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

Priyanka Jamwal* and Shahana Shirin

Centre for Environment and Development, Ashoka Trust for Research in Ecology and the Environment (ATREE), Jakkur 560064

*Corresponding author. E-mail: priyanka.jamwal@atree.org

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

This is an Open Access article distributed under the terms of the Creative Commons Attribution Licence (CC BY 4.0), which permits copying, adaptation and redistribution, provided the original work is properly cited (http://creativecommons.org/licenses/by/4.0/).
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 (Almuktar 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 (Almuktar 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

![Flow diagram of the Lab setups](http://iwaponline.com/wpt/article-pdf/doi/10.2166/wpt.2021.017/850681/wpt2021017.pdf)

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

**Table 1** Composition of synthetic wastewater

| Compounds       | Quantity/100 L |
|-----------------|----------------|
| Urea            | 2.4 g          |
| Sugar           | 8.0 g          |
| NH₄Cl           | 0.4 g          |
| K₂HPO₄          | 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/m²/day) NO₃-N NH₄-N PO₄-P Total COD | Overhead tank refilling frequency |
|-------------|----------------------------|-----------------------------------|-------------|-----------|---------------------------------------------------------------|----------------------------------|
| Control     | 30                         | 33                                | 5           | 27        | 2.20, 0.57, 0.42, 4.83                                        | Once in two days |
| Setup 1     | 34                         | 34                                | 6           | 41        | 2.30, 0.59, 0.43, 5.05                                        |                                  |
| Setup 2     | 31                         | 31                                | 5           | 30        | 2.08, 0.54, 0.39, 4.56                                        |                                  |
| Control     | 100                        | 95                                | 16          | 8         | 4.38, 3.22, 1.33, 9.56                                        |                                  |
| Setup 1     | 98                         | 98                                | 16          | 6         | 4.50, 3.31, 1.36, 9.83                                        | Once in a day                   |
| Setup 2     | 98                         | 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).

\[
\text{CFU/g dry weight} = \frac{\text{Plate count} \times \text{Dilution factor}}{\text{Dry weight of soil} \times \text{Volume of sample plated}}
\]  

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 1050 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 Quanty 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)).

\[
\text{Removal efficiency} (\%) = \frac{C_i - C_o}{C_i} \times 100
\]

\[
\text{Mass Loading Rate (g/m}^2\text{/day)} = C \times \text{HLR}
\]

\[
\text{Mass Removal Rate (MRR) (g/m}^2\text{/day)} = (MLR}_i - MLR_o
\]

\[
\text{Mass Removal Rate (\%)} = \frac{(MLR}_i - MLR_o}{MLR}_i \times 100
\]
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$_2$A 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
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 geniculate 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
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 effective 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      | R2A     | 1.18 × 10^5   |
| Setup 1      | R2A     | 1.97 × 10^5   |
| Setup 2      | R2A     | 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     | • Bacillus sp.  
• Pseudomonas sp. (40%)  
Limited Microbial influence. Removal of Ammonium-N dominated by plant uptake | - | - | • Staphylococcus sp.  
• Corynebacterium sp.  
• Pseudomonas sp.  
• Proteus sp.  
• Enterococcus sp. (80%) | • Bacillus sp.  
• Pseudomonas sp. (40%) |
| Plant setup-1 | • Bacillus sp.  
• Pseudomonas sp. (62%) | - | - | • Bacillus sp.  
• Pseudomonas sp.  
• Corynebacterium sp. (50%) | • Bacillus sp.  
• Pseudomonas sp. (62%) |
| Plant setup-2 | • Bacillus sp.  
• Pseudomonas sp. (66%) | - | - | • Bacillus sp.  
• E. coli  
• Staphylococcus sp.  
• Pseudomonas sp. (77%) | • Bacillus sp.  
• Pseudomonas sp. (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 phosphorus (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](http://iwaponline.com/wpt/article-pdf/doi/10.2166/wpt.2021.017/850681/wpt2021017.pdf)

