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Research Paper

Removal of nitrogen and phosphorous from blackwater via an integrated nature-based system: hybrid-vertical flow constructed wetland, upflow anaerobic fixed biofilm reactor, and granular activated charcoal column along with in situ electrochlorination disinfection

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ABSTRACT

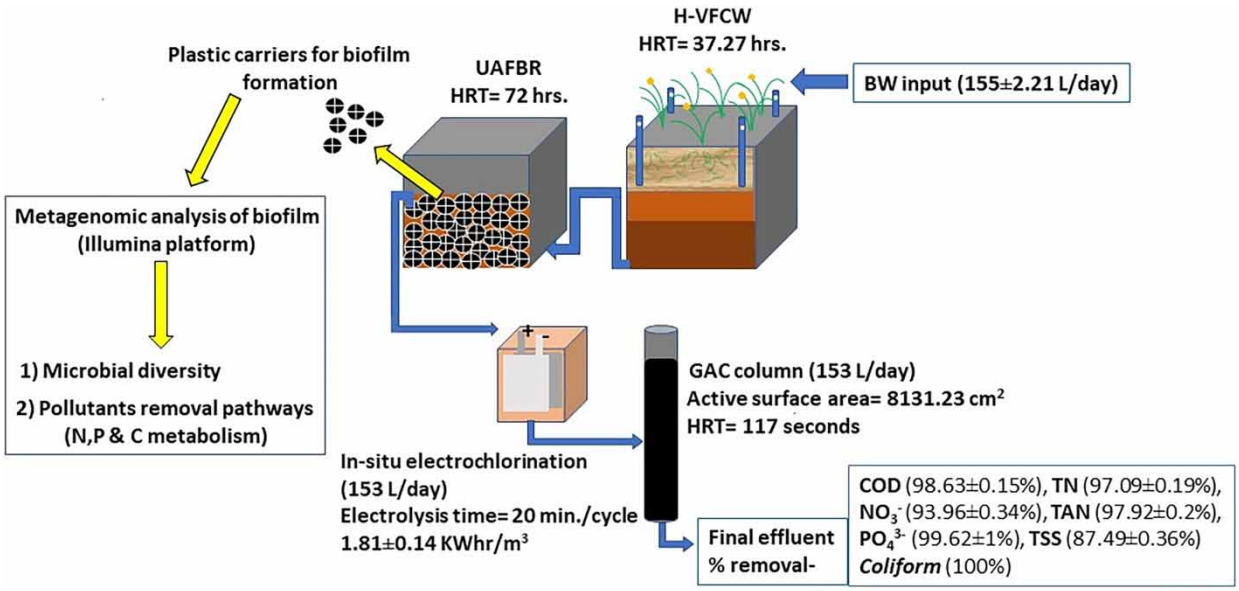
The hybrid vertical-flow constructed wetland (H-VFCW) sequenced with an upflow anaerobic fixed biofilm reactor (UAFBR) was developed to treat blackwater for single households. The H-VFCW filter bed layer's redox zonation and hydraulic retention time (HRT) were designed optimally with the potential of simultaneous nitrification, denitrification, and enhanced biological phosphorous removal. The UAFBR optimisation for residual nitrate removal was based on the carbon -nitrogen ratio, HRT, and biofilm growth on carriers. Complete disinfection was achieved by optimised in situ electrochlorination by dosing 0.16% sodium chloride, with 20 min of electrolysis and 44.48 Am^{-2} of current density. The power consumption was 1.81 ± 0.14 $KWhm^{-3}$. Ammonia volatilisation was studied on a granular activated charcoal column (GACC) column to remove residual TAN as the final treatment. The integrated treatment system removed 98.63 $\pm$ 0.15% chemical oxygen demand (COD), 97.09 $\pm$ 0.19% total nitrogen (TN), 93.96 $\pm$ 0.34% nitrate (NO_3^-), 97.92 $\pm$ 0.2% TAN, 99.62 $\pm$ 1% orthophosphate (PO_4^{3-}), 87.49 $\pm$ 0.36% total suspended solids (TSS), and 100% *Coliform*, with the final effluent pH of 8.4 $\pm$ 0.08. The microbial and functional annotation diversity of the UAFBR biofilm was studied. The treatment systems achieved the Central Pollution Control Board (India) effluent discharge standards.

