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# Pathogen Treatment in Single and Two-Stage Vertical Flow Wetland as a Potential Sanitation Technology for Rural India

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## ABSTRACT

Vertical flow-constructed wetlands (VFCW) are well-established, cost-effective, and sustainable options for wastewater treatment. Along with organic matter removal, wetlands are helpful in the removal of microbial pathogens. This study focuses on understanding the bacterial pathogen removal efficacy of three different design types of VFCWs and understands the best designs for the efficient removal of pathogens in a tropical climate. The three wetlands studied for removal efficiency were (a) two-stage vertical flow constructed wetland (TSVFCW), (b) Single-stage vertical flow constructed wetland (SSVFCW), and (c) single-stage saturated vertical flow constructed wetland (SSSVFCW). Results revealed that all three types of wetlands were effective in removing pathogenic bacteria. Still, SSVFCW was found to be more efficient in pathogen removal (Total Coliforms, *Shigella* spp., *Salmonella* spp., *Pseudomonas* spp., *Vibrio* spp., *Enterococcus faecalis*) 2.90 ± 2.56, 2.86 ± 2.39, 2.39 ± 0.61, 1.38 ± 0.65, 2.22 ± 2.06, 4.81 ± 1.00 $Log_{10}$ reductions respectively compared to TSVFCW (0.76 ± 0.02, 1.22 ± 0.39, 2.04 ± 1.56, 0.31 ± 0.07, 3.52 ± 2.89 and 0.44 ± 0.11 $Log_{10}$ reductions respectively) and SSSVFCW (0.434 ± 0.37, 1.31 ±0.09, 1.45 ± 0.85, 0.95 ± 0.16, 2.55 ± 1.27, 5.03 ± 1.2 $Log_{10}$ decreases respectively). For abiotic factors (Chemical oxygen demand, total Kjeldahl nitrogen, and phosphorus) also TSVFW shows better efficiency (45 ± 8.7, 24.7±4.5 and 3.1, ± 0.2 $g.m^2$, respectively) than SSVFCW (12 ± 1.3, 7.6 ± 0.4 and 1.8 ± 0.2 $g.m^2$ respectively) and SSVFCW (6.3 ± 1.1, 7.7 ± 0.1 and 1.2 ± 0.1 $g.m^2$ respectively). However, the removal efficiency of both single-stage wetlands was comparable.

## INTRODUCTION

India, the second most populated country in the world, has a huge water demand, mainly for drinking, agricultural and industrial uses (Goel 2006). In Class I and II cities of India, an estimated 72368 million liters per day (MLD) of sewage is generated, but the sewage treatment capacity is only available for 12197 MLD. Similarly, just 60% of industrial wastewater is treated, especially in large-scale enterprises (CPCB 2021). Also, most treated water doesn't meet the slandered for discharge, especially pathogen concentration (Kaur et al. 2012, Schellenberg et al. 2020).

Under the new government scheme of India, "Swachh Bharat Abhiyan," each household will have a toilet in all the country's rural areas (Jain et al. 2020). As these villages are situated far from the city, and the population density is low compared to cities, it will not be possible to provide and sewerage network (Chandana & Rao 2021, Yadav et al. 2018, Axelrood et al. 1996, Talekar et al. 2018, Massoud et al. 2009, Morvannou et al. 2015, Badejo et al. 2018, Singh et al. 2019). Without a suitable decentralized wastewater treatment system, sewage will end up in local rivers or fields, adversely affecting the environment.

Vertical flow-constructed wetlands (VFCW) are a well-established technology for the nutrient treatment and other contaminant removals. However, the system's pathogen removal efficacy is still being studied (Dzakpasu et al. 2015, Pang et al. 2015). The effectiveness of constructed wetlands (CW) for the treatment of microbiological pollutants depends on various physical (sedimentation, retention time of the system, temperature and pH of water), chemical (UV, oxidation, biocides), and microbiological parameters (antibiosis, predation, lytic bacteria or viruses) (Díaz et al. 2010, Agrawal 1999). One of the factors, which play an important role in the removal of pathogenic bacteria from CW, is the retention time. In Horizontal flow and other types of sub-surface or surface flow wetlands, the residence time is usually long to get optimal output. Still, in the case of VFCW, the residence time is very short, which does not provide sufficient time for the natural die-off and removal of pathogens by other mechanisms (Franceys et al. 1992, Arias et al. 2003). The sedimentation process also plays a vital role in treating various pathogens (Gersberg et al. 1989). Several studies have found that *Salmonella*, protozoans, and virus tend to stay in sediment (Gerba & McLeod 1976, Hendrick 1971, Van Donsel & Geldreich 1971). Viruses get adsorbed on larger particles and settle at the bottom (Gersberg et al. 1987).

VFCWs' chances of sedimentation are very low due to low retention time; this can be improved by designing saturated vertical flow constructed wetlands, which will help increase residence time. This study aimed to understand the application of VFCW for pathogen treatment and the removal efficiency of different types of VFCW.

## MATERIALS AND METHODS

### Study Site and Wetland Characterisation

Three VFCWs were chosen for the study, located in different parts of the BITS Pilani KK Birla Goa Campus, Goa, India. (a) Two-stage vertical flow constructed wetland (TSVFCW), situated in the STP (sewage treatment plant) of campus, is fed with raw domestic sewage from campus. (b) Single-stage vertical flow constructed wetland (SSVFCW), located near one of the hostels. The system is fed with black water from the output of two chambered septic tanks connected to hostel toilets. (c) Single-stage saturated vertical flow wetland (SSSVFCW) is fed black water from single-chambered septic tank wastewater. Fig. 1 shows the schematics of the wetlands, and table 1 shows the characteristics of VFCWs.

### Design and Working

The beds of all three wetlands (TSVFCW, SSVFCW, and SSSVFCW) were constructed using laterite stones, plastered and leak-proof. 40 mm pipes passively aerated beds at the bottoms. For TSVFCW, the surface area of 1st bed was four $m^2$ and 2nd bed had a surface area of 1.9 $m^2$. The gravel size and depth of different gravel layers are given in Table 2. The hydraulic loading rate (HRL) for the 1st stage is 0.1 $m.day^{-1}$. Wastewater from the storage tank (300 $m^3$ tank, where the water from campus is stored and aerated continuously for further large-scale treatment SBR) is fed to the 1st stage, and from 1st stage, it percolates down through gravity to the 2nd storage tank; from this, it is provided to the 2nd stage. The SSVFCW has a surface area of 40 $m^2$. The HLR of the wetland was 0.036 $m.day^{-1}$ with no HRT. The black water from the hostel toilets was collected into two chambered septic tanks. From the tank, it was transferred to the wetland with the help of a submersible pump twice a day. The SSSVFCW is a single-stage saturated VFCW of 3 $m^2$ surface area. The hydraulic loading rate for the wetland was 0.067 $m^2.day^{-1}$. The wetland was fed wastewater from a single-chambered septic tank directly connected to the toilet. So, whenever the toilet is flushed, the wastewater from the septic tank flows down to the wetland through gravity. No pumping system was used for this wetland. The system has a hydraulic retention time of 24 h to develop a partial anaerobic condition at the bottom of the system.

