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Enhanced nutrient (N&P) removal by partially saturated and hybrid vertical flow constructed wetland from single household greywater

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Abstract

Constructed wetlands (CWs) are a group of novel, natural wastewater treatment technologies which principally house microbes with help of plants and filter media. The denitrification rates in vertical flow constructed wetland (VFCW) are sensitive and in most cases complete nitrogen removal does not occur. In current study, two systems were evaluated, 1st was partial saturated up-flow VFCW and the 2nd was hybrid pilot scale setup of VFCW and floating treatment wetland (FTW) in series. Both these systems were implemented at single household scale for treatment of greywater. VFCW filter media were river sand and gravels with *Canna indica* as macrophyte. FTW rafts were made of thermocol sheets covering 100% of water surface in tank. System sampling was done for every 3rd day and pollutant removal performance was measured in terms of COD, total nitrogen (TN), ammoniacal nitrogen (NH_4-N), nitrate (NO_3-N), nitrite (NO_2-N), and o-phosphate (PO_4-P) concentrations of influent and effluent. Saturated VFCW system removed 89.09%, 78.7%, 67%, 68.74%, 94.6%, and 86.01% whereas hybrid wetland removed 91.5%, 92.32%, 82.2%, 71.13%, 97.25%, and 89.37% of COD, TN, NH_4-N, NO_3-N, NO_2-N and PO_4-P respectively. After stable pollutant removal trend, FTW macrophyte roots were harvested and subjected to metagenomic analysis on Illumina platform to identify microbial diversity and functional annotation related to nitrogen and phosphate removal. Microbial diversity showed *Pseudomonas, Bacteroides, Clostridium, Azoarcus, Achromobacter* as dominant top 5 genus. This group of genus highlights denitrifying microbial abundance greater than VFCW metagenomes microbial population. The presence of significantly higher denitrifying microbes in FTW confirms higher denitrification rate than VFCW, resulting in overall more Nitrogen removal by the system.

Introduction

Greywater (GW) is increasingly accountable as an important alternative water source for non-potable water recycling because it is consistently produced in large volumes, close enough to satisfy the demand. Yet, it has nutrients (N&P) at concentrations that pose a health hazard to aquatic ecosystem if discharged untreated. Excessive nutrients like nitrogen and phosphorus in the untreated discharge wastewater can cause eutrophication and lower the dissolved oxygen if released into fresh water bodies (Saeed and Sun 2012). Constructed wetlands (CWs) specifically vertical flow constructed wetlands (VFCWs) are widely used where low footprint of treatment system is required compared to other CWs. VFCWs are unsaturated wetlands that employ intermittent feeding of wastewater, resulting in efficient nitrification while exhibiting poor denitrification. Thus, many times nitrate levels are elevated in the effluent (Vymazal 2007; Kadlec, R.H. & Wallace 2009). In CWs, subsequent nitrification and denitrification by microbes is primary mechanism of nitrogen removal (Stottmeister et al. 2003). Denitrification is carried out abundantly by heterotrophic microbes. These microbes use NO_3^- electron acceptor under anoxic or anaerobic conditions (Li et al. 2016). Denitrification rates are affected by presence of oxygen or aerobic conditions, carbon sources and electron donors (Fan et al. 2013). Given the issues in VFCWs of poor denitrification and overall nitrogen removal efficiency, various researchers have modified operating conditions like recirculation of effluent, use of external carbon source (natural – wheat straw, paddy straw, paper, plant

litter, biochar and synthetic – methanol, sulfamethoxazole), partial saturation, intermittent and /or artificial aeration including assessment of long term effects (Fan et al. 2013, 2016; Wang et al. 2016; Zhou et al. 2017, 2018; Kraiem et al. 2021).

A recent variation of CWs based on Free Water Surface (FWS) are called as floating treatment wetlands (FTW) in which naturally emerging plants are fixed in the buoyant support like raft or sheets leaving their roots floating in the wastewater to be treated (Lucke et al. 2019). These systems are without filter media (gravel and sand) as in conventional wetlands. Thus, possess advantage of clogging risk and subtracting the cost of filter media. These systems thrive on microbial interaction with plant roots for the removal of pollutants from wastewater. FTW systems are applicable in tertiary clean-up of wastewater and has been implemented in stormwater runoff, polluted water canals, acid mine drainages and water supply reservoirs (Dotro et al. 2017). (Gao et al. 2017) investigated application of autotrophic, heterotrophic and enhanced FTWs for nitrogen removal from secondary effluent. They also identified *Dechloromonas, Thiobacillus and Nitrospira* as major genus in FTWs with concluding assessment that autotrophic and heterotrophic denitrification occur simultaneously in FTWs.

In current study, the objective was to increase nitrogen & phosphate removal efficiency of VFCW based treatment system treating single household GW as compared to our previous study (Punyapwar and Mutnuri, 2020). The system from previous study was modified by making operational and minor infrastructural changes in VFCW. Also, a new pilot scale system of VFCW and FTW in series was implemented. Both the systems were monitored over 6 months to collect data and analyse the pollutant removal efficiency. Further, metagenomic study was conducted on FTW macrophyte roots to understand the role of microbes in nutrient removal from GW in hybrid wetlands.

Material and Methods

Up-flow saturated stage II VFCW

This system was conventional French two stage VFCW setup previously evaluated and investigated for microbial community (Punyapwar and Mutnuri 2020). There was modification made in the flow of 2nd stage VFCW in the upward direction making the bed saturated. The loading rate/day was kept same with daily capacity of 300 l/day treatment as that of previous study (Punyapwar and Mutnuri 2020). The schematics of up-flow saturated VFCW is given in the Fig. 1. The system was monitored for standard wastewater parameter removal especially Nitrogen and Phosphorus.

Figure 1

Hybrid wetlands – VFCW and FTW in series

In the 2nd approach to enhance nutrient removal, the new pilot system was hybrid wetlands which consisted of stage II configuration of VFCW (Paing et al. 2015a) followed by FTW in series. The schematics of the system is showed in Fig. 2. FTW and VFCW reactors were made in similar chemical

tank used in saturated VFCW setup. VFCW stage II was at an elevation of height 1m from the ground level on which the FTW system was placed so that GW flows through gravity into FTW tank. In the first week, treated water was loaded to wetland bed so as to remove solids and soil from the filter media. Loading rates and volume of GW treated/day was kept same as up-flow saturated VFCW. Plants used for FTW and VFCW both were *Canna indica.* Rafts for FTW were made by double layer of thermocol sheets readily available **(**Fig. 3. **A-B)**. Holes were plunged into it and young plants with root bulbs and no hairs were inserted upside down so as to provide grip to stem part of plant. System was monitored for standard wastewater parameter removal, especially Nitrogen and Phosphorus.

