonium-N (mg/L) (<i>n</i> = 21)	10.4 ± 3.1	20.3 ± 6.3	7.2 ± 2.5	13.1 ± 2.1	28	31	1.2 ± 1.3	9.6 ± 2.6	87	49	0.8 ± 0.7	8.3 ± 2.9	92	55
Total nitrogen (mg/L) (<i>n</i> = 8)	55.1 ± 11.9	43.9 ± 7.7	25.1 ± 6.6	17.2 ± 4.0	52	60	15.0 ± 6.9	14.9 ± 6.1	71	65	15.1 ± 6.4	15.6 ± 6.6	71	63
Ortho-P (mg/L) (<i>n</i> = 29)	7.6 ± 0.4	8.3 ± 0.3	5.3 ± 0.5	5.81 ± 0.8	30	30	5.0 ± 0.5	7.1 ± 0.5	35	15	5.0 ± 0.6	7.0 ± 0.6	35	16
Total COD (mg/L) (<i>n</i> = 14)	88.6 ± 15.9	60.1 ± 8.3	69.0 ± 14.1	48.3 ± 10.6	20	20	48.8 ± 17.4	39.7 ± 10.2	43	34	64.5 ± 12.9	48.4 ± 11.0	25	20
<i>E. coli</i> (MPN/100 mL) (<i>n</i> = 6)	$7.0 \times 10^2 \pm 7.0 \times 10^2$	$8.1 \times 10^3 \pm 6.4 \times 10^3$	$3.8 \times 10^2 \pm 2.9 \times 10^2$	$2.9 \times 10^3 \pm 3.5 \times 10^3$	–	–	$1.8 \times 10^1 \pm 1.2 \times 10^1$	$8.9 \times 10^2 \pm 6.4 \times 10^2$	–	–	$0.8 \times 10^1 \pm 0.6 \times 10^1$	$8.4 \times 10^2 \pm 4.2 \times 10^2$	–	–
<i>E. coli</i> log reduction (<i>n</i> = 6)	–	–	0.22 ± 0.53	0.47 ± 0.35	–	–	1.62 ± 0.67	0.89 ± 0.38	–	–	1.90 ± 0.55	0.77 ± 0.65	–	–

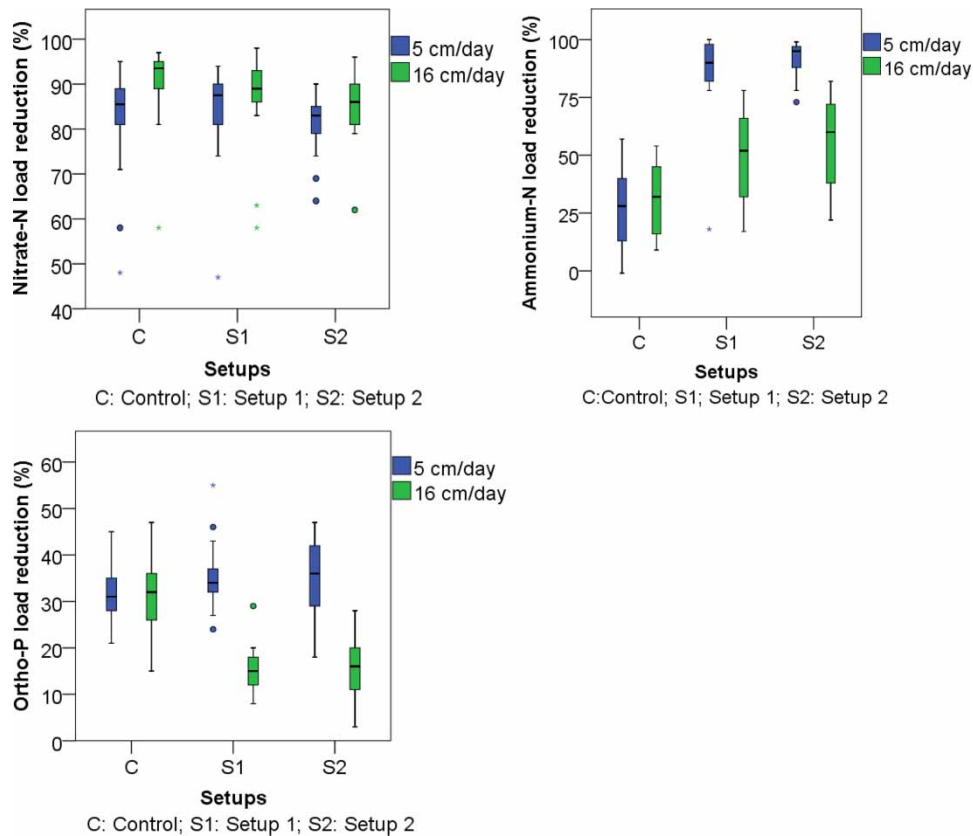


Figure 7 | Contaminant load reduction in control and plant setups at different HLRs.