### Water Sample Collection and Analysis of Abiotic and Biotic Parameters

The samples were collected from the inlet and outlet of SSVFCW and SSSVFCW. In the case of the TSVFCW wetland, it was collected from the inlet of the 1st stage, outlet 1st stage, and 2nd stage. Five liters of the sample were collected from each collection point and analyzed for abiotic

Table 1: Design characteristics of all three types of wetlands.

| Wetland Name | TSVFW | SSVFW | SSSVFW |
|---|---|---|---|
| Wetland Type | Two-stage vertical flow | Single-stage vertical flow | Single-stage saturated vertical flow |
| Age (Functional years) | 4 | 3 | 3 |
| No. of stages | 2 | 1 | 1 |
| Depth [m] | 1 | 1 | 1 |
| Saturation level [cm] | 0 | 0 | 7 |
| HRT [day] | - | - | 1 |
| Septic Tank Volume | Aerated tank of 300 $m^3$ | 6 $m^3$ | 1.5 $m^3$ |
| Vegetation type | *Canna indica* | *Canna indica* | *Canna indica* |
| Area [$m^2$] | 6 | 40 | 3 |
| Average inflow [$l.day^{-1}$] | 600 | 1500 | 200 |
| HLR [$m.day^{-1}$] | 0.1 | 0.036 | 0.067 |
| No. of batches/day | 3/day | 2/day | It depends on the no. of time toilet used |

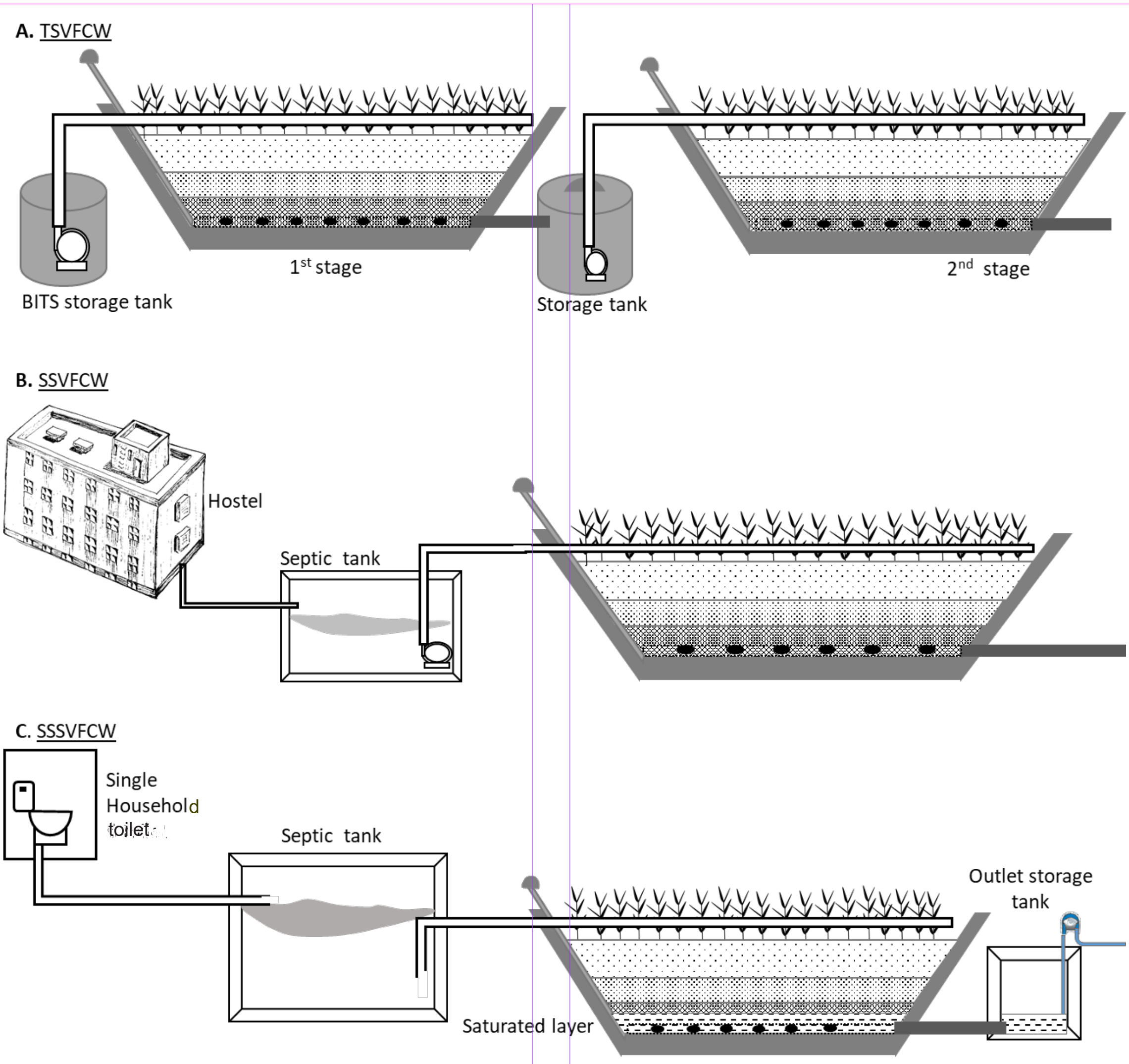


Fig. 1: Design of different wetlands (A) Two-stage vertical flow constructed wetland (TSVFCW), (B) Single-stage vertical flow constructed wetland (SSVFCW), and (C) Single-stage saturated vertical flow constructed wetland (SSSVFCW).

