Monitoring efficacy of constructed wetland for treating domestic effluent – microbiological approach

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Water scarcity and elevated potential in wastewater treatment in the last decades raise attention towards constructed wetlands (CWs). The present study was carried out to evaluate the efficacy of CW for faecal coliform (FC) expulsion and to isolate and characterize the microbial communities. Significant differences were observed between influent and effluent microbial counts of vegetated and control cells (without vegetation) of wetland. FC reduction ranged from 64% to 81%; however, total bacterial, fungal and actinomycetes average poll ranged from 66.67×10^5 cfu/g to 142.67×10^5 cfu/g, 1.67×10^2 cfu/g to 10.33×10^2 cfu/g and 16.00×10^3 cfu/g to 53.33×10^3 cfu/g respectively, isolated from vegetated and control cells. Results further indicated that bacteria were most abundant, followed by actinomycetes, whereas the number of fungi was least among three groups of microbes, which could be attributed to wide tolerance to the properties of CW. Removal of FC was less apparent initially compared to the later stages of operation, which is of concern for long-term efficiency and stability of wetland. Also, diversity of identified bacterial strains is beneficial for growth and yield enhancement of agriculture crops. The results also demonstrate that CWs are the key habitats for bioactive actinomycetes with paramount medical, scientific and economic potential significance globally in general and developing countries like India in particular. Overall, backwash imparts the baseline compilation of CWs for its management for sustainable agriculture.

Keywords: Actinomycetes, bacteria, constructed wetland, faecal coliform, MPN.

Introduction

ENVIRONMENTALLY sustainable and cost-effective treatment system for the renovation of wastewater (industrial/domestic) by biological means is addressed through the use of constructed wetlands (CWs). They reduce total suspended solids (TSS), biochemical oxygen demand (BOD_5), total Kjeldahl nitrogen (TKN), and/or total phosphate[1,2]. Main factors influencing are BOD, pH, suspended solids, presence of toxic compounds and other compositions that decide selection of wastewater to be treated[3]. However, synergy among plants, soil, microorganisms, wastewater characteristics and operational conditions is important for the functioning of CW[4–6]. Although pollutant removal efficiency in CW is altered by macrophyte species, plant roots and rhizomes are the key factors affecting microbial transformation processes and subsequently wastewater treatment[7]. In general, the reduction of pollutants is due to various physical, chemical and biological processes encompassing sedimentation, filtration, absorption/adsorption, precipitation, microbial interactions, biofilm formation and bacteria/plant interactions[8,9]. As far the reduction of enteric microorganisms from domestic effluents is concerned, studies were limited to detection of faecal coliforms (FC) assays[10], and relatively less attention has been paid to the isolation and characterization of microorganisms (bacteria, fungi and actinomycetes, i.e. BFA) from CW. Therefore, the present study was conducted to evaluate the potential removal of pathogenic microorganisms (FC) along with diversity of microbial population developed to better understand the long-term efficiency and potential stability of wetlands.

Materials and methods

A constructed wetland is located at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) farm. It treats the domestic wastewater released by a community of approximately 1000 inhabitants. Influent wastewater pass through a primary treatment pond followed by 12 treatment cells – 6 sub surface (SS) and 6 free surface (FS) comprising 2 as controls (non-vegetated) as shown in Figure 1. CW contained gravel and sand as filter media in B and C cells with varied vegetation, viz. napier bajra (*Pennisetum purpureum*), lemongrass (*Cymbopogon citratus*), bamboo (*Bambuseae* spp.), cattails (*Typha* spp.), billy goat (*Ageratum conyzoides*), para-grass (*Brachiaria mutica*); also water hyacinth (*Eichhornia crassipes*) and water lettuce (*Pistia stratiotes*) as emergent macrophytes (Figure 1). Entire