Key words: blackwater, granular activated charcoal column, hybrid vertical-flow constructed wetland, in situ electrochlorination, nature-based, upflow anaerobic fixed biofilm reactor

HIGHLIGHTS

- The H-VFCW redox potential impacted the simultaneous removal of TAN, NO_3^-, and PO_4^{3-} from BW.
- Investigated research gaps in microbial and functional diversity of the UAFBR biofilm in BW treatment.
- The carbon–nitrogen ratio significantly impacted the UAFBR nitrate removal regardless of HRT.
- The GAC column showed efficient ammonia volatilisation application.
- In situ electrochlorination showed 100% disinfection in just 0.058 GBP/m^3.

GRAPHICAL ABSTRACT



INTRODUCTION

Groundwater nitrate pollution is a critical concern faced by most parts of India, e.g., Rajasthan with 4,750 mg/L (Ozha 2010), Andhra Pradesh with 879.65 mg/L (Brindha *et al.* 2012), etc. This can cause a threat to the environment, human health, and economy. There are various conventional techniques available for nitrogen and phosphorous pollutants removal, e.g., ion exchange, reverse osmosis, electrodialysis, and air stripping, but are expensive. Hence, switching towards nature-based decentralised treatment methods can be environmentally and economically sound as discussed in the following.

Nitrification is a two-step process that oxidises ammonia to nitrate by two groups of chemoautotrophic bacteria in a strict aerobic condition. The nitrifying bacteria group uses oxygen as a terminal electron acceptor for energy production, e.g., a genus of *Nitrosomonas, Nitrosococcus, Nitrobacter*, etc. (Burghate & Ingole 2013). Denitrification is a process where bacteria reduce nitrate to nitrogen in strictly anaerobic conditions by utilising nitrate as an alternative terminal electron acceptor during respiration. However, some species of *Pseudomonas* can also perform denitrification in aerobic conditions (Lv *et al.* 2017). Most denitrifying bacteria are heterotrophic; hence the external carbon source is a reaction rate-limiting factor (Kostrytsia *et al.* 2018). However, some bacteria can also perform denitrification by a facultative or obligate autotrophic pathway, e.g., *Paracoccus, Thiobacillus, and Ferrobacillus ferrooxidans*, respectively (Carboni *et al.* 2021). The enhanced biological phosphorous removal (EBPR) is carried out by a group of polyphosphate-accumulating organisms (PAOs) with the ability to take up extracellular orthophosphate and store it as polyphosphate reserve (polyhydroxyalkanoates (PHA) or polyhydroxy butyrate (PHB)) (Kim & Lenz 2001). The process is favoured by the sequential exposure of anaerobic to aerobic conditions, e.g., *Acinetobacter, Corynebacterium, Pseudomonas,* etc. (Yuan *et al.* 2012).

The phytoremediation process is commonly used in the constructed wetland (CW) for the removal of total ammoniacal nitrogen (TAN), NO_3^-, PO_4^{3-}, etc. (Gogoi *et al.* 2022). The major criteria for phytoremediation of wastewater (WW) are its resistance to anoxic and water stress and long fibrous root types to enhance its absorption, aeration, and the rhizosphere zone. The commonly used plants are *Canna indica* and *C. glauca* (Yadav *et al.* 2018).

The vertical-flow constructed wetland (VFCW) is a human-engineered form of natural wetlands in controlled conditions. The VFCW types are popularly used for secondary WW treatment, with a high potential for biological oxygen demand (BOD), chemical oxygen demand (COD), TAN, and total suspended solids (TSS) removal (Yadav *et al.* 2018; Gogoi *et al.* 2022). The treatment is achieved through various mechanisms, including filter media-physical filtration and sedimentation, chemical adsorption, as well as biological treatments, such as phytoremediation and microbial remediation. However, the VFCW treatment is limited by a hike in nitrate and less phosphorous removal (Gogoi *et al.* 2022; Yaragal & Mutnuri 2022).