Figure 2

Figure 3

Analytical Methodology

The treatment system water quality in terms of pollutant concentration was measured and analysed at each sampling points for both the systems. The contaminant removal efficiency of the VFCW was analysed by measuring wastewater parameters like COD, o-phosphates, nitrates, nitrites, ammoniacal-N, pH and TN. GW samples were collected in the morning and were prepared by filtering through 0.45µm filter. The prepared sample was stored for further analysis. The COD and orthophosphates concentration were measured by the APHA methods (Eaton et al. 2005). The TN concentration were measured using Shimadzu TOC-TNM-L ROHS analyser. The nitrates, nitrites and ammoniacal nitrogen concentration were obtained by Spectroquant® cell test kits using Spectroquant® Prove 100 spectrophotometer. The pH was measured by Oakton-PC 450 pH meter.

Extraction, qualitative and quantitative analysis of total DNA

Metagenomic DNA was isolated from the *Canna indica* root biofilm samples using c-TAB and Phenol: chloroform extraction method followed by RNase A treatment. The quality and quantity of isolated metagenomic DNA were checked on NanoDrop and agarose gel electrophoresis, respectively.

Library preparation and sequencing

The paired-end sequencing libraries were prepared from the quality checked (QC) passed metagenomic DNA samples using Illumina TruSeq Nano DNA Library Prep Kit. Approximately 200 ng of DNA was fragmented by Covaris M220 to generate a mean fragment distribution of 350bp. Covaris shearing generates ds-DNA fragments with 3' or 5' overhangs. The fragments were then subjected to end-repair. This process converts the overhangs resulting from fragmentation into blunt ends using End Repair Mix. The 3' to 5' exonuclease activity of this mix removes the 3' overhangs and the 5' to 3' polymerase activity fills in the 5' overhangs followed by adapter ligation to the fragments. This strategy ensures a low rate of chimera (concatenated template) formation. The ligated products were size selected using AMPure XP beads. The size-selected products were PCR amplified with the index primer from the kit protocol and indexing adapters were ligated to the ends of the DNA fragments, preparing them for hybridization onto a

flow cell. The library was then sequenced on Illumina platform (2 x 150 bp chemistry) to generate ~ 2.5 GB data/sample.

Post sequencing bioinformatic analysis

The MG-RAST (Meta Genome Rapid Annotation using Subsystem Technology) server is one of the widely used online tools for metagenomic analysis (Meyer et al. 2008).The raw sequence reads were uploaded on the MG-RAST (Version 4) for the automated annotation and analysis. MG-RAST provided accession numbers mgm4968733 and mgm4968734 for FTW root samples. Also, raw sequences were uploaded on NCBI GenBank with accession numbers SRR20771454 and SRR20771454. The sequences then were subjected to QC and trimming of adapters in the MG-RAST pipeline. In diversity profiling, the taxonomy was predicted by using Ref Seq database with minimum E-value cut-off of 1e-5, the minimum length of 50 bp and 80% identity with the lowest common ancestor (LCA) algorithm in the MG-RAST. The functional proteins annotation and their role in metabolism was done by using the SEED subsystems database. The metagenomic abundance of microbial community performing metabolism of Nitrogen cycle, and Phosphorus intake was assessed with parameters E-value cut-off 1e-5, 60% identity and 15bp amino acid length.

Statistical analysis

Taxonomical diversity of microbial community in a metagenome were represented by heatmap made by Statistical Analysis of Metagenomic Profiles (STAMP) (Parks et al. 2014) with tables extracted from MG-RAST server. The functional annotation heat maps and the extended error plots were generated using STAMP for detailed representation with ANOVA test and significance level of $p < 0.05$ with post hoc test analysis by Tukey Kramer.

Results

In this study, two modified VFCW based treatment systems were investigated for enhanced nutrient removal. The influent and effluent streams were sampled and analysed to evaluate the system performance in terms of COD, TN, nitrate, nitrite, ammoniacal nitrogen and o-phosphate removal. The sampling was carried out for 2 months with sampling done on every 3rd day. Further, after stable pollutant removal trend FTW root biofilm metagenomes were sequenced on Illumina platform and subjected for microbial diversity and their role in nitrogen, phosphate removal. The results were compared with previous study VFCW and GW samples (Punyapwar and Mutnuri 2020).

Up-flow saturated stage II VFCW

In this system, operational change was made in classic French VFCW to make 2nd stage of VFCW saturated with water flowing in up-flow direction. Sampling was done at two points i.e. inlet and outlet to maintain anoxic conditions in the saturated bed. Figure 4 shows the pollutant removal trend and removal efficiency of the system in terms of wastewater parameters. COD removal by the system was averaged at

89.09% over 48 days. Maximum inlet COD concentration was 237 mg/l and mean concentration was 150.63 mg/l whereas outlet maximum COD was 38 mg/l with mean concentration of 15 mg/l and 0 mg/l in one of the sampling points. Similar trend of significant removal was followed in terms of o-phosphate. Overall, o-phosphate removal efficiency was 86.1% over 48 days. Inlet maximum o-phosphate concentration was 12.582 mg/l with mean concentration of 9.28 mg/l. Outlet water concentration of o-phosphate was averaged at 1.2 mg/l with maximum concentration of 2.8 mg/l on the last sampling day. In case of total nitrogen, removal efficiency was significantly improved over conventional French VFCW system. TN removal in this system averaged 78.7% almost double (34%) as shown by previous system (Punyapwar and Mutnuri 2020). Inlet average TN concentration was 9.54 mg/l with maximum concentration of 12.75 mg/l. Both wetland beds of system collectively removed the TN with average outlet concentration of 1.88 mg/l. Better TN removal efficiency was possible with significant improvement in nitrate removal by the system. Nitrate removal mean over 48 weeks was 68.74% which is again huge improvement over previous system's 26%. Maximum outlet nitrate concentration was 6.3 mg/l with average being 5.08 mg/l. Similar patterns of ammoniacal-N and nitrite removal were in line with previous system (Punyapwar and Mutnuri 2020). Maximum nitrogen form removed was nitrite (94.6%) and nitrogen removal as ammonia was 67%. Negative removal was observed on the 1st sampling day. There were certain sampling days where nitrite concentration was nil. The average outlet concentration of ammoniacal nitrogen and nitrite was 2.88 mg/l and 2.172 mg/l respectively.