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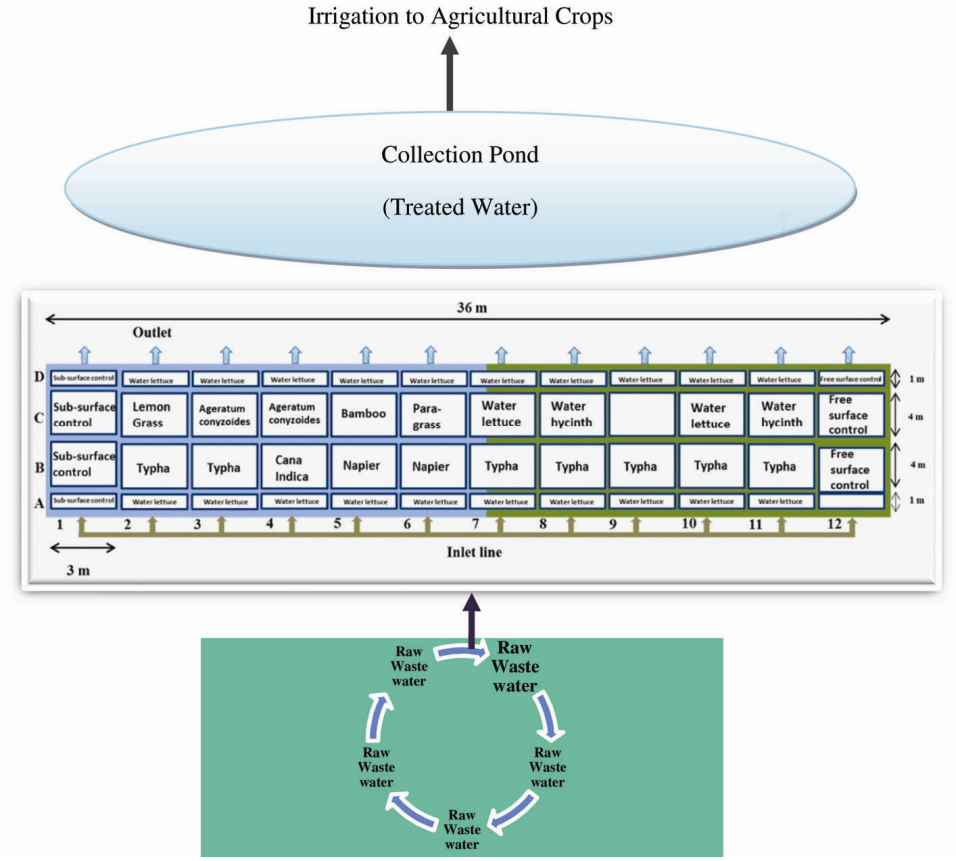

Figure 1. Schematic presentation of constructed wetlands showing wastewater treatment process.

system is operated beneath gravity feed and flow of water. Treated water is stored in collection pond and later used for irrigation of agricultural crops.

Sampling procedure and FC analysis

A total of 13 water samples were collected at the manifold sample ports, i.e. one inflow and twelve outflows of CW. Samples were subjected to the multiple tube fermentation technique using most probable number (MPN) analysis to determine the concentration of FC by adopting standard procedure of APHA[11]. The technique consists of three steps: presumptive, confirmed and completed. For presumptive test, diluted samples were inoculated into fermentation tubes of lactose broth following incubation at 35°C for 48 h. Samples recognized positive through gas production were then reassigned to fermentation tubes of brilliant green lactose bile broth and again incubated at 35°C for 48 h. Samples showing growth and gas were considered as positive for the confirmation test. Microorganisms from positive-confirmed tubes were isolated in pure culture on agar plates of differential/selective media (Eosin Methylene Blue Agar) and then tested for growth and gas production in fermentation tubes of lactose or lauryl tryptose broth incubated at 35°C for 48 h; and a negative reaction in the Gram stain. For a positive completed test, the organisms must show growth plus gas production in the fermentation tubes and be Gram negative.

Isolation of microorganisms

Samples collected from all the vegetated and control cells to study diversity of microbial assembly developed in CW were selectively isolated, enumerated and characterized. Microorganisms were isolated by serial dilution method[12]. Stock solution was prepared by diluting 1 ml of sample in 9 ml of sterile water and shaken well by using vortex mixer. From the stock solution, 1 ml was used to prepare the final volume of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}, finally, 0.1 ml of suspension was used to spread on nutrient agar (bacteria), potato dextrose agar (fungi) and actinomycetes isolation agar (actinomycetes) medium aseptically. For each sample three plates were used and incubated at 28 ± 2°C for 24–48 h in case of bacteria and 72–120 h for fungi and actinomycetes. The plates were observed periodically for the growth of microbes (BFA). The pure colonies developed on plates were selected, isolated and maintained in nutrient agar slants/PDA plates at 4°C for subsequent studies.