The upflow anaerobic fixed biofilm reactor (UAFBR) is an anaerobic WW treatment method, saturated with biocarriers (commonly of plastic and rubber material). Biofilm is grown on carriers and its treatment is based on the contact time with WW either in batch or continuous mode. The biofilm formed on carriers prevents the washout of the microbial inoculum, hence preventing the time gap of inoculum stabilisation for the next treatment cycle (Song *et al.* 2004). There are very few studies of UAFBR on blackwater (BW) treatment and its metagenomic study of the biofilm.

The in situ electrochlorination disinfection is based on treating WW in the reactor itself by the produced oxidising agents like chlorine species, free hydroxyl radicals, and active oxygen species (Gogoi *et al.* 2022), thus, reducing the cost of transportation and storage of chlorine species. The mixed metal oxide (MMO) – titanium plate coated with 70% ruthenium, 20% iridium, and 10% titanium is preferred for chlorine production. The in situ electrochlorination disinfection was previously studied by Gogoi *et al.* (2022) and Talekar *et al.* (2018); however, the scope of reducing power consumption cost was required.

The ammonia volatilisation method can be used to remove ammonium from WW. Ammonium under alkaline pH ($\geq$7.8) converts to gaseous ammonia and it volatilises with an increase in the temperature, wind speed, and alkalinity (Abrol *et al.* 2007; Agri-facts 2008). Thus, theoretically, this method would not be saturated in the process of ammonium removal. Thus, practical investigation with WW needs to be studied.

This work aims to develop a sustainable decentralised integrated treatment technology for removing prominently nitrogen (NO_3^-, TAN, and TN) and phosphorous (orthophosphate) pollutants from BW up to Central Pollution Control Board (CPCB – India) discharge limits. The novelty of the study was to innovate the H-VFCW with an optimum redox potential to facilitate simultaneous nitrification, denitrification, and EBPR. To study a carbon–nitrogen ratio (C:N) and HRT effects on UAFBR treatment efficiency of nitrogen and phosphorous. Also, to investigate the biofilm formation efficiency on polypropylene carriers and its microbial and functional annotation diversity. The previously studied in situ electrochlorination (ECl_2) disinfection system was optimised to reduce power consumption costs. The granular activated charcoal column (GACC) was investigated for ammonia volatilisation method efficiency.

MATERIALS AND METHODS

Site description and research design

The single household-Aqua privies (SHH-AP) toilet with an integrated treatment system for regular six people equivalent (PE), was demonstrated at BITS Pilani, KK Birla Goa campus (15.39° N, 73.88° E), India. The toilet has two separate chambers designed for different purposes, as illustrated in Figure 1. The toilet commode flush generated ~4.6 L/flush that goes into the septic tank, whereas the handwash basin and urinal flush directly go into the H-VFCW. The WW feeding in H-VFCW and UAFBR was based on the liquid overflow output of the septic tank and H-VFCW by gravity, respectively (to avoid the water pump cost) (Figure 1). The overflow was based on users per day generated WW displacement volume. The complete study was documented for ~7 months.

Design and optimisation of H-VFCW

The treatment performance of pre-integrated unsaturated-VFCW (U-VFCW) of our previous study (Gogoi *et al.* 2022) was monitored for 47 days. Then, the U-VFCW treatment was enhanced by the newly designed H-VFCW. The river-based gravels were used for the filter media and their size and layering height distribution of U-VFCW and H-VFCW are shown in Table 1. Initially, the pilot-scale H-VFCW with different saturation heights of 40 and 60 cm, respectively, or the HRT effect on BW treatment was studied for 67 days. The three ventilation PVC pipes (1.9 inches) were laid to 39 cm of filter layer for passive aeration. Then, the optimised pilot-scale H-VFCW parameters were scaled up to full-scale H-VFCW in a 1-m^3 high-density polyethylene (HDPE) tank, which was studied for 77 days (Figure 1). The optimised H-VFCW filter media profile was almost the same as U-VFCW, except the drainage layer height was increased by 20 cm (Table 1). The *C. indica* was planted with an initial density of 12 plants per m^2.