**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.

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-5 cm/day Mean ± SD | HLR-16 cm/day Mean ± SD | HLR-16 cm/day Mean ± SD | HLR-5 cm/day Mean ± SD | HLR-16 cm/day Mean ± SD | HLR-16 cm/day Mean ± SD |
| pH (n = 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 (μS/cm) (n = 27) | 1,517 ± 65  | 1,358 ± 35 | 1,500 ± 82 | 1,333 ± 34 | - -           | 1,549 ± 136 | 1,569 ± 56 | - -           | 1,552 ± 107 | 1,361 ± 53 | - -           |
| Temperature (°C) (n = 27) | 24.7 ± 1.8  | 28.8 ± 1.9 | 28.0 ± 1.9 | 24.5 ± 1.9 | 28.2 ± 2.2 | - -           | 23.9 ± 1.8 | 28.2 ± 2.0 | - -           |
| Dissolved Oxygen (mg/L) (n = 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) (n = 26)  | 40.4 ± 9.7  | 27.5 ± 6.2 | 6.6 ± 2.8 | 2.7 ± 3.0 | 82 90         | 5.7 ± 2.5 | 3.5 ± 3.1 | 85 87         | 7.2 ± 1.6 | 3.9 ± 1.8 | 81 85         |
| Ammonium-N (mg/L) (n = 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) (n = 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) (n = 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 | 55 16         |
| Total COD (mg/L) (n = 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         |
| E.coli (MPN/100 mL) (n = 6) | 7.0 × 10^7  | 7.0 × 10^3 | 8.1 × 10^3 | 2.9 × 10^3 | - -           | 1.8 × 10^3 | 8.9 × 10^2 | - -           | 0.8 × 10^3 | 8.4 × 10^2 | - -           |
| E.coli log reduction (n = 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 | - -           |
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.

**ACKNOWLEDGEMENTS**

This research was supported by a research grant from the Royal Norwegian Embassy (RNE) and the Ashoka Trust for Research in Ecology and the Environment (ATREE). The authors would like to thank Pavan Muttepawar (Trainee) and Sumita Bhattacharyya (Doctorate student) at ATREE for helping us with the analysis of water samples and maintenance and upkeep of the experimental setup.

**DATA AVAILABILITY STATEMENT**

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

**REFERENCES**

Adebayo, G. B., Jimoh, A. A., Odebunmi, E. O. & Oke, M. A. 2013 Kinetics study of bioremediation of industrial effluents by *Pseudomonas* spp and *Bacillus* spp using chemical oxygen demand (COD). *Research Journal of Agriculture and Environmental Management* 2(9).
Kramer, S. 2016 Nitrosomonas eutropha: a study of the effects of nitrosomonas on pathogenic bacterium and the effects of current hygiene habits on the colonization of nitrosomonas within our normal flora. *JCCC Honors Journal* 7(1), 3.

Lee, C., Fletcher, T. D. & Sun, G. 2009 Nitrogen removal in constructed wetland systems. *Engineering in Life Sciences* 9(1), 11–22.

Llanos-Lizcano, A., Barraza, E., Narvaez, A., Varela, L. & Caselles-Osorio, A. 2019 Efficiency of pilot-scale horizontal subsurface flow constructed wetlands and microbial community composition operating under tropical conditions. *International Journal of Phytoremediation* 21(1), 34–42.

Massoud, M. A., Tarhini, A. & Nasr, J. A. 2009 Decentralized approaches to wastewater treatment and management: applicability in developing countries. *Journal of Environmental Management* 90(1), 652–659.

McMullen 2018 *How to Grow Cattails From Seeds | Home Guides | SF Gate*. Available from: https://homeguides.sfgate.com/grow-cattails-seeds-70962.html (Accessed April 17, 2020).

Mesquita, C., Albuquerque, A., Amaral, L. & Nogueira, R. 2018 Effectiveness and temporal variation of a full-scale horizontal constructed wetland in reducing nitrogen and phosphorus from domestic wastewater. *ChemEngineering* 2(1), 3.

Nasr, F. A. & Mikhaeil, B. 2013 Treatment of domestic wastewater using conventional and baffled septic tanks. *Environmental Technology* 34(16), 2357–2363.

Nautiyal, C. S., Rehman, A. & Chauhan, P. S. 2010 Environmental Escherichia coli occur as natural plant growth-promoting soil bacterium. *Archives of Microbiology* 192(3), 185–193.

Oljira, T., Muleta, D. & Jida, M. 2018 Potential applications of some indigenous bacteria isolated from polluted areas in the treatment of brewery effluents. *Biotechnology Research International* 2018, 1–13.

Pham, V. & Kim, J. 2016 Improvement for isolation of soil bacteria by using common culture media. *Journal of Pure and Applied Microbiology* 10.

Philip, L., Murty, B. S. & Sundaramoorthy, S. 2012 *Guidelines for Decentralized Wastewater Management*. MoUD Centre of Excellence in DWM, Department of Civil Engineering, Indian Institute of Technology, Madras – Chennai.

Philippot, L., Piutti, S., Martin-Laurent, F., Hallet, S. & Germon, J. C. 2002 Molecular analysis of the nitrate-reducing community from unplanted and maize-planted soils. *Applied and Environmental Microbiology* 68(12), 6121–6128.