Figure 4

Hybrid wetland – VFCW and FTW in series

Apart from modifying existing system of VFCW, there was new system (which was parallelly run) that consisted of stage II configuration of VFCW and FTW to treat GW from single household in series which together formed hybrid wetland system. Figure 5 highlights the difference in the pilot scale system before and after the operation. *Canna indica* plants have grown to its full potential exhibiting phytoremediation and clean-up of pollutants from GW of single household.

Figure 5

Performance of this system was also evaluated in terms of COD, o-phosphate, TN, ammoniacal-N, nitrate and nitrite removal over 57 days with sampling on every 3rd day. Figure 6 represents pollutant removal trend across each sampling point and sampling day along with inlet and outlet concentrations over system operating period. COD removal by VFCW was 40–50% during initial 18 days and it accelerated to 42–76% during rest of days. FTW showed 91.5% removal of COD over 57 days. Variation in COD removal by VFCW was compensated by FTW to achieve overall removal above 90% with outlet concentration of 10.14 mg/l. o-phosphate had similar fate as that of COD. For initial 18 days, VFCW removed 28–43% while FTW summed up the removal as 58–90% of o-phosphate. After 21 days of operation, removal varied from 59–79% by VFCW whereas FTW cleaned up the remaining phosphates with efficiency of 94–99% removal with average outlet concentration of 0.284 mg/l after 18 days of operation. Maximum inlet phosphate concentration was 12.55 mg/l with mean of 9.31 mg/l. Outlet concentration over operation

period averaged at 0.91 mg/l. o-phosphate removal is most consistent removal trend after 18 days. Total nitrogen removal efficiency was also improved over the saturated system. From Fig. 6-C it's clearly visible that VFCW can remove just over 50% of TN while deficiency of denitrification in VFCW is compensated by FTW. Wetland bed (stage II) with passive aeration had variable TN removal efficiency ranging from 13–58% whereas FTW summed up overall efficiency with 92.32% over 57 days with minimum and maximum removal of 68% and 98% respectively. Inlet maximum TN concentration was 9.547 mg/l and mean concentration was 8.143 mg/l, which came down to 5.36 mg/l after VFCW while FTW cleaned up the residues to result in an effluent concentration of 0.61 mg/l. Major contributor to overall TN removal was nitrate. Nitrate over 57 days with maximum concentration of 19.95 mg/l and mean concentration of 16.91 mg/l in the influent was removed to average concentration of 4.61 mg/l in the effluent. As expected from previous system, poor nitrate removal efficiency was exhibited by VFCW but FTW due to its anoxic conditions and dense, hairy plant roots was able to remove nitrates. VFCW removal efficiency ranged from 6–59% with negative removal on a single sampling day. FTW had efficiency of 29–89% with mean being 71% over 57 days of operation. The maximum influent Ammoniacal-N concentration was 10.75 mg/l. And its removal by the system steadily improved over the operating period achieving 95–98% removal in the last 15 days of operation. VFCW contributed 18–83% removal while FTW summed up 59–98% removal of ammonia in GW. Similar trend of nitrite removal was followed in this study as well. Maximum concentration of nitrite influent was 61.3 mg/l and mean concentration was 26.15 mg/l. Final effluent averaged at 1.77 mg/l with maximum concentration of 5.95 mg/l on 6th day.

Figure 6

Both the systems (up-flow saturated and hybrid wetlands) were more efficient than conventional French VFCW studied previously (Punyapwar and Mutnuri 2020). Table 1 shows overall comparative efficiency of both the system in terms of physicochemical wastewater parameters. Hybrid wetland system outlet shows highest pollutant removal with significant contribution by FTW stage in terms of nitrogen and phosphorus. Hybrid wetlands pollutant level in the discharge is under permissible US-EPA discharge limits.

Table 1
Comparative performance of up-flow stage II VFCW, Hybrid system of VFCW with FTW in series

Parameter	Up-flow saturated stage II VFCW (48 days)		Hybrid wetlands: VFCW and FTW in series (57 days)			US-EPA Standards limits for reuse in mg/l
	Inlet in mg/l (Mean ± SD)	Outlet in mg/l (Mean ± SD)	Inlet in mg/l (Mean ± SD)	FTW Inlet in mg/l (Mean ± SD)	Outlet in mg/l (Mean ± SD)	
COD	150.63 ± 4.5	15.03 ± 5.3	153.55 ± 7.2	55.27 ± 6.14	10.14 ± 2.2	10
Ammoniacal-N	9.18 ± 0.58	2.89 ± 0.77	7.97 ± 0.46	3.63 ± 0.64	1.13 ± 0.2	10
Nitrate	16.72 ± 0.5	5.08 ± 0.33	16.91 ± 0.33	12.94 ± 0.58	4.63 ± 0.24	< 5
Nitrite	33.36 ± 0.25	2.17 ± 0.1	26.15 ± 0.22	10.41 ± 0.16	1.77 ± 0.15	10
o-phosphate	9.27 ± 0.007	1.181 ± 0.004	9.31 ± 0.004	3.8 ± 0.002	0.913 ± 0.001	1
TN	9.54 ± 0.11	1.2 ± 0.01	8.143 ± 0.14	5.36 ± 0.015	0.61 ± 0.01	10
pH (no unit)	7.026 ± 0.1	6.90 ± 0.1	7.04 ± 0.1	6.93 ± 0.15	6.69 ± 0.2	5.5–9.0

Table 1

Metagenomic Analysis

Since, hybrid wetlands were most efficient system to treat GW in which significant nutrient removal was contributed by FTW. Thus, it was important to look into driving force of FTW which is microbial community in the roots. After stable pollutant removal trend, FTW roots biofilm was subjected to metagenomic analysis to investigate microbial diversity and functional annotation with respect to nitrogen and phosphate removal pathways. Table 2 shows preliminary sequence data from MG-RAST server for FTW samples. It represents low QC error and good mean sequence length. Functional annotation is dependent on protein features identified by the pipeline tool which in this case is very huge.