Design and optimisation of UAFBR

The full-scale UAFBR comprised of 1-m^3 black-coloured HDPE tank, filled with carriers (Figure 2). The pilot-scale optimisation for the time required for stable biofilm formation on carriers, C:N ratio (total carbon (TC):total nitrogen (TN)), and HRT for effective denitrification was performed in a 200-L HDPE barrel. It was filled with 60 L of H-VFCW (60 cm saturation) effluent and was saturated with carriers (7,320 pieces). Then, 60 L of H-VFCW effluent was fed every day using a peristaltic

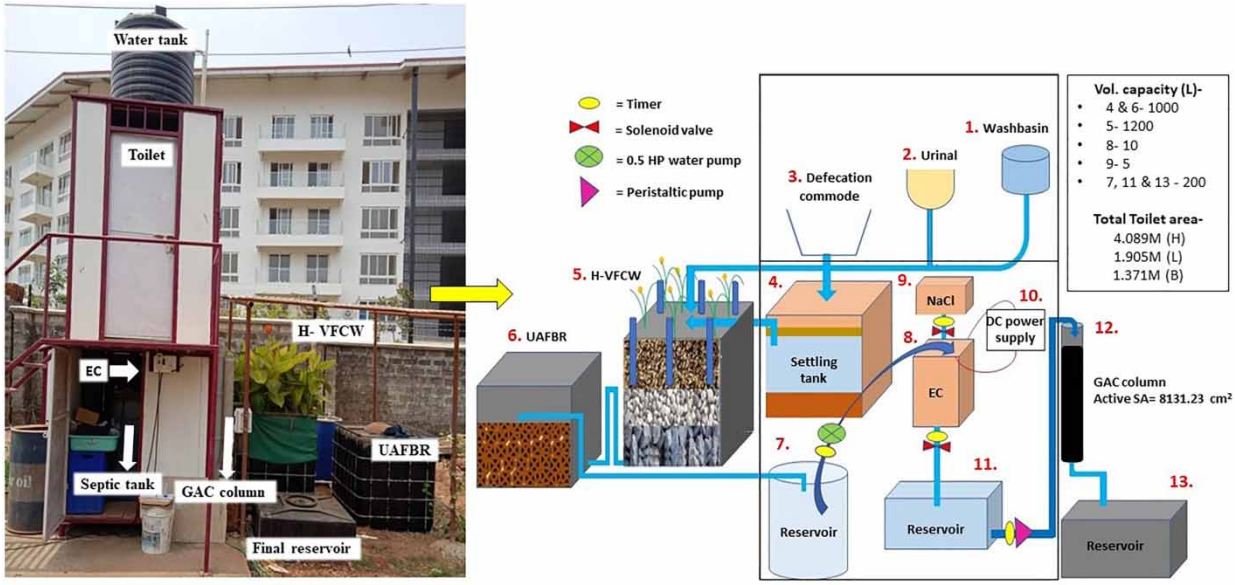


Figure 1 | Field-scale image and schematic of the SHH-AP toilet with an integrated treatment system showing the feed flow-path.

Table 1 | Comparative VFCW filter media profile

Filter media (river gravels)	U-VFCW Layer depth (m)	H-VFCW	U-VFCW Gravel size (mm)	H-VFCW
The top layer (filter layer)	0.4	0.4 (unsaturated)	4–8	
The middle layer (transition layer)	0.2	0.2 (saturated)	18–20	
The bottom layer (drainage layer)	0.2	0.4 (saturated)	30–40	

Model	BI 22
Media effective specific surface area (SSA)	400 m^2/m^3
Colour	black
Body material	Polypropylene
Media height	16 mm
Media diameter	22 mm
Structure	Cylindrical with external fins

Figure 2 | Properties of the carrier.

pump (NFP-03, Flowtech, India) from the bottom of the UAFBR. After confirming the biofilm formation, the outlets for 1-, 2-, and 3-day HRT were made, respectively, in reference to the C:N ratio and nitrate removal efficiency parameters. The same procedure of feeding H-VFCW effluent in UAFBR was followed as the above. The respective HRT effluents were analysed twice a week for 22 days. Then, the optimised pilot-scale UAFBR parameters were scaled up to the full-scale UAFBR,

with the capacity to treat 155 L/day. The pilot-scale UAFBR matured biocarriers were used as an inoculant for full-scale UAFBR startup. The basic properties of the carriers are shown in Figure 2.