low HLR (5 cm/day) ($p < 0.05$). This could be attributed to the longer average HRT at low HLRs. Studies suggests that high HRTs encourages nutrient uptake and contributes to higher removal efficiencies in planted systems. Several studies have reported increased Ammonium-N, Phosphorous and organic matter removal efficiency at low HLRs (Çakir *et al.* 2015; Mesquita *et al.* 2018). Dan *et al.* (2011) reported 1.1% to 8.2% of Nitrogen mass removal and 0.6% to 2.2% of Phosphorus mass removal by plant uptake with highest removal observed at 8 cm/day HLR. An average of 50% decline in the Ortho-P and Ammonium-N load reduction at 16 cm/day was observed in plant setups. Also, as compared to conventional septic tank systems (anaerobic process), significant decrease in Ammonium-N levels was observed in both planted and unplanted systems. Studies have reported significant increase in Ammonium-N levels in the effluent from septic tanks (Nasr & Mikhaeil 2013).

Both unplanted and planted systems showed increased or similar Nitrate-N removal efficiency at high HLR (16 cm/day) respectively. Exposure to high organic load (16 cm/day) promoted the growth of microbes leading to the formation of thick biofilms and anoxic sites, which favours denitrification activity (Dalahmeh *et al.* 2014), thereby contributing to increased denitrification rates and Nitrate-N removal (Shen *et al.* 2015). In addition to this, the organic carbon present in the soil contributed to higher C/N ratio that might have promoted the growth of denitrifying bacteria. Hence, both the increased loading rate and organic carbon in soil might have contributed to the high Nitrate-N load reduction at high HLR in control setup.

CONCLUSION

This study demonstrated suitability of unplanted/control setup for enhancing the growth of anaerobic and facultative bacteria. In addition, the ability of anaerobic and facultative bacterial colonies to grow

on NA (pour plate method) indicates its suitability for identifying and quantifying microflora in soils and other media.

Both control and plant setups exhibited similar Nitrate-N removal efficiency at both HLRs. Control setup provided suitable conditions for the growth of denitrifying bacteria. Eighty percent of the bacterial species identified in control setup contributed to denitrification process. In case of planted setups, additional nutrient uptake by plants might have contributed to Nitrate-N removal at high HLR. Both the soil organic carbon and thick anoxic biofilms within the systems significantly contributed to Nitrate-N removal.

Both control and plant setups showed absence of nitrifying bacteria. Low oxygen and high organic carbon inhibited the growth of nitrifying bacteria in control and plant setups. Physical processes dominates removal of Ammonium-N, Ortho-P and COD in control setup. Similar removal efficiency was observed at both HLRs in control setup indicating presence of sorption sites rather than HLRs as a limiting factor for contaminant removal. At low HLR biological processes (uptake by plants) dominated the removal of Ammonium-N in plant setup. Ammonium-N removal efficiency decreased with increase in HLR.

Presence of diverse phosphate solubilizing bacterial species (66%–68%) in plant setup contributed to high Ortho-P removal at low HLRs. The uptake of Ortho-P by plants is limited by the HRT as a result lower Ortho-P removal efficiencies (50% less) were observed at lower HRTs (high HLR).

Higher *Bacillus spp.* and *Pseudomonas spp.* levels (diversity and density) (62%–66%) contributed to higher COD removal in plant setups as compared to control setup. Whilst HLR did not have any effect on the COD removal efficiency in control setup (dominated by physical process), COD removal efficiency decreased at high HLR in the plant setups.

This study demonstrated that unplanted constructed wetlands are suitable to treat effluents from secondary wastewater treatment systems that are based on the aerobic process such as activated sludge process. The secondary treated effluent from such systems are low in COD and have high Nitrate-N and Ortho-P levels that can be easily treated by unplanted CW provided additional carbon source is made available in filter media. Whereas, planted wetlands are suitable to treat effluents from treatment systems based on anaerobic processes such as septic tanks. The effluent from such systems are low COD and have high Ammonium-N and Ortho-P levels that can be easily treated with planted CW systems.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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