Table 2: Gravel size and depth of layers of all three types of wetlands.

| Layers (bottom to top) | TSVFCW | | SSVFW | SSSVFW |
|---|---|---|---|---|
| | 1st Stage | 2nd stage | | |
| 20 cm | Gravel 20-30 mm | Gravel 10-20 mm | Gravel 10-20 mm | Gravel 10-20 mm |
| 20 cm | Gravel 10-20 mm | Gravel 2-8 mm | Gravel 2-8 mm | Gravel 2-8 mm |
| 40 cm | Gravel 2-8 mm | Gravel 0-2 mm | Gravel 0-2 mm | Gravel 0-2 mm |

and biotic factors (No precipitation effect). The pathogenic bacteria from wastewater were quantified using specific (selective and differential) media. All the media plates were prepared per the manufacturer's instructions on the media bottle (HiMedia). A list of bacteria quantified (referred to as biotic factors), along with the media used and colony characters, are listed in Table 3.

Wastewater samples were serially diluted in bacteriological saline and spread-plated (0.1 mL) in replicates on respective media plates. Plates were incubated at 28 ± 2ºC (Nutrient Agar plates) and 37 ± 2ºC (Specific media plates) for 24-48 h till the colonies were observed. After incubation, colonies were counted, and numbers were represented as CFU (Colony Forming Unit).

Parameters like pH, Chemical Oxygen Demand (COD), Total Kjeldahl Nitrogen (TKN), Ammonical Nitrogen ($NH_4$-N), Phosphorus (P) (APHA 2005), and Nitrates ($NO_3^-$), Nitrites ($NO_2^-$) (Merk analysis Kit) were considered as abiotic factors and analyzed for all the samples, pH was measured by using Oakton pH meter.

The system's size and hydraulic loading rate are different for each, making it difficult to compare all three types of wetlands. So, to make the basis equal for comparison, load reduction per meter square of each wetland was calculated for biotic and abiotic parameters.

$$\text{Load removal rate (CFU/m}^2\text{ d)} = \left(\frac{Q(Ci - Co)}{A}\right) \times 10$$

Where Q is the inflow rate ($l.d^{-1}$), Ci and Co are is the concentration in the influent and effluent, respectively ($CFU.100mL^{-1}$), and A is the area of the bed ($m^2$).

### Statistical Analysis

All data were checked for normality and homogeneity of variances. ANOVA was performed to check the variation between the abiotic and biotic parameters with respect to wetlands. Further, Pearson correlation and Principal Component analysis (PCA) were performed to assess the relationship between the abiotic and biotic parameters. All the statistical analysis was performed using SPSS software. PCA analysis was done using Primer software.

## RESULTS

### Removal Efficiency Of Different VFCW

**Two-stage vertical flow constructed wetland (TSVFCW):** TSVFCW showed significant pathogen removal efficiency. The values of biotic factors reduction are given in Table 4 (both logarithmic units and load reduction per meter square). The total coliform number significantly reduced from $1.42 \times 10^7$ $CFU.100mL^{-1}$ to $1.65 \times 10^6$ $CFU.100mL^{-1}$ after the second stage with a 0.76 $log_{10}$ reduction (Table 4). TSVFCW showed a 2.04 $log_{10}$ reduction of *Salmonella* spp. The number of *Salmonella* spp. in inlet water was $2.3 \times 10^4$ $CFU.100mL^{-1}$, which was reduced to $7 \times 10^3$ $CFU.100mL^{-1}$ in outlet water. *Shigella* spp. number reduced from $3.7 \times 10^6$ $CFU.100mL^{-1}$ (Inlet water) to $3 \times 10^5$ $CFU.100mL^{-1}$ (outlet water), with 1.22 $log_{10}$ reduction. Pseudomonas's inlet water concentration was $9.9 \times 10^7$ $CFU.100mL^{-1}$, which was reduced to $8.8 \times 10^7$ $CFU.100mL^{-1}$ after the first stage and further reduced to $5.2 \times 10^7$ $CFU.100mL^{-1}$ in outlet water resulting in 0.31 $log_{10}$ reduction. *Enterococcus* spp. number reduced from $1.06 \times 10^6$ $CFU.100mL^{-1}$ to $3.08 \times 10^5$ $CFU.100mL^{-1}$ from inlet to outlet water, with 0.44 $log_{10}$ reduction. TSVFCW showed a 3.52 $log_{10}$ reduction for *Vibrio* spp. The number of *Vibrio* spp. reduced from $1.37 \times 10^6$ $CFU.100mL^{-1}$ to $1.65 \times 10^4$ $CFU.100mL^{-1}$ from inlet to outlet water (Table 4).

TSVFCW efficiently reduced COD from 877 $mg.L^{-1}$ to 600 $mg.L^{-1}$ after the first stage and further decreased to 427 $mg.L^{-1}$ with 45 $g.m^{-2}$ average load reduction in outlet water. Nitrogen removal in TSVFCW was found to be significantly efficient in terms of TKN and $NH_4$-N reduction. TKN values reduced from 394 $mg.L^{-1}$ to 217 $mg.L^{-1}$ after the first stage and further reduced to 147 $mg.L^{-1}$ (24.7 $g.m^{-2}$ average load reduction) in outlet water. $NH_4$-N concentration significantly decreased from 59 $mg.L^{-1}$ to 34 $mg.L^{-1}$ after the first stage and further reduced to 17 $mg.L^{-1}$ after the second stage. Thus, resulting in a 4.2 $g.m^{-2}$ average load reduction after the second stage. $NO_3^-$ as well as $NO_2^-$concentration got increased in outlet water than in inlet water. The average load reduction of phosphorous of TSVFCW was 3.1 $g.m^{-2}$. The pH of the inlet water was acidic (6), which was neutralized (7) after the first stage and became acidic (6) again after passing from the second stage (Table 5 and Fig. 3).