Optimisation of in situ ECl_2 disinfection

The design concept and electrodes of the electrolytic cell (EC) were in reference to our previous study of the membraneless EC, with dosed non-iodized 0.16% sodium chloride (NaCl) for disinfection (Gogoi *et al.* 2022). The anode and cathode were made of titanium plate coated with 6-μm-thick MMO and stainless steel (SS 316 grade) plate, respectively, with 1 mm thickness. The present study's novelty was to lower the EC power consumption by decreasing the electrolysis time (focused only on disinfection rather than other parameters), increasing the treatment volume capacity to 9L/cycle, optimising current density and reducing the TOC and TSS feed load. The optimisation of electrolysis time was done for 10, 15, 20, and 30 min, respectively, in reference to *Coliform* disinfection and residual chlorine (WHO limits of <5 mg/L).

Design optimisation of ammonia volatilisation column (AVC):

The GAC of 4–8 mesh and pH of 9.1 (Honeywell, catalogue C2764) was selected for the AVC study due to its adsorptive property, market availability and cost-effectiveness. The GACC optimisation was based on the TAN removal efficiency. The lab-scale optimisation was performed in the polypropylene tube, with a pre-fixed active height of 60 cm and a diameter of 2.8 cm. The full-scale UAFBR-treated effluent was pumped into the GACC (540.1 cm^2) at different flow rates of 12, 13 and 14- mL/min, respectively, for 5 h a day by a peristaltic pump. Then, the lab-scale optimised GACC surface area (SA) to the maximum feed flow rate ratio was scaled up to the field scale. The field-scale GACC was set up using PVC pipe, with a total SA of 8,131.23 cm^2. The UAFBR effluent was pumped with a flow rate of 200 mL/min for 12 h 45 min, with a target to treat 153 L per day (Figure 1).

Sampling and analysis

BW analysis

The overall experimental samples were collected twice a week and analyzed within 24 h, for ~7 months. The samples were analyzed using standard methods for various WW parameters i.e., COD- 5220 D closed reflux, colorimetric method, PO_4^{3-}- Vanado-molybdophosphoric acid colorimetric method, TSS- 2540 D method, *Coliform* counts by spread plate method on a differential media- MacConkey agar using standard methods (APHA/AWWA/WEF 2012), TN and TC- Shimadzu TOC-TNM-L ROHS analyzer, NO_3^- and TAN- Spectroquant® nitrate test kit (1.01842.0001) and ammonium test kit (1.00683.0001) using Spectroquant® Prove 100 spectrophotometer, respectively, and pH- Oakton pH 510 Series Meter P/N 54X002608. The standard deviation analysis was performed for all the experimental analyses.

Biofilm analysis

The carriers with slimy layers were selected for the biofilm analysis in duplicate from three respective HRT zones. The carriers were gently washed with distilled water to remove any loosely bound sludge matter. Carriers were soaked in phosphate buffer saline solution and vortexed at 4,000 rpm for 10 min. The above-extracted biofilm suspension was used for crystal violet (CV) assay, scanning electron microscopy (SEM), and metagenomic analysis.

The CV assay was carried out in duplicates with 1 h of incubation interval of 4 h at 37 °C with constant mixing at 100 rpm. Then the biofilm growth rate was confirmed by spectrophotometry absorbance at 570 nm. Then the SEM analysis was done by using a Quanta 250 FEG scanning electron microscope (sputter coated with gold–palladium alloy) and operated at 15 kV. The above analysis was carried out for both pilot and full-scale UAFBR, except the metagenomic analysis was done only for the full-scale UAFBR (after 60 days of treatment).

Metagenomic analysis of biofilm

The extracted biofilm samples in duplicates were stored at −80 °C for further molecular analysis. It was carried out in three major steps:

Isolation, qualitative and quantitative analysis of DNA

The metagenomic DNA was isolated from liquid biofilm samples using the c-TAB and Phenol: chloroform extraction method and was followed by the RNase A treatment. The quality and quantity of isolated metagenomic DNA were checked on Nano-Drop and agarose gel electrophoresis (0.8%), respectively.

Preparation of library and sequencing

The paired-end sequencing libraries were prepared using the Illumina TruSeq Nano DNA Library Prep Kit (as per the manufacturer's protocol), with ~200 nanograms of DNA. Then all the prepared libraries were processed for sequencing on the Illumina NovaSeq6000 platform using 2×150 base pairs (bp) chemistry to generate raw reads of ~2 GB data/sample. Sequence raw reads were submitted to *NCBI SRA (GenBank)*, with accession numbers *SRR20737934, and SRR20737933*.