Table 2
Preliminary characteristics of FTW metagenome sequences samples as predicted by MG-RAST analysis pipeline

Sample name	Total sequences identified	Sequences after quality check	Mean Sequence Length post QC	Mean GC content post QC	Identified Protein Features	Identified rRNA Features
FTW_1	14,022,296	13,252,927	180 ± 44 bp	56 ± 13%	5,221,592	25,166
FTW_2	13,770,473	12,950,150	172 ± 41 bp	55 ± 13%	5,180,346	23,577

Table 2

Microbial Diversity

Microbial diversity of metagenomes in FTW were compared with French VFCW stage II filtration layer and GW samples from previous study (Punyapwar and Mutnuri 2020). The metagenomic profiles were extracted by classifying metagenomes against the GenBank database in the MG-RAST portal by the best hit classification. Figure 7 represents dominant 10 genus from all three group of metagenomes. These 10 genera together contribute 51% of abundance with *Pseudomonas* as most abundant species in FTW samples with 27.7% proportion as that of averaging 13% in VFCW samples. Other genera followed by *Pseudomonas* were *Azoarcus* (12.6%), *Achromobacter* (10.65%), *Thauera* (8%), rounding with *Clostridium, Dechloromonas* around 5% in FTW samples. The unweighted pair group method with arithmetic mean (UPGMA) dendrogram of Fig. 7 exhibits overall microbial community relationship between FTW and VFCW samples. VFCW metagenomes had *Flavobacterium* and *Sphingopyxis* whereas GW metagenomes had *Enterobacter* and *Bacteroides as* dominant genera completely differentiating from FTW ecosystem.

Figure 7

Functional Annotation of microbial metabolism

Functional annotation analysis was aimed to investigate the role of microbes in the nitrogen cycle and its metabolism, phosphate metabolism in FTW metagenomes. In this, similar comparison was made with previous metagenomes of VFCW and GW (Punyapwar and Mutnuri 2020). Microbial functional pathways of nitrogen and phosphate metabolism analysed by protein features from all three metagenomes uploaded on MG-RAST against SEED subsystems database. **Figure S1** shows primary metabolism abundance like carbohydrate metabolism, amino acid production, protein metabolism exhibiting growth and amplification of microbial community. In case of functional annotation, there is closest UPGMA relationship between FTW and GW samples quite antagonistic to microbial diversity. Figure 8-A highlights the nitrogen pathways involved in all three group of metagenomes resulting in removal of nitrogenous pollutants from GW. The dominant pathway is ammonia assimilation followed by nitrate-nitrite ammonification in all group of metagenomes. Denitrification is significantly more abundant in FTW and

GW samples (around 12–14%) compared to 7% abundance in VFCW metagenomes. Similar proportion and trend were followed by nitrogen fixation pathway as well. The extended error plot from Fig. 8-B shows significant abundance difference between VFCW and FTW metagenomes. Denitrification and nitrogen fixation pathways are dominant in FTW metagenomes where $p < 0.05$ for double side t-test. In case of other nutrient pollutant (phosphate), there is only one metabolic pathway which dominated in all three metagenomes with maximum abundance of 62–64% **(figure S2)**. The UPGMA relationship is following same trend as subsystems level I, nitrogen metabolism between FTW and GW.

Figure 8

Discussion

French VFCWs are very well established to treat raw wastewater in France (Paing et al. 2015b) but when implemented in Goa, India, there was insufficient pollutant removal especially in terms of nitrogen (Talekar et al. 2018; Yadav et al. 2018; Punyapwar and Mutnuri 2020) in treating GW, exclusive blackwater and domestic sewage. The major issue faced was low denitrifying rates and thus, nitrate levels were increased in outlet after certain period of operation. This moved the approach from conventional CWs setup to hybrid setup and operational changes focussed on specific pollutant removal like nitrogen and phosphorus. In this study, two pilot scale systems of modified saturated VFCW and hybrid wetland including FTW was investigated for GW treatment. Up-flow saturated system had stage II bed as saturated which was a minor infrastructural change made to already running system. Pollutant removal in VFCW based system with saturated VFCW stage II significantly removed organic matter and suspended solids; were represented by removal of COD with similar efficiency (89%) of previous conventional French VFCW system (90%) (Punyapwar and Mutnuri 2020). There was improvement in o-phosphate removal by 10% (80% previous to 90% in current). Increased pollutant removal efficiency is backed by increased hydraulic retention time (HRT) of the system. Saturated stage II provides more time for GW to reside in the system improving interaction between microbes and filter media. Thus, due to more HRT of the stage II VFCW, o-phosphate and COD were removed by sedimentation, filtration and biochemical degradation by microbes. Classic French VFCW showed poor denitrification and thus resulted in low nitrogen and nitrate removal efficiency. Due to up-flow and saturated nature of stage II of VFCW, there was increased anoxic/anaerobic conditions and increased HRT in wetland bed promoting denitrification (Vymazal 2007). Hence, there is improvement in nitrate removal from 26–68% which ultimately improved TN removal efficiency to 78.7%. Similar enhanced nitrogen removal was reported by studies of (Silveira et al. 2015) in partially saturated single stage French VFCW, (Kraiem et al. 2021). Overall, hybrid wetland system consisting of VFCW and FTW showed more pollutant removal than saturated VFCW system. HRT of each system (stage II saturated VFCW and hybrid wetlands) was 3 days with similar volume treated/day. VFCW as established removes pollutants by help of microbes, plant uptake and sedimentation (Vymazal 2007) whereas floating wetlands depend on plant root trapping, intake by plants as bioaccumulation, microbes (endophytic and rhizospehric), fungi and their mutualistic relation (Zhu et al. 2011; Gao et al. 2017; Shahid et al. 2020). Most pollutants in water are trapped by roots have two fates i.e., intake by plants or degradation by microbes in the vicinity of roots. To have

efficient removal of pollutants, plant roots should be dense (Chen et al. 2016). Another role of plants roots is the release of exudates which balances water pH, increase in the humic content of water which assists sedimentation of soluble pollutants to insoluble particles (Chen et al. 2016). Dense roots region provide surface for biofilm formation of microbes and provide oxygen to aerobic bacteria and thus increasing pollutant removal efficiency by degradation of organic matter. Further, these biofilms accumulate more particulate matter from water. Simple nutrients are removed by uptake of plants and export through biomass in upper parts (Shahid et al. 2020). In current study, *Canna indica* played vital role in pollutant removal in GW. Plant height was maxed to its potential (1-1.2 m) **(**Fig. 5.**B)** with root length growth from 5 cm at start to 40–45 cm after 57 days of operation (Fig. 5.**D-E).** Anoxic conditions are maintained because of 100% coverage of rafts and dense roots hosting denitrifying microbes. This enhances the nitrate removal in the roots vicinity ultimately improving TN removal over conventional VFCW system. VFCW in the current system takes care of ammonification and subsequent nitrification along with COD and o-Phosphate removal. Similar reduction of nitrogen and phosphorus by use of floating wetland system is documented by various authors (Stewart et al. 2008; Sun et al. 2009; Gao et al. 2017; Spangler et al. 2019a, b) for agricultural runoff and municipal wastewater which are inclined with the reduction pattern observed in our system. Microbial diversity revealed organotroph *Pseudomonas***(**Fig. 7**)** as dominant genera in FTW samples which is model organism to study denitrification (Ergas and Aponte-Morales 2014). Various species of *Pseudomonas* have been reported for their denitrifying activity in wastewaters (Deng et al. 2014; Zhang et al. 2022). *Achromobacter, Azoarcus, Thauera, Clostridium* also contribute significantly in denitrification where the C/N ratio is usually low (Liu et al. 2006; Su et al. 2018; Cui et al. 2022). This abundant microbial population in FTW samples showed metabolic activity related to nitrogen and phosphates in Fig. 8**& figure S2** respectively. Figure 8-B observations clearly indicate there is significant higher denitrification rate in FTW samples compared to VFCW samples. This can be correlated with the relative more abundance of denitrifying bacteria in FTW root biofilms. Phosphate is actively used in microbial metabolism as non-reactive form(Punyapwar and Mutnuri 2020; Shen et al. 2022) and majority intake is by the *Canna indica* with translocation in within the tissues (Haritash et al. 2017). In current system, there was increased HRT time and hence, phosphate concentrations were able to be consistently under discharge limits provided by US-EPA.