**Single-stage vertical flow constructed wetland (SSVFCW):** Pathogen removal was observed in SSVFCW. It showed a 2.90 $log_{10}$ reduction of total coliform from inlet water ($2.5 \times 10^5$ $CFU.100mL^{-1}$) to outlet water ($5.37 \times 10^4$

Table 3: Identification of different bacteria based on characteristics of colony formed on specific media.

| | Bacteria | Media Used (HiMedia) | Characteristics of the colony selected |
|---|---|---|---|
| 1. | Total Coliforms (TC) | MacConkey's Agar | Pink-red with bile precipitate |
| 2. | *Pseudomonas* spp. (Pseu) | *Pseudomonas* agar | All the colonies were counted |
| 3. | *Salmonella* spp. (Sal) | SS Agar | Colorless with black centre |
| 4 | *Shigella* spp. (Shi) | SS Agar | Colorless |
| 5. | *Vibrio* spp. (VS) | Thiosulphate-Citrate-Bile Salts (TCBS) | Yellow and bluish green |
| 6. | *Enterococcus faecalis* (EF) | *Enterococcus* Agar | Pink to dark red |

CFU.100mL$^{-1}$). *Salmonella* spp. numbers reduced to 2.5x10$^{2}$ CFU.100mL$^{-1}$ from initial count 1.57x10$^{4}$ CFU.100mL$^{-1}$ with 2.39 log$_{10}$ reduction. *Shigella* spp. count of inlet water was 1.83x10$^{5}$ CFU.100mL$^{-1}$ which reduced to 3.15x10$^{4}$ CFU.100mL$^{-1}$ in outlet water with 2.86 log$_{10}$ reduction. *Pseudomonas* spp. cell numbers reduced from 1.05x10$^{7}$ CFU.100mL$^{-1}$ to 1.12x10$^{6}$ CFU.100mL$^{-1}$ after treatment showing 1.38 log$_{10}$ reduction. SSVFW showed a 4.81 log$_{10}$ reduction of *Enterococcus* spp. Total *Vibrio* spp. It was reduced by 2.22 log$_{10}$ from the inlet (1.7x10$^{4}$ CFU.100mL$^{-1}$) to outlet water (5.2x10$^{3}$ CFU.100mL$^{-1}$) (Table 4).

SSVFCW reduced COD with 12 g.m$^{-2}$ average load reduction from inlet water (470 mg.L$^{-1}$) to outlet water (198 mg.L$^{-1}$). SSVFCW showed a reduction in TKN and $NH_4$-N concentration, whereas $NO_3^-$ and $NO_2^-$ increased. TKN of the inlet was 448 mg.L$^{-1}$ which was reduced to 222 mg.L$^{-1}$ in outlet water (7.6 g.m$^{-2}$ average load reduction). $NH_4$-N was decreased to 50 mg.L$^{-1}$ from 174 mg.L$^{-1}$ showed 4.7 g.m$^{-2}$ average load reduction. However, $NO_3^-$ and $NO_2^-$ values increased from 2 mg.L$^{-1}$ and 0.07 mg.L$^{-1}$ to 22 mg.L$^{-1}$ and 2.59 mg.L$^{-1}$ after treatment by SSVFCW. The average load reduction of phosphorous was 12 g.m$^{-2}$ from inlet water (65 mg.L$^{-1}$) to outlet water (17mg.L$^{-1}$). The pH of the inlet was neutral (7), which became acidic (5) after treatment (Table 5 and Fig. 3).

**Single-stage saturated vertical flow constructed wetland (SSSVFCW):** Similar to other wetlands, SSSVFCW also showed effective removal of pathogenic bacteria. Total coliform showed a reduction in numbers by 0.43 log$_{10}$ decrease from the inlet (9.07x10$^{4}$ CFU.100mL$^{-1}$) to outlet water (4.35x10$^{4}$ CFU.100mL$^{-1}$).

*Salmonella* spp. cell numbers reduced from 1.11 × 10$^{3}$ CFU.100mL$^{-1}$ to 2.50 × 10$^{2}$ CFU.100mL$^{-1}$ after treatment showing 1.45 log$_{10}$ reduction. SSSVFCW showed a 1.31 log$_{10}$ reduction in *Shigella* spp. from the inlet (7.5 × 10$^{4}$ CFU.100mL$^{-1}$) to outlet water (4 × 10$^{3}$ CFU.100mL$^{-1}$). *Pseudomonas* spp. number reduced to 7.25 × 10$^{5}$ CFU.100mL$^{-1}$ in outlet water from 5.8 × 10$^{6}$ CFU.100mL$^{-1}$ resulting in 0.95 log$_{10}$ reduction. The removal efficiency for *Enterococcus* spp. was 5.03 log$_{10}$. The CFU.100mL$^{-1}$ reduced from 9.09 × 10$^{5}$ to 0 number in the outlet leading to the complete removal. *Vibrio* spp. also showed a 2.55 log$_{10}$ reduction from the inlet (3.65 × 10$^{4}$ CFU.100mL$^{-1}$) to the outlet (1.75x10$^{3}$ CFU.100mL$^{-1}$) after treatment (Table 4).

SSSVFCW reduced the concentration of COD by 6.3 g.m$^{-2}$ from the inlet (190 mg.L$^{-1}$) to outlet water (108 mg.L$^{-1}$). Nitrogen removal in SSSVFCW was also observed, wherein TKN was significantly removed by 7.7 g.m$^{-2}$ average load reduction. $NH_4$-N concentration reduced to 22 mg.L$^{-1}$ from 86 mg.L$^{-1}$ with 4.5 g.m$^{-2}$ average load reduction. However, $NO_3^-$ and $NO_2^-$ concentrations increased after treatment by SSSVFCW as observed in other wetlands, $NO_3^-$ and $NO_2^-$ concentrations increased to 45 mg.L$^{-1}$ and 0.37 mg.L$^{-1}$ from 4 mg.L$^{-1}$ and 0.02, respectively. Phosphorous was removed efficiently with 1.2 g.m$^{-2}$ average load reduction from the inlet (25 mg.L$^{-1}$) and outlet (7 mg.L$^{-1}$). The inlet water had neutral (7) pH that became acidic (6) after treatment by wetland (Table 5 and Fig. 3).

**Removal efficiency of biotic and abiotic parameters by different VFCWs:** All wetlands removed the abiotic and biotic factors with varying degrees. In terms of pathogenic bacterial removal, all three wetlands showed pathogen reduction, but SSVFCW was found to be more efficient in removing all the pathogens studied (Table 5).

Among the three types of wetlands, TSVFCW was found to be efficient in removing COD, TKN, and phosphorous with 45, 24.7, and 3.1 g.m$^{-2}$ average load reduction, respectively. $NH_4$-N (4.7 g.m$^{-2}$) was removed efficiently by SSVFCW compared to the TSVFCW and SSSVFCW. The pH of the outlet water of TSVFCW and SSSVFCW was 6, and of SSVFCW, it was 5.

TSVFCW was found to be efficient in the removal abiotic (Table 5) as compared to the single stage wetlands but between the single stage wetland the removal efficiency of SSVFCW was higher than SSSVFCW.