Post-sequencing bioinformatics analysis

The obtained raw sequenced data of 2.39 and 2.72 Gb, respectively, were uploaded to the online MG-RAST (Meta Genome Rapid Annotation using Subsystem Technology- (Version 4)) server for the automated taxonomic and function annotation (Punyapwar & Mutnuri 2020). The generated accession numbers are *mgm4968861 and mgm4968862*. The taxonomy and functional annotation were decoded in Ref Seq and SEED subsystems database pipeline, respectively. The associated enzyme abundance was also investigated in the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases pipeline (Punyapwar & Mutnuri 2020).

RESULTS AND DISCUSSION

Optimised H-VFCW design

The BW generated per day by SHH toilet users (6 PE) was ~155 L. The optimised H-VFCW was sequentially divided into aerobic (20 cm unsaturated) and anaerobic (60 cm saturated) zonation (Figure 1), hence was termed as hybrid-VFCW. Thus, the H-VFCW design efficiently facilitated nitrification, denitrification, and EBPR, simultaneously. The comparative treatment performance analysis of the U-VFCW (HRT of 2.24 min) and the pilot-scale H-VFCW of 40 cm (HRT of 25 h 18 min) and 60 cm (HRT of 37 h 16 min) saturation, respectively, were studied, as shown in Figure 3.

Thus, from Figure 3, it can be seen that the overall comparative treatment efficiency was higher for the H-VFCW than the U-VFCW, except for COD and TAN removal was slightly more for U-VFCW by 2.44 and 14.49%, respectively. However, the hike in nitrate was observed in U-VFCW by 34.18%. The U-VFCW removed slightly more TAN and COD due to passive aeration of the 80 cm filter layers, which favoured nitrification and better organic matter degradation in aerobic conditions rather