Characterization of isolates

Morphological characters of the isolates were studied. The macroscopic studies of microorganisms growing on agar medium were useful and for rapid identification of their respective genus, which includes characters such as colony characteristics (configuration, margin, elevation, surface, pigment, shape, colour and arrangement), absence or presence of aerial mycelium and extent of spore formation. *Bergey's Manual of Determinative Bacteriology* was followed for the structure resemblance and comparison for genus identification of purified isolates[13].

Results and discussion

Faecal coliform study

FC analysis was continuously carried out (from February 2015 to May 2015) after stabilization of CW. Quantitative estimation of microflora via the number of colonies (cfu/ml) of water sample showed a significant reduction of FC as water pursued the route from inlet to outlet. Figure 2 shows MPN index for all the wetland outlets. In the box-plot dotted line represents average of MPN index observed during 100 days (12 data points). The MPN index of inlet wastewater samples was always greater than 1600. The FC reductions varied from 64% to 81% in different treatment cells having varied macroflora and subsurface (SS) as well as free-surface (FS) control. Least FC reduction was observed in nonvegetated cells, i.e. control SS (64%) and FS (65.50%) followed by increase in cell 9 having typha alone (70.16%). However maximum coliform reduction was observed in cell 7 (81.30%) and 10 (81.76%) followed by cell 11 (80.88%) and cell 8 (78.08%), all having typha (cell B) in combination with water lettuce (cell 7 and 10) and water hyacinth (cell 8 and 11). Moreover, FC percentage reduction was higher in the cells having combination of typha with other vegetation (ranged from 77.7% to 81.8%) compared to those having other plant combinations, which varied from 74.0% to 75.7%. Results indicated that treatment cells having dual vegetation are more efficient in reduction of FC compared to nonvegetated (control) and univegetated cells. Although reduction in FC is attributed to various physical and biological processes occurring in CW, viz. sedimentation, aggregation, oxidation, filtration, solar irradiation, antibiosis, predation and competition along with diverse vegetation[14]; in this study, typha showed higher removal efficacy in conjunction with other vegetation/plant species. Greater removal of FC in extensively vegetated wetland systems was also studied compared with nonvegetated systems[15]. The use of CWs as a sole treatment process for water containing initially high levels of microorganisms may accomplish final effluent FC standard as required for discharge of treated domestic effluent for agricultural use.

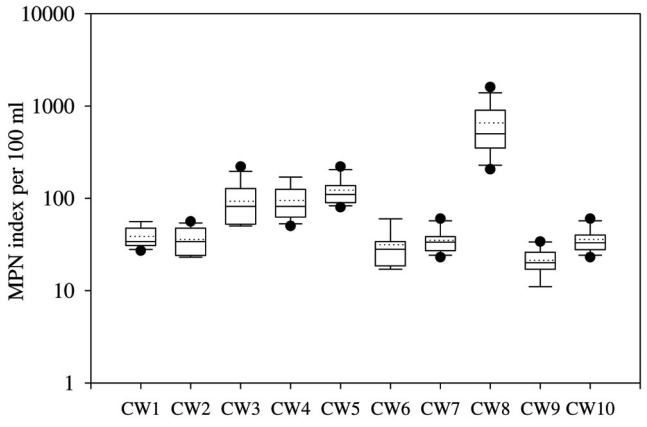

Figure 2. Box-and-whisker plot of most probable number index per 100 ml observed for outlets of 10 CWs. Dotted lines in box represent average value.

Microbial poll

Comparative analysis of bacteria, fungi and actinomycetes poll from rhizosphere sample was done for vegetated wetland (CW 2 to 11) and non-vegetated wetlands (CW 1 and 12). The results of samples collected from corresponding B and C cells of CW (Figure 3) showed that bacterial poll in cell B (78×10^5 cfu/g–134×10^5 cfu/g), cell C (74×10^5 cfu/g–137×10^5 cfu/g) overtopped in the