The correlation of different water quality parameters showed a positive correlation, indicating that these parameters favor the growth of pathogenic bacteria. However, from the correlation matrix of the water quality parameters, it is evident that there is a reduction in abiotic and biotic parameters. PCA analyses revealed a significant inverse relationship between all the parameters and $NO_3^-$ and $NO_2^-$ (Fig. 4). There is a significant reduction of all the abiotic and biotic parameters however increase in $NO_3^-$ and $NO_2^-$ in all the wetlands (Table 5). All the pathogenic bacteria showed a significant positive correlation with pH, TKN, $NH_4$-N, and COD.

## Areal Removal Rates of Biotic Factors

TSVFCW had the greatest areal removal rate for all pathogens compared to other wetlands. When the first and second stages of TSVFCW are compared, the second stage (TC, Shi, Sal, Pseu, and Vibrio species: -10.1, 7.2, 9.5, 10.7, 8.9 and 9.1Log$_{10}$CFU.m$^{-2}$.d$^{-1}$ reduction respectively) has a higher removal efficiency than the first (TC, Shi, Sal, Pseu, and Vibrio specie: -10.0, 7.0, 9.4, 10.0, 7.5, and 9.0Log$_{10}$ CFU.m$^{-2}$.d$^{-1}$ reduction respectively) (Fig. 2). Table 4 shows that SSVFCW wetland has higher removal efficacy for TC, Shi, Sal and Pseu, species than SSSVFCW. SSVFCW

Table 4: Pathogen concentration at inlet and outlet and removal rates for different wetlands.

| Wetland Type | | Total Coliform (TC) | *Shigella* spp. (Shi) | *Salmonella* spp.(Sal) | *Pseudomonas* spp. (Pseu) | *Vibrio* spp. (VS) | *Enterococcus faecalis* (EF) |
|---|---|---|---|---|---|---|---|
| TSVFCW (HLR 100 $l.m^{-2}d^{-1}$) | Influent concentration [$CFU.100mL^{-1}$] | $1.4 \times 10^7 \pm 1.2 \times 10^6$ | $3.7 \times 10^6 \pm 8.8 \times 10^4$ | $2.3 \times 10^4 \pm 4.7 \times 10^3$ | $9.9 \times 10^7 \pm 8.0 \times 10^6$ | $1.4 \times 10^6 \pm 3.1$ x$10^5$ | $1.1 \times 10^6 \pm 2.6$ x$10^5$ |
| | Effluent concentration [$CFU.100mL^{-1}$] | $2.4 \times 10^6 \pm 1.9 \times 10^5$ | $3.0 \times 10^5 \pm 5.0 \times 10^4$ | $7.0 \times 10^3 \pm 1.7 \times 10^3$ | $5.2 \times 10^7 \pm 5.9 \times 10^6$ | $1.7 \times 10^4 \pm 4.1$ x$10^3$ | $3.1 \times 10^5 \pm 7.3$ x$10^4$ |
| | $Log_{10}$ reduction [$CFU.100mL^{-1}$] | $0.76 \pm 0.02$ | $1.22 \pm 0.39$ | $2.04 \pm 1.56$ | $0.31 \pm 0.07$ | $3.52 \pm 2.89$ | $0.44 \pm 0.11$ |
| | Areal load removal rate [$CFU.m^2.d^{-1}$] | $1.18 \times 10^{10} \pm 5.41 \times 10^8$ | $3.40 \times 10^9 \pm 6.88 \times 10^7$ | $1.60 \times 10^7 \pm 3.0 \times 10^6$ | $4.70 \times 10^{10} \pm 1.06 \times 10^9$ | $1.35 \times 10^9 \pm 1.56$ x$10^8$ | $7.59 \times 10^8 \pm 9.26$ x$10^7$ |
| SSVFCW [HLR 37.5 $l. m^2.d^{-1}$] | Influent concentration [$CFU.100mL^{-1}$] | $2.60 \times 10^5 \pm 6.38 \times 10^3$ | $1.84 \times 10^5 \pm 6.88 \times 10^2$ | $1.58 \times 10^4 \pm 3.69 \times 10^3$ | $1.06 \times 10^7 \pm 2.13 \times 10^5$ | $1.70 \times 10^4 \pm 5.00$ x$10^2$ | $3.27 \times 10^5 \pm 8.07$ x$10^4$ |
| | Effluent concentration [$CFU.100mL^{-1}$] | $5.38 \times 10^4 \pm 1.34 \times 10^4$ | $3.15 \times 10^4 \pm 7.87 \times 10^3$ | $2.51 \times 10^2 \pm 6.24 \times 10^1$ | $1.12 \times 10^6 \pm 2.58 \times 10^5$ | $5.25 \times 10^3 \pm 1.31$ x$10^3$ | 0.00 |
| | $Log_{10}$ reduction [$CFU.100mL^{-1}$] | $2.90 \pm 2.56$ | $2.86 \pm 2.39$ | $2.39 \pm 0.61$ | $1.38 \pm 0.65$ | $2.22 \pm 2.06$ | $4.81 \pm 1.00$ |
| | Areal load removal rate [$CFU.m^2.d^{-1}$] | $7.72 \times 10^7 \pm 3.71 \times 10^6$ | $5.71 \times 10^7 \pm 1.35 \times 10^6$ | $5.81 \times 10^6 \pm 6.80 \times 10^5$ | $3.54 \times 10^9 \pm 8.44 \times 10^6$ | $4.41 \times 10^6 \pm 3.40$ x$10^5$ | $1.23 \times 10^8 \pm 1.51$ x$10^7$ |
| SSSVFCW [HLR 66.7 $l. m^2.d^{-1}$] | Influent concentration [$CFU.100mL^{-1}$] | $9.08 \times 10^4 \pm 2.31 \times 10^3$ | $7.50 \times 10^4 \pm 6.25 \times 10^3$ | $1.11 \times 10^3 \pm 2.25 \times 10^2$ | $5.80 \times 10^6 \pm 1.50 \times 10^5$ | $3.65 \times 10^4 \pm 8.31$ x$10^3$ | $9.09 \times 10^5 \pm 2.23$ x$10^5$ |
| | Effluent concentration [$CFU.100mL^{-1}$] | $4.35 \times 10^4 \pm 6.88 \times 10^3$ | $4.00 \times 10^3 \pm 5.00 \times 10^2$ | $2.50 \times 10^2 \pm 6.25 \times 10^1$ | $7.25 \times 10^5 \pm 8.13 \times 10^4$ | $1.75 \times 10^3 \pm 4.38$ x$10^2$ | 0.00 |
| | $Log_{10}$ reduction [$CFU.100mL^{-1}$] | $0.43 \pm 0.37$ | $1.31 \pm 0.09$ | $1.45 \pm 0.85$ | $0.95 \pm 0.16$ | $2.55 \pm 1.27$ | $5.03 \pm 1.22$ |
| | Areal load removal rate [$CFU.m^2.d^{-1}$] | $3.15 \times 10^7 \pm 3.06 \times 10^6$ | $4.74 \times 10^7 \pm 1.92 \times 10^6$ | $5.67 \times 10^5 \pm 1.08 \times 10^4$ | $3.39 \times 10^9 \pm 2.29 \times 10^7$ | $2.32 \times 10^7 \pm 4.71$ x$10^6$ | $6.06$x $10^8 \pm 1.49$ x$10^8$ |