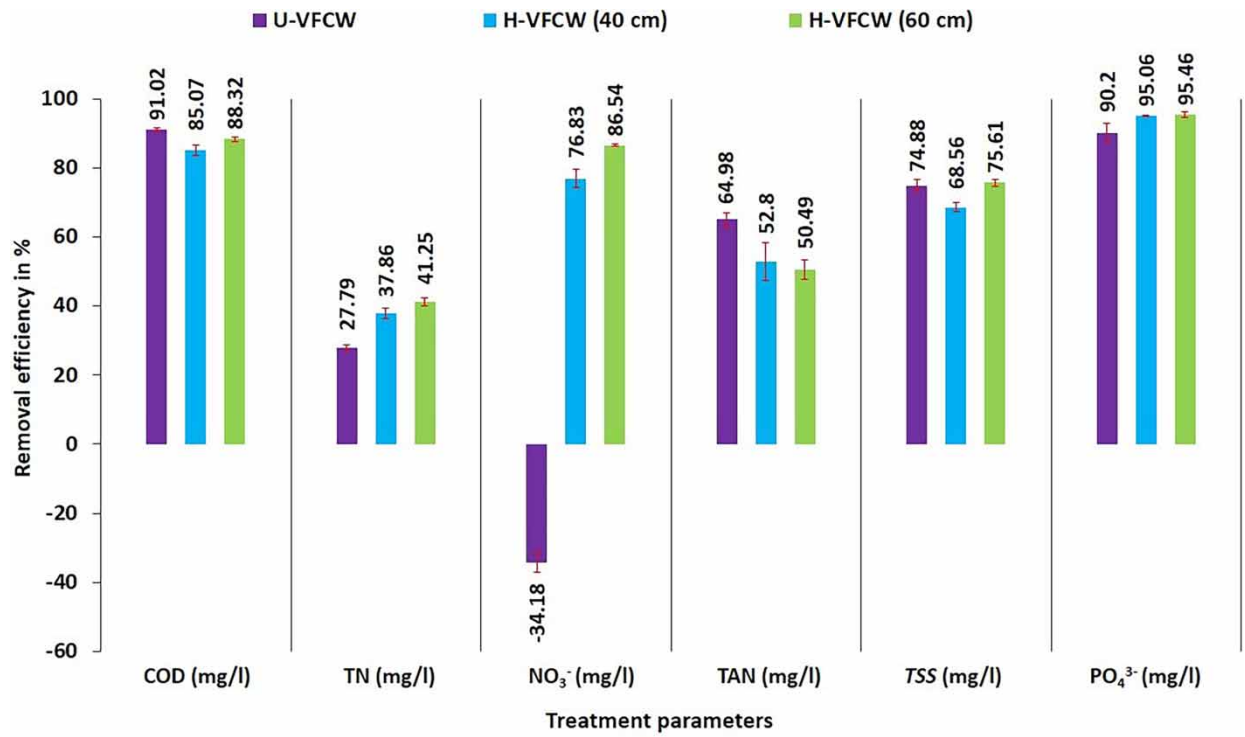


Figure 3 | Comparative optimisation of VFCW types for nitrogen and phosphorous removal.

than anaerobic. Hence, the absence of anaerobic conditions did not favour denitrification. Instead, the nitrification metabolism dominated, leading to an increase in nitrate concentration in the U-VFCW effluent.

The optimised pilot-scale H-VFCW; with 60 cm saturation height showed the overall highest removal efficiency than the 40 cm saturation, except the TAN removal was less by 2.31% (Figure 3). The major reason can be due to the extra 20 cm drainage layer filtration and more HRT in 60 cm saturation height, facilitating more microbial-BW contact time for bioremediation. Lower TAN removal at 60 cm saturation (–2.31%) was due to increased anaerobic height (20 cm) and HRT (12 h 2 min), allowing for more organic-nitrogen degradation time and thus releasing more ammonium. It also might be due to the dissimilatory nitrate reduction to ammonium (DNRA) process due to less nitrate concentration than in the H-VFCW of 40 cm saturation (DNRA process has higher nitrate affinity than the denitrification process in nitrate limited condition (van den Berg *et al.* 2016)).

The optimised full-scale H-VFCW showed the highest treatment efficiency- for COD, NO_3^-, PO_4^{3-}, and TSS than the other integrated treatment units (Figure 4). The input C:N ratio was 2.01 $\pm$ 0.13 (septic tank effluent). The *C. indica* plant was selected due to its high tolerance under the waterlogged and tropical monsoon climate of Goa. Hence, the VFCW limitations of hike in nitrate and poor phosphorous removal were resolved by the optimised design of H-VFCW as shown in Figures 3 and 4.

The critical drawback of H-VFCW as the secondary treatment for BW is the clogging issues if the primary treatment (sedimentation tank) fails or is filled up.

Optimised UAFBR for nitrate removal

The UAFBR was designed to remove the nitrate from H-VFCW effluent $\leq$10 mg/L (CPCB limits). The C:N ratio of U-VFCW and pilot-scale H-VFCW (40 cm) and H-VFCW (60 cm) was 0.96 $\pm$ 0.88 mg/L, 1.25 $\pm$ 1.03 mg/L, and 1.6 $\pm$ 0.12 mg/L, respectively. Hence, the pilot-scale optimisation of UAFBR was preferably post-integrated to H-VFCW (60 cm) due to the higher C:N ratio. The polypropylene carriers (model of BI 22) were used due to their high durability and stable properties and are easily available. The pilot-scale UAFBR optimisation study found the visible slimy layer (suspected biofilm) after 34 days. The biofilm was initially confirmed by the CV assay as shown in Figure 5, highlighting the increase in absorbance after every 1 h of incubation, revealing the multiplication of the actively growing biofilm-forming bacteria on the glass coverslip surface.

Then, the biofilm morphology was confirmed by the SEM analysis at different magnifications as shown in Figure 6(a)–6(d). The clumps of different shapes (cocci, rod, and racket shapes) and sizes of bacteria were observed (Figure 6(a)–6(d).