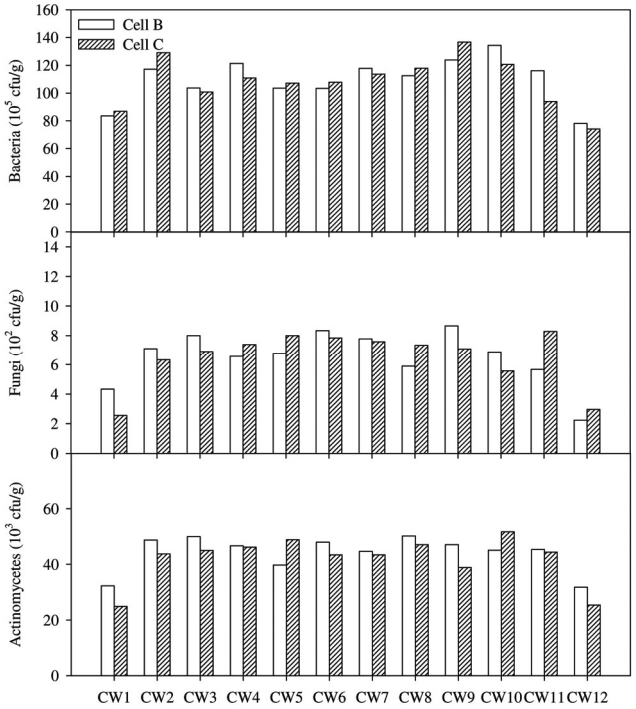

Figure 3. Enumeration of microbial population in the rhizosphere soil collected from cell B and C of all 12 CWs.

Table 1. Morphological characteristics of the selected isolates

Colony morphology	Isolates															
	MK_1	MK_2	MK_3	MK_4	MK_5	MK_6	MK_7	MK_8	MK_9	MK_{10}	MK_{11}	MK_{12}	MK_{13}	MK_{14}	MK_{15}	MK_{16}
Configuration	Round	Round	Round	Circular	Circular	Round	Round	Round	Circular	Round	Round	Circular	Round	Circular	Circular	Round
Edge/margin	Entire	Undulate	Entire	Entire	Entire	Lobate	Smooth	Undulate	Entire	Entire	Lobate	Entire	Entire	Undulate	Entire	Smooth
Elevation	Flat	Flat	Pulvinate	Convex	Umbonate	Convex	Flat	Raised	Flat	Convex	Flat	Raised	Pulvinate	Convex	Umbonate	Raised
Surface	Smooth	Round	Smooth	Smooth and shiny	Round	Round	Round	Smooth and shiny	Round	Smooth	Smooth	Round	Smooth	Smooth and shiny	Smooth and shiny	Smooth
Pigment	Pale yellow	Cream	Creamish white	–	Yellow	–	–	Cream	Cream	–	–	Pale yellow	–	Cream	Pale yellow	–
Opacity/colour	Opaque	Shiny	Opaque	Opaque	Opaque	Dull	Shiny	Trans-lucent	Trans-lucent	Shiny	Opaque	Trans-lucent	Dull	Opaque	Trans-lucent	Shiny
Gram's reaction	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Cell shape	Rods (bacilli)	Rods (bacilli)	Rods (bacilli)	Ellip-soidal	Rods (bacilli)	Irregular	Rods (bacilli)	Irregular	Circular	Rods (bacilli)	Rods (bacilli)	Rods (bacilli)	Circular	Rods (bacilli)	Ellip-soidal	Oval
Arrangement	Occurring singly and in chains	Occurring singly mostly	Mostly singles and pairs	Occurring singly and in chains	Occurring singly mostly	Occurring singly mostly	Mostly singles and pairs	Occurring and singly mostly	Mostly singles and pairs	Occurring singly mostly	Mostly singles and pairs	Mostly singles and pairs	Mostly singles and pairs	Occurring singly mostly	Mostly singles and pairs	Mostly singles and pairs
Spore (s)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility	+	+	–	+	+	–	+	–	+	+	+	+	–	+	+	–

MK, Bacterial isolates, –, Absent, +, Present.

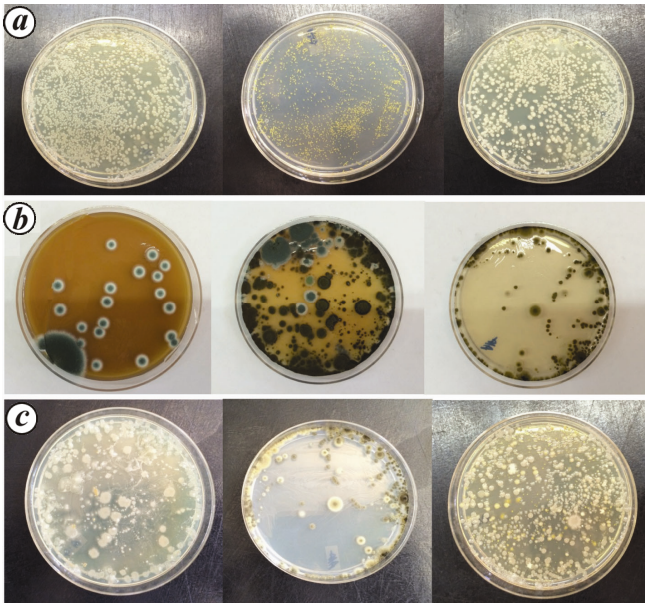

Figure 4. Growth and morphology of (***a***) bacteria, (***b***) fungi and (***c***) actinomycetes.