Conclusion

Partial nitrogen removal by VFCW is major and well documented issue which infer that the standalone systems were not able to achieve complete nitrogen removal with higher concentration of nitrate in the effluent in certain cases. Similar low TN and nitrate removal efficiency was observed in French VFCW system in previous study. To solve this issue, various experiments were conducted in existing VFCW system with some operational and infrastructural change, and new hybrid wetlands. Saturated stage II wetland system removed overall COD, ammoniacal-N, nitrate, nitrite, TN and o-phosphate with efficiency of 89.09%, 67%, 68.74%, 94.6%, 78.7% and 86.01% respectively over 48 days of sampling period. Hybrid wetland having two different components (VFCW and FTW) collectively removed overall COD, ammoniacal-N, nitrate, nitrite, TN and o-phosphate with efficiency of 91.5%, 82.2%, 71.13%, 97.25%,

92.32% and 89.37% respectively over 57 days of sampling period. Thus, hybrid wetland just nicks slightly with more pollutant removal efficiency. FTW metagenomic samples revealed abundance of denitrifying bacteria like *Pseudomonas, Achromobacter, Azoarcus, Clostridium* and *Thauera.* Functional annotation of nitrogen and phosphate metabolism justify the role of microbial community in efficient removal as compared to previous conventional VFCW treatment systems. Effluent quality of both stage II saturated VFCW and hybrid wetland is under CPCB, India and US-EPA permissible discharge limits. Hence, the treated water has potential non-potable application like toilet flushing, gardening and agricultural, etc.