outperforms SSVFCW in terms of Vibrio removal efficiency. Both wetland exhibit 100 percent EF removal with 0.0 CFU.100mL$^{-1}$ concentration in the outlet, indicating that both types of wetlands might have similar EF removal efficiency as the difference in removal efficiency is dependent on the inlet concentration in this case (Fig. 2).

## DISCUSSION

The present study revealed the importance of constructed wetlands in reducing pathogens in wastewater. However, the type of pathogen removal is dependent on the constructed wetland used for the treatment (Weber & Legge 2008, Wu et al. 2016) also demonstrated that *Salmonella* spp. is bacteria of great concern as well as a good representative of the reduction of other bacterial pathogens because they are typically present in higher densities than other bacterial pathogens and can survive for a long time in the environment. Most of the pathogenic bacteria are significantly reduced by

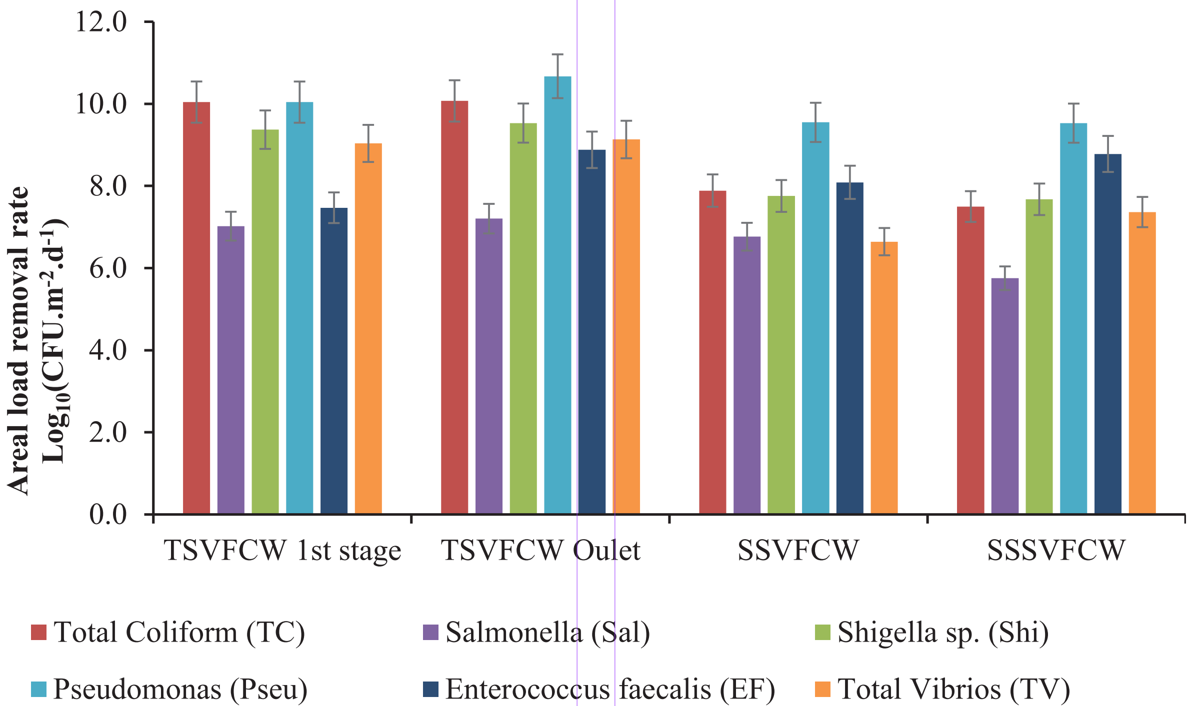


Fig. 2: Areal load reduction of Biotic parameters

Table 5: Concentration of abiotic parameters in all three types of wetlands.

| | COD [ppm] | TKN [ppm] | Nitrate [ppm] | Nitrite [ppm] | $NH_3$-N [ppm] | $PO_4$ [ppm] | pH |
|---|---|---|---|---|---|---|---|
| TSVFW | | | | | | | |
| Inlet | 877 ± 18 | 394 ± 66 | 1.23 ± 0.61 | 0.17 ± 0 | 59 ± 10 | 61 ± 3 | 6 ± 0.05 |
| 1st stage | 600 ± 64 | 217 ± 35 | 1.05 ± 0.53 | 0.17 ± 0.02 | 34 ± 7 | 42 ± 2 | 7 ± 0.11 |
| 2nd stage | 427 ± 68 | 147 ± 21 | 14 ± 0.34 | 2 ± 0.45 | 17 ± 3 | 31 ± 5 | 6 ± 0.08 |
| SSVFW | | | | | | | |
| Inlet | 470 ± 8 | 448 ± 7 | 2 ± 0.95 | 0.07 ± 0.01 | 174 ± 7 | 65 ± 0.37 | 7 ± 0.02 |
| outlet | 198 ± 20 | 222 ± 1 | 22 ± 3 | 3 ± 0.25 | 50 ± 4 | 17 ± 4 | 5 ± 0.7 |
| SSSVFW | | | | | | | |
| Inlet | 190 ± 12 | 273 ± 4 | 4 ± 2 | 0.07 ± 0.02 | 86 ± 13 | 25 ± 3 | 7 ± 0.07 |
| outlet | 108 ± 33 | 152 ± 3 | 45 ± 4 | 1.27 ± 0.37 | 22 ± 4 | 7 ± 1 | 6 ± 0.09 |