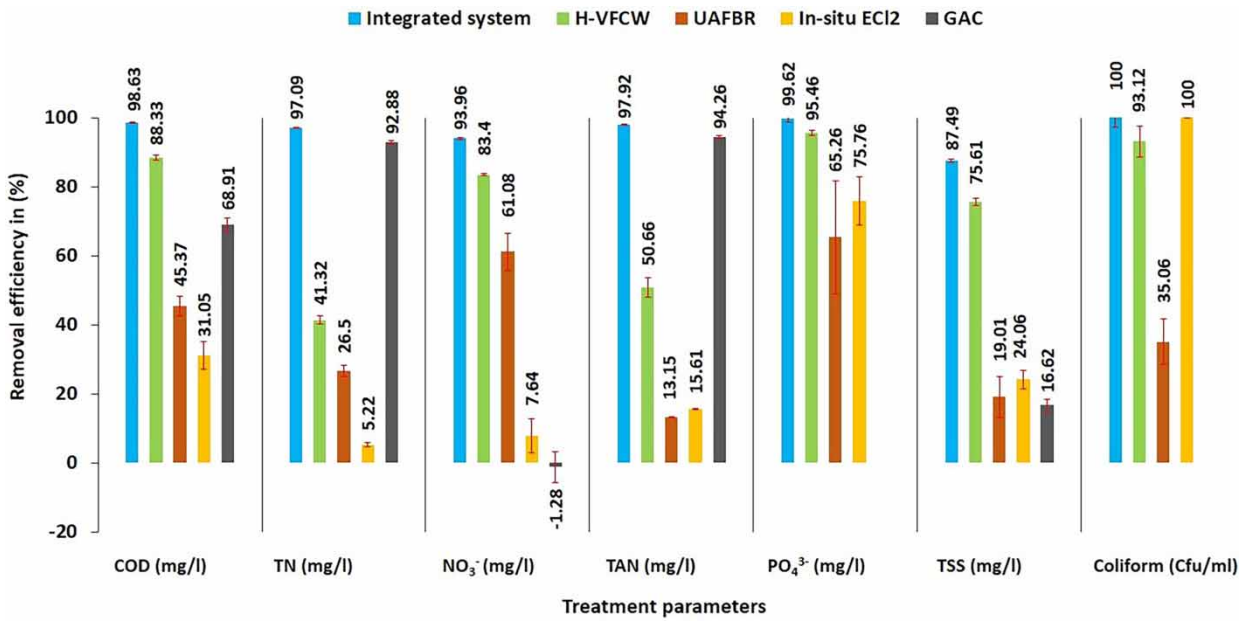


Figure 4 | The sequential wastewater treatment efficiency by an integrated treatment system.

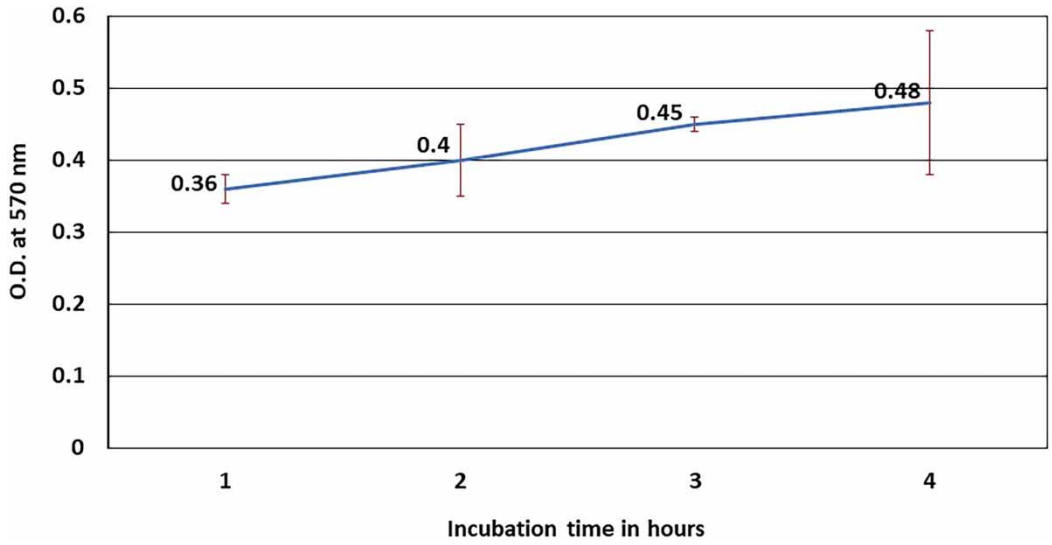


Figure 5 | Biofilm determination by a CV assay.