Declarations

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Figures

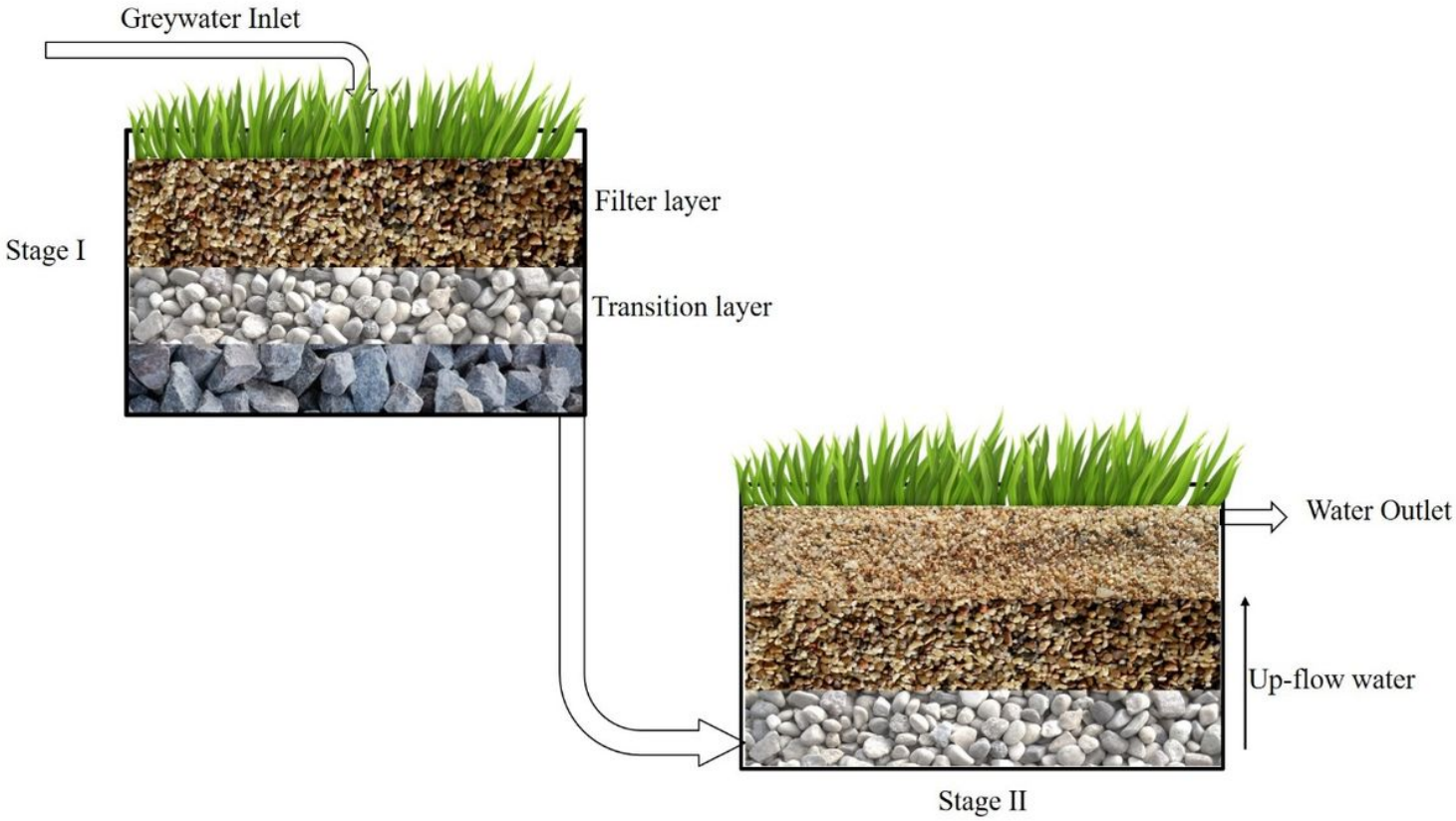


Figure 1

Schematics of 2nd stage saturated VFCW

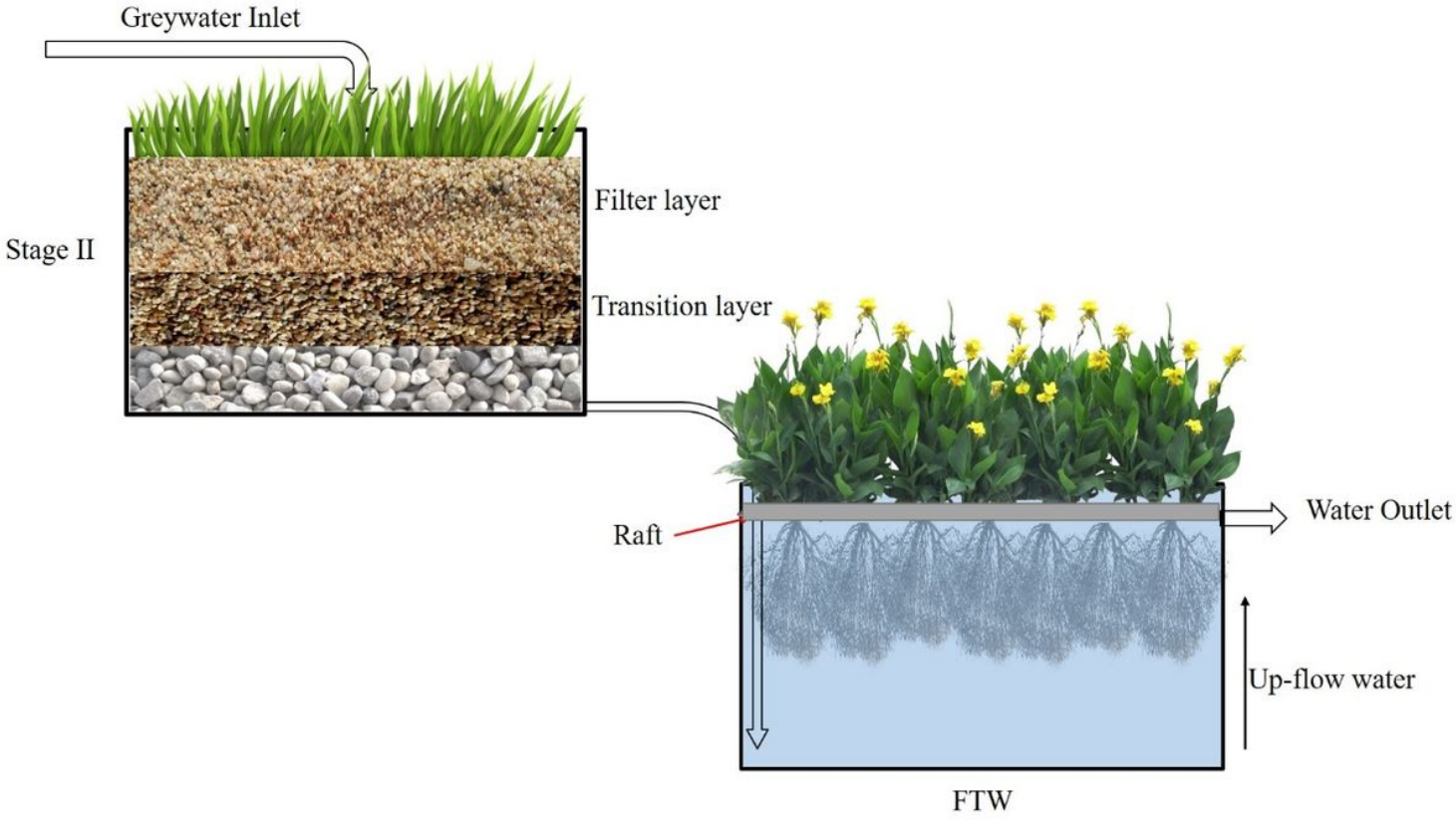


Figure 2

Schematics of hybrid wetlands - VFCW and FTW in series



Figure 3

A-B. Side view and top view of floating rafts, **C.** Hair roots development after 2 weeks of operation of FTW **D-E.** Root further densifying to form anoxic region and biofilm surface for microbes (57^{th} day)