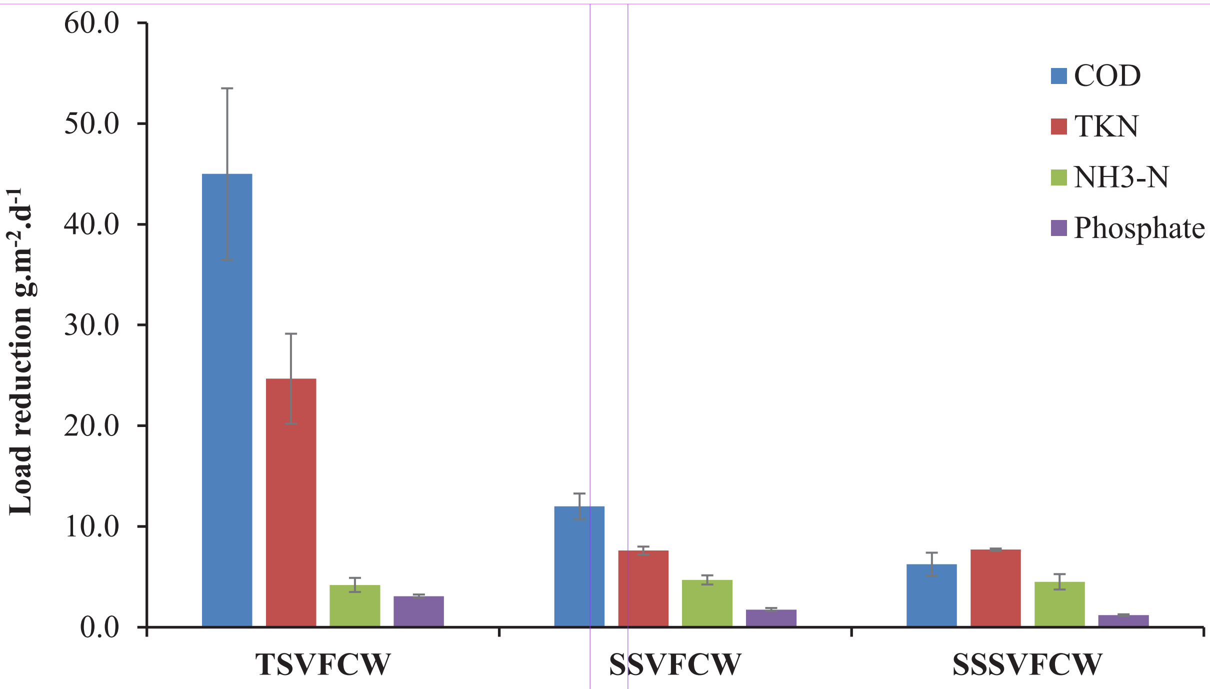


Fig. 3: Areal load reduction of abiotic parameters.

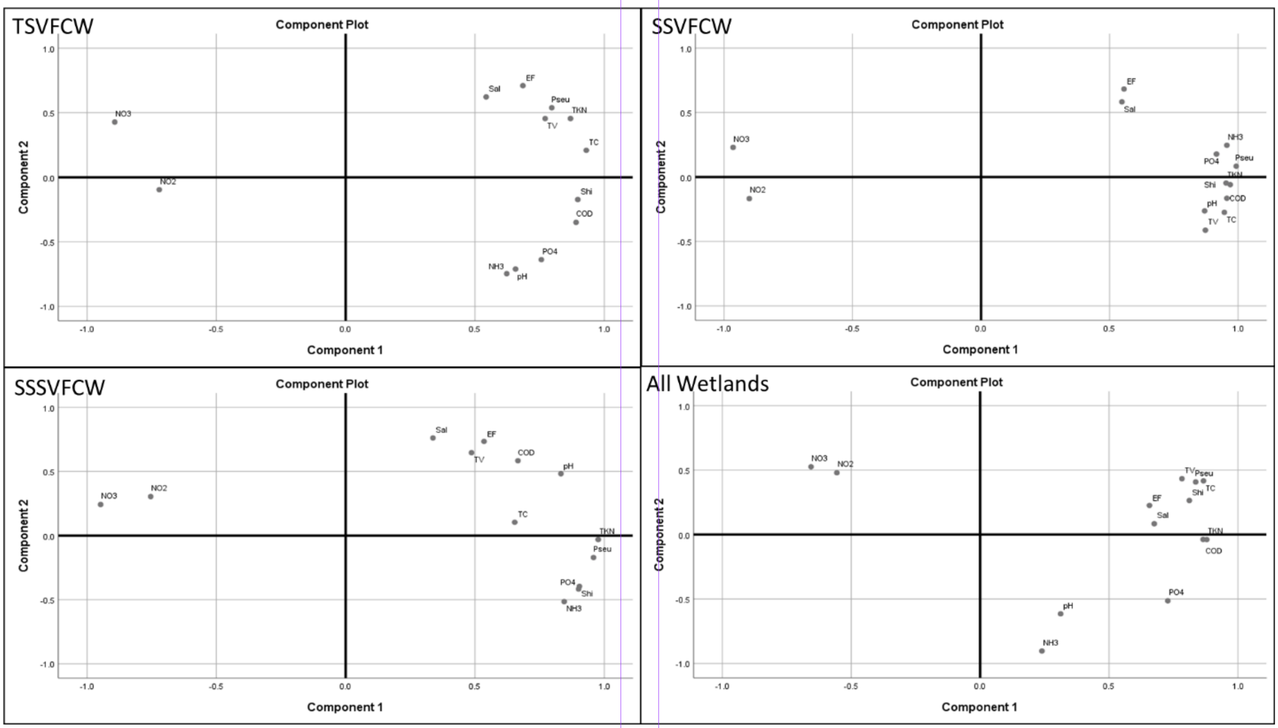


Fig. 4: Principal component analysis for all the TSVFCW, SSVFCW, SSSVFCW, and All wetlands together.

SSVFCW (Total Coliforms, *Shigella* spp., *Salmonella* spp., *Pseudomonas* spp. (2.90 ± 2.56, 2.86 ± 2.39, 2.39 ± 0.61, 1.38 ± 0.65, $Log_{10}$ reductions respectively) thus suggesting its highest removal efficiency from wastewater compared to other two wetlands TSVFCW (0.76 ± 0.02, 1.22 ± 0.39, 2.04 ± 1.56, 0.31 ± 0.07, and $Log_{10}$ reductions respectively) and SSSVFCW (0.43 ± 0.37, 1.31 ± 0.09, 1.45 ± 0.85, 0.95 ± 0.16, , $Log_{10}$ reductions respectively). Although SSSVFCW found to be efficient in the *Enterococcus faecalis* removal than SSVFCW and TSVFCW (5.03 ± 1.22, 4.81 ± 1.00, 0.44 ± 0.11 $Log_{10}$ reduction respectively). Removal efficiency of *Vibrio* spp. was higher for TSVFCW than SSVFCW and SSSVFCW (3.52 ± 2.89, 2.22 ± 2.06 and 2.55 ± 1.27 $Log_{10}$ reduction respectively).