ecosystem followed by actinomycetes in cell B (32×10^3–50×10^3 cfu/g), cell C (25×10^3–52×10^3 cfu/g) and it was least for fungi in cell B (2×10^2–9×10^2 cfu/g) and cell C (3×10^2–8×10^2 cfu/g). Least microbial poll was observed in control SS (CW 1 in Figure 3) and FS (CW 12 in Figure 3), which may be due to the absence of vegetation (Figure 1). Moreover, CW 1 and 12 are newly constructed wetlands compared to the one-year old wetlands (CW 2 to CW 11). The difference in microbial poll between cell B and C for all CWs was less. Relative abundance of these species in the cells may also be due the filtering media (gravel and sand), high moisture content along with humus and plant debris level present, which supposedly had a qualitative and/or quantitative influence on the microflora developed in CW ecosystem[16]. In addition to bacteria and actinomycetes, relatively small fungal populations are also present and significantly afflicted by the plant species in gravel filled wetlands[17,18].

Characterization of isolates

A notable array of macroscopic features (colony and other morphological characteristics) was displayed by different strains. Maximum strains were creamish whereas others were of various hues such as creamish white, cream, pale yellow, pinkish yellow and yellow (Table 1 and Figure 4). Results also indicate that nutrient-rich medium favours rapid growth and sporulation of isolates. All selected bacterial isolates were Gram +ve in nature and identified as *Bacillus* spp. according to *Bergey's Manual of Determinative Bacteriology*[13]. Identified genus (*Bacillus* spp.) is beneficial as it promotes P-solubilization, N_2-fixation and antagonistic activities. The multifarious plant growth-promoting attributes of identified genus (*Bacillus* spp.) suggest that it can be used to increase agricultural crop productivity and also to sustain soil health (as an alternative of chemical fertilizers). Thus biofertilizer formulations can be made and commercialized that emphasizes more towards sustainable agriculture. It was also observed that various genera of fungus were involved in P-solubilization converting fixed form of phosphorus to available form and thus reducing the use of various chemical fertilizers providing benefits in terms of increasing crop production and sustenance of soil health. Actinomycetes (ray-fungi) are producers of more than 70% antibiotics[19], bioactive compounds and other secondary metabolites having biological activities[20]. Thus various strains of actinomycetes have gene clusters involved in the biosynthesis of melanin, carotenoids, siderophores, polyketide and peptide compounds[21]. Most of the colonies that grew on agar plates belong to the genus *Streptomyces* as the colonies were slow growing, aerobic, glabrous (Figure 4) or chalky, heaped, folded and with aerial and substrate mycelia of distant colours[22]. In addition, all colonies possessed an earthy odour. All *Streptomyces* strains were acid-fast negative and Gram-stain positive. The diverse microbial community stabilized within these systems also enhances nitrification and denitrification processes[23]. Thus vegetation in CW and their influence on microbial assemblages alters the quality of water to be treated and use for agricultural purpose.

Conclusions

Most probable number studies confirmed a reduction in FC in CW due to various factors including the extent of plant coverage, hydraulic retention time and settling of microorganisms. The study indicates removal efficiency of FC with different treatment combinations (plant species of cell B and C) in CW, and this suggests that CW-treated water will greatly reduce percentage of FC, making it safer to be used for irrigation purposes. In addition, bacterial strains isolated and identified during the study might be beneficial for crops as they have multifarious plant growth-promoting properties. Further work at molecular level is in progress to identify beneficial bacterial strains for the development of consortium for biological treatment of wastewater and also to develop an efficient bio fertilizer formulation. Results also support and highlight the need for searching diverse and unexplored actinomycetes strains with novel antibacterial activity.

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ACKNOWLEDGEMENTS. We thank Water4Crops (EU) and Department of Biotechnology (DBT), Govt. of India 'integrating bio-treated wastewater reuse and valorization with enhanced water use efficiency to support the Green Economy in Europe and India' for financial support to carry out this work.

doi: 10.18520/cs/v110/i9/1710-1715