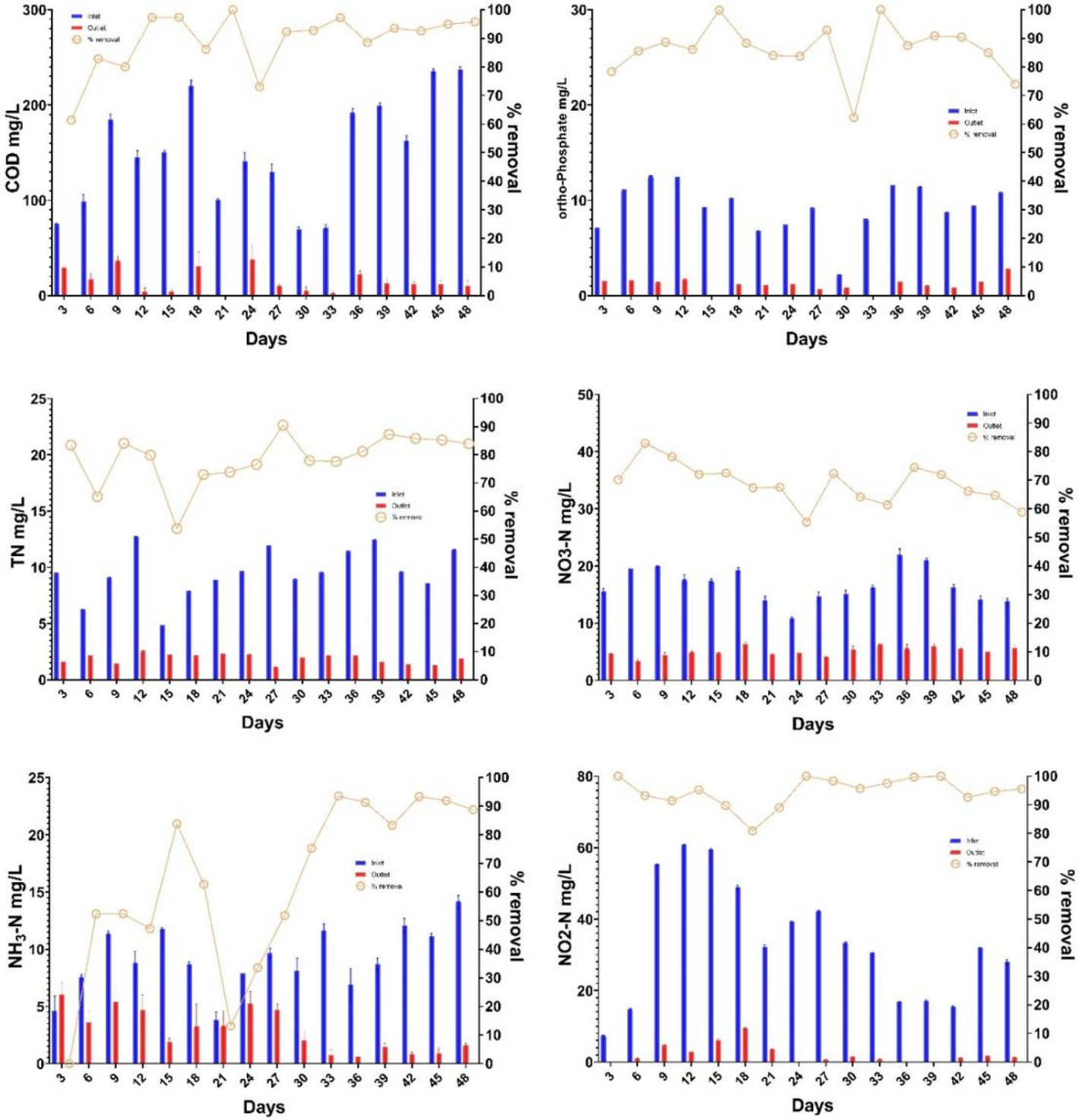


Figure 4

Pollutant removal by up-flow stage II VFCW **A.** COD, **B.** o-phosphate, **C.**Total nitrogen, **D.** Nitrate, **E.** Ammoniacal-N, **F.** Nitrite over 48 days



Figure 5

Hybrid wetlands - **A.** Before start of operation, **B.**After 57 days of operation

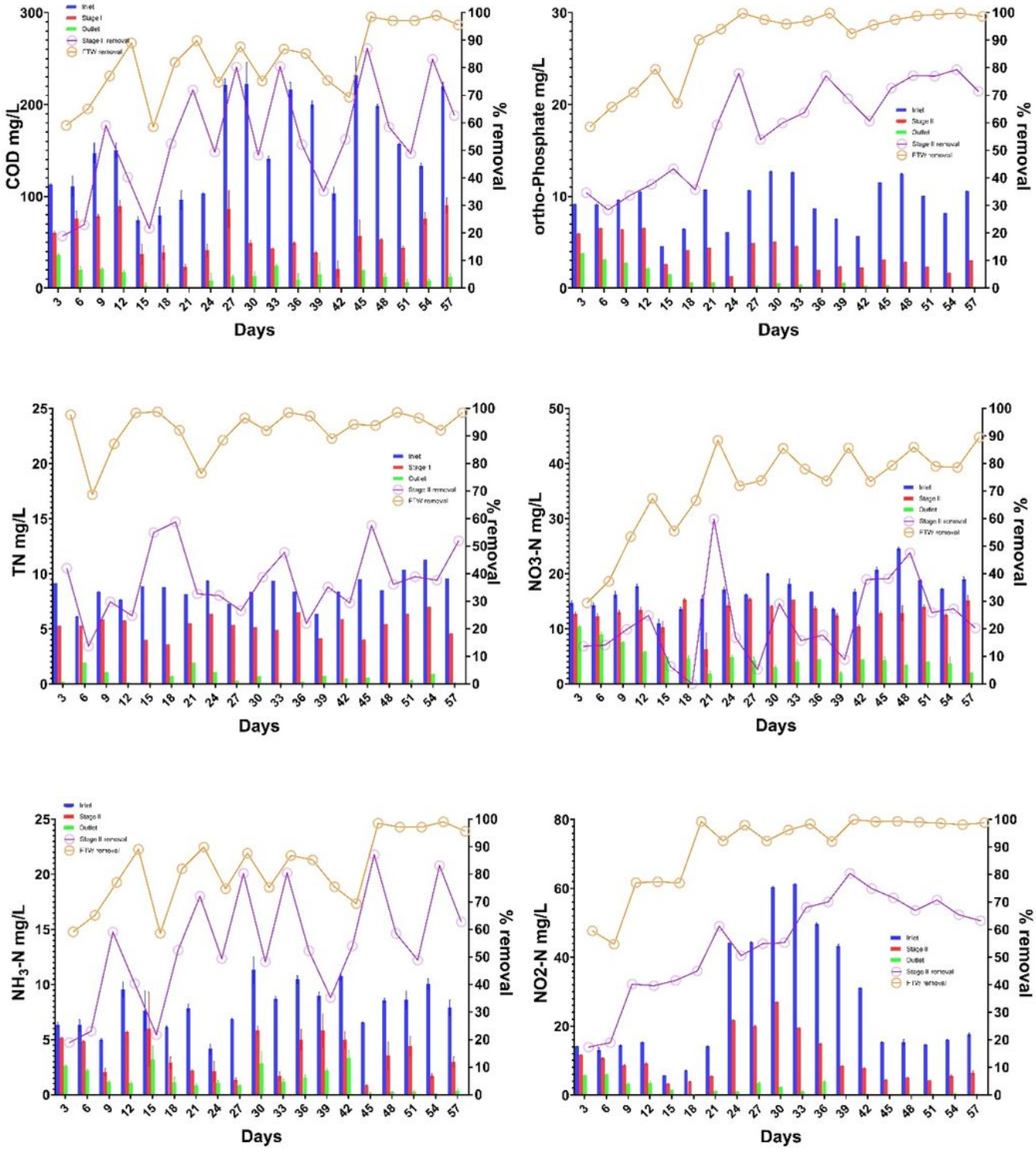


Figure 6

Pollutant removal by Hybrid wetlands – VFCW and FTW across 3 sampling points **A.** COD, **B.** o-phosphate, **C.** Total nitrogen,**D.** Nitrate, **E.** Ammoniacal-N, **F.** Nitrite over 57 days