Pathogen removal in VFCW depends on many physical, chemical, and biological processes like filtration, adsorption, sedimentation, residence time, oxidation, predation activity, and biolytic processes. But in the case of TSVFCW and SSVFCW, the residence time is very low, which will not provide sufficient time for sedimentation and other chemical or biological processes (Alexandros & Akratos 2016). According to Arias et al. (2003), the first stage of TSVFCW wetland was more efficient in the removal of fecal coliform (0.8-1.7$Log_{10}$ unit) than that of 2nd stage (0.5-1.1$Log_{10}$ unit) and suggested that the removal efficacy of the system depends on inlet concentration rather than hydraulic loading, in the present study single stage wetland found to be efficient in the pathogen removal than two stage. The result shows that TSVFCW has a higher inlet concentration of pathogens than single-stage wetlands (Table 4), even though the areal removal rate of TSVFCW is higher the overall log reduction of pathogen is lower. The current study also observed that both stages of TSVFCW have the same loading rate. Even though receiving a lower inlet concentration of pathogens for 2nd stage (as it receives water after the 1st stage treatment), it still shows an equivalent removal efficiency (Fig 2). This suggests the removal efficiency also depends on the size (0-2 mm sand layer on the top for 2nd stage) of the media used in the bed (Arias et al. 2003, Pundsack et al. 2001, Wang et al. 2021). Pundsack et al. (2001) were able to reduce *Salmonella* in the order of 1-9$Log_{10}$ from winter to summer using different types of sand and peat filters, which is also comparable to the result obtained in the current study (*Salmonella*: 1.45-2.44 $Log_{10}$ reduction). So, it can be concluded that the pathogen removal in VFCWs is mainly filtration through the media or adsorption of the pathogen to the surface of the media. After filtration or adsorption, the pathogens are neutralized by natural die-offs or other chemical and biological processes.

SSSVFCW also showed comparatively lower pathogen removal than other unsaturated wetlands (TSVFCW and SSVFCW), as unsaturated flow is likely to provide better removal conditions than saturated flow (Stevik et al. 2004). Higher the size and density, the higher the sedimentation rate. This allows the bacteria with a higher settling velocity, such as fecal coliforms, fecal *streptococci*, and helminths, to be removed more efficiently than other microorganisms (e.g., protozoa cysts) and viruses (Alexandros & Akratos 2016).

The overall organics (COD) removal efficiency of all three types of wetlands (TSVFCW, SSVFCW, and SSSVFCW are 45, 12, and 6.3 $g.m^{-2}.d^{-1}$ was found to be lower as compared to other reported studies by Abdelhakeem et al. (2016) which was around 60.8 $g.m^{-2}.d^{-1}$ in gravel and vermiculite media and in the study reported by Herouvim et al. (2011) which was around 217 $g.m^{-2}.d^{-1}$. Also, in the study on two experimental combined wetlands in tropical climates reported by Kantawanichkul et al. (2003), the average COD removal rate was 53.3 $g.m^{-2}.d^{-1}$ for the first system (combined). For the other two-stage system, it was 82.9 $g.m^{-2}. d^{-1}$. This may be due to the low batch frequency of the wetland (Kengne et al. 2019), which was just three times per day in the case of TSVFCW and SSVFCW. In the case of the SSSVFCW wetland, it was purging (mainly in the morning and very few times during the whole day) the water only when the toilet was in use, so per batch volume was also low. The hydraulic aspect of VFCW is significant; one important factor is the number of batches applied per day (batch frequency), which can influence the oxygen transfer rate (Molle 2014, Green et al. 1997, Abou-Elela et al. 2013). The high number of batches applied daily can reduce the volume per batch, favoring more organic removal (Kengne et al. 2019, Brix & Arias 2005).

French VFCW are designed for a higher oxygen transfer rate, which helps in the growth of nitrifying bacteria favoring the nitrification of organic nitrogen in the wastewater. In this study, the batch frequency per day was low (Table 1). So, the batch volume was significant, which favored a high oxygen transfer rate in the wetland, resulting in a high nitrification rate (Molle et al. 2006, Brix & Arias 2005), leading to high TKN and $NH_3$-N removal per $m^2$ of the area as compared to study Abdelhakeem et al. (2016) ($NH_3$-N:-1.1-2.1 $g.m^{-2}.d^{-1}$). The decrease in NO3- and NO2- after the first stage of TSVFCW indicates the development of anaerobic areas in the wetland, favoring the denitrification of available nitrates and nitrites (Table 5) (Sirivedhin & Gray 2006). The $NO_3^-$ and $NO_2^-$ concentrations increased in the outlet of SSVFCW, SSSVFCW, and after the second stage of TSVFCW, indicating better aerobic conditions in the wetland. This is also evident from PCA analysis. SSSVFCW was designed to provide an anaerobic condition at the bottom by adding a saturated layer to enhance denitrification. Since the hydraulic loading rate was very low and water was pumped out continuously throughout the day with a peristaltic pump shown in Fig. 4 (for post-wetland treatment, which is not included in the current study). It may develop a suction at the bottom of the wetland, resulting in more air entering and helping to make the wetland aerobic (Brix & Arias 2005).

## CONCLUSION

The study points out significant pathogenic bacterial removal efficiency by VFCWs. All three types of wetlands studied effectively removed pathogenic bacteria and abiotic parameters with varying degrees despite short residence time. The study concludes with a few key points below.

1. The pathogen removal efficiency of VFCW constructed depends on the filtration media and the size of the media used.
2. The removal efficiency of VFCW is not solely depends on the inlet pathogen concentration of wastewater.
3. The study also concluded that removal efficacy does not depends on the number of stages used to treat wastewater.
4. Unsaturated VFCW have higher removal efficacy than saturated VFCW

VFCW can be a very efficient technology for treating raw sewage in rural areas of India, as it does not require higher operating costs or any particular skill. Still, further treatment is required to ensure the safe reuse of wastewater for the complete inactivation of pathogens.

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## DECLARATION

The authors also confirm that the analyzed data supporting the findings of this study can be obtained from the corresponding author (SM) on request.

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