Quan, Q., Chen, B., Zhang, Q. & Ashraf, M. A. 2016 Research on phosphorus removal in artificial wetlands by plants and their photosynthesis. *Brazilian Archives of Biology and Technology* 59(spe). Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1516-89132016000200701&lng=en&tlng=en (Accessed April 17, 2020).

Rajan, R. J., Sudarsan, J. S., Nithiyanantham, S., Rajan, R. J., Sudarsan, J. S. & Nithiyanantham, S. 2018 Microbial population dynamics in constructed wetlands: review of recent advancements for wastewater treatment. *Environmental Engineering Research* 24(2), 181–190.

Riley, K. A., Stein, O. R. & Hook, P. B. 2005 Ammonium removal in constructed wetland microcosms as influenced by season and organic carbon load. *Journal of Environmental Science and Health, Part A* 40(6–7), 1109–1121.

Rodriguez, H. & Praga, R. 1999 Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology Advances* 17(4–5), 319–339.

Saeid, A., Prochownik, E. & Dobrowolska-Iwanek, J. 2018 Phosphorus solubilization by bacillus species. *Molecules* 23(11), 2897.

Samsó, R. & García, J. 2013 Bacteria distribution and dynamics in constructed wetlands based on modelling results. *Science of The Total Environment* 461–462, 430–440.

Sharma, C. & Sharma, S. 2018 *Centralized Versus Decentralized Wastewater Treatment and Reuse: A Feasibility Study for NITITRR Campus, Chandigarh*.

Shehzadi, M., Afzal, M., Khan, M. U., Islam, E., Mobin, A., Anwar, S. & Khan, Q. M. 2014 Enhanced degradation of textile effluent in constructed wetland system using Typha domingensis and textile effluent-degrading endophytic bacteria. *Water Research* 58, 152–159.

Shen, Z., Zhou, Y., Liu, J., Xiao, Y., Cao, R. & Wu, F. 2015 Enhanced removal of nitrate using starch/PCL blends as solid carbon source in a constructed wetland. *Bioresource Technology* 175, 239–244.

Singh, S., Haberl, R., Moog, O., Shrestha, R. R., Shrestha, P. & Shrestha, R. 2009 Performance of an anaerobic baffled reactor and hybrid constructed wetland treating high-strength wastewater in Nepal – A model for DEWATS. *Ecological Engineering* 35(5), 654–660.

Soma, B. N. & Srinivasa, C. V. 2015 Location identification for water conservation and quality assurance. *Aquatic Procedia* 4, 1134–1141.

Vaish, M., Price-Whelan, A., Reyes-Robles, T., Liu, J., Jereen, A., Christie, S., Alonzo, F., Benson, M. A., Torres, V. J. & Krulwich, T. A. 2018 Roles of staphylococcus aureus Mnh1 and Mnh2 antiporters in salt tolerance, alkali tolerance, and pathogenesis. *Journal of Bacteriology* 200(5). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5809693/ (Accessed May 4, 2020).

Wang, Q., Xie, H., Ngo, H. H., Guo, W., Zhang, J., Liu, C., Liang, S., Hu, Z., Yang, Z. & Zhao, C. 2016a Microbial abundance and community in subsurface flow constructed wetland microcosms: role of plant presence. *Environment Sci Pollut Res* 10.

Wang, W., Ding, Y., Ullman, J. L., Ambrose, R. F., Wang, Y., Song, X. & Zhao, Z. 2016b Nitrogen removal performance in planted and unplanted horizontal subsurface flow constructed wetlands treating different influent COD/N ratios. *Environmental Science and Pollution Research* 23(9), 9012–9018.
Weerakoon, G., Jinadasa, K., Herath, G. B. B., Mowjood, M. I. M. & Van Bruggen, J. J. A. 2013 Impact of the hydraulic loading rate on pollutants removal in tropical horizontal subsurface flow constructed wetlands. *Ecological Engineering* **61**, 154–160.

Wu, Y., He, T., Chen, C., Fang, X., Wei, D., Yang, J., Zhang, R. & Han, R. 2019 Impacting microbial communities and absorbing pollutants by canna indica and cyperus alternifolius in a full-scale constructed wetland system. *International Journal of Environmental Research and Public Health* **16**(5), 802.

Yang, L., Chang, H.-T. & Huang, M.-N. L. 2001 Nutrient removal in gravel- and soil-based wetland microcosms with and without vegetation. *Ecological Engineering* **18**(1), 91–105.

Zhu, H., Yan, B., Xu, Y., Guan, J. & Liu, S. 2014 Removal of nitrogen and COD in horizontal subsurface flow constructed wetlands under different influent C/N ratios. *Ecological Engineering* **65**, 58–63.