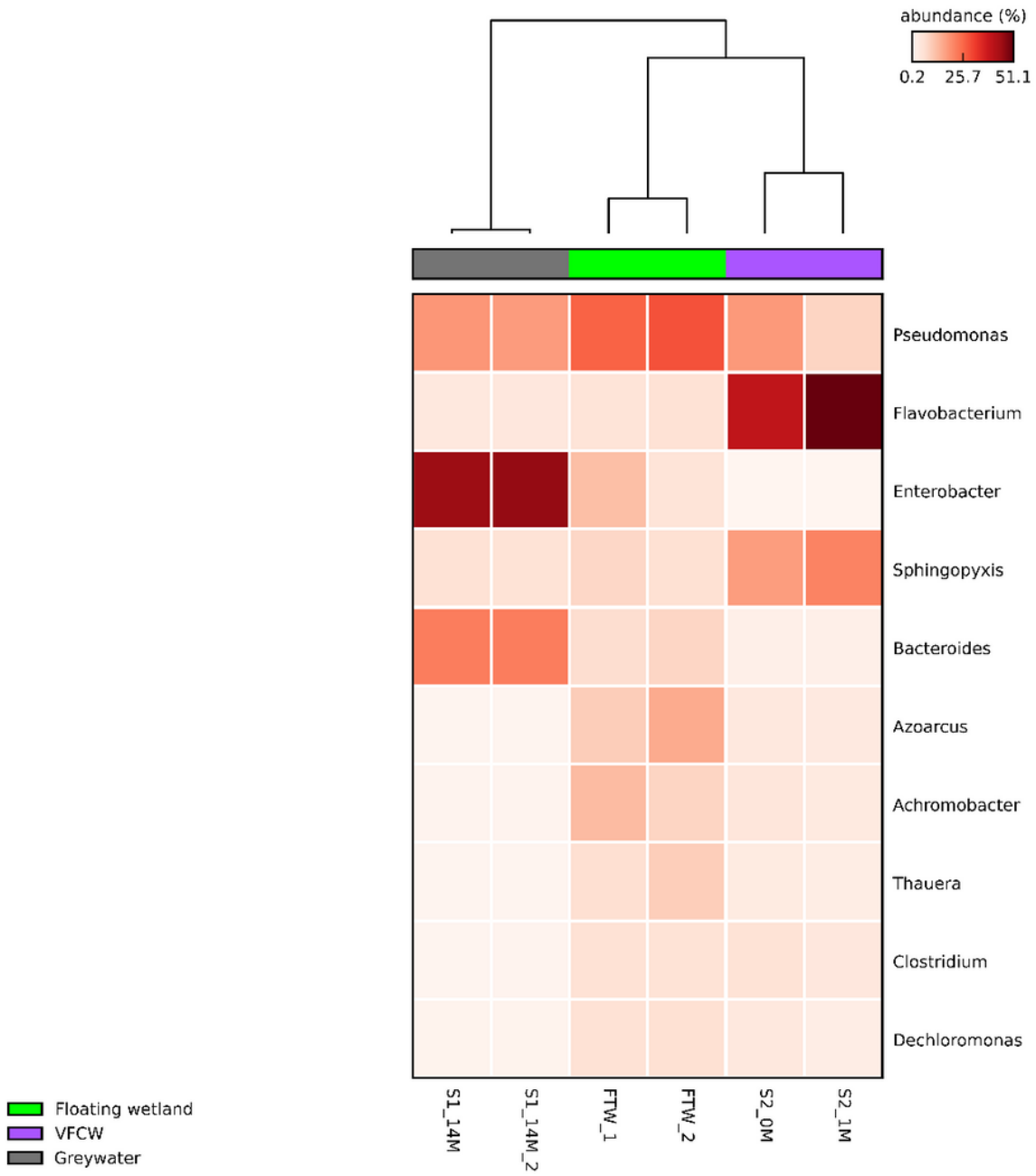


Figure 7

Heatmap of dominant 10 microbial genera abundance in FTW, VFCW and GW metagenomes.

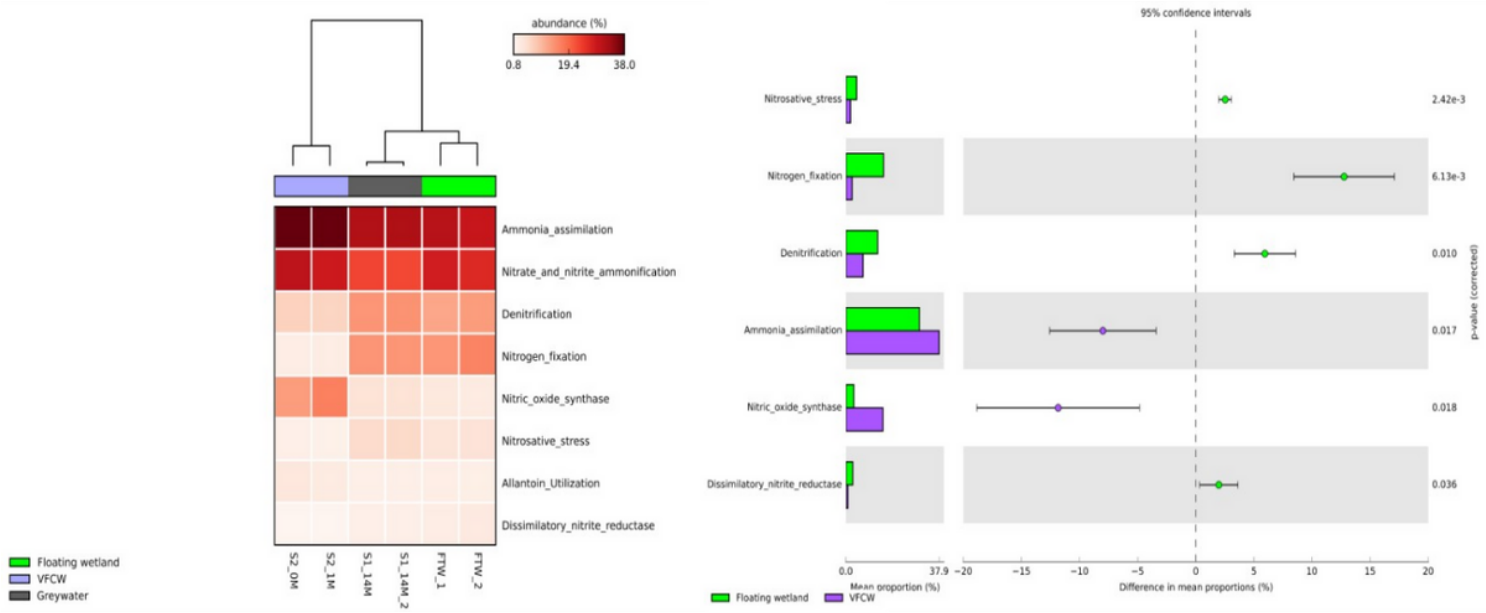


Figure 8

Functional annotation of metagenomes classified against Nitrogen metabolism in SEED subsystems level III. A. Heat map depicting abundance of reads in FTW, GW and VFCW, B. Extended error plot showing statistical difference and abundance between FTW and VFCW.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryData.docx
- floatimage1.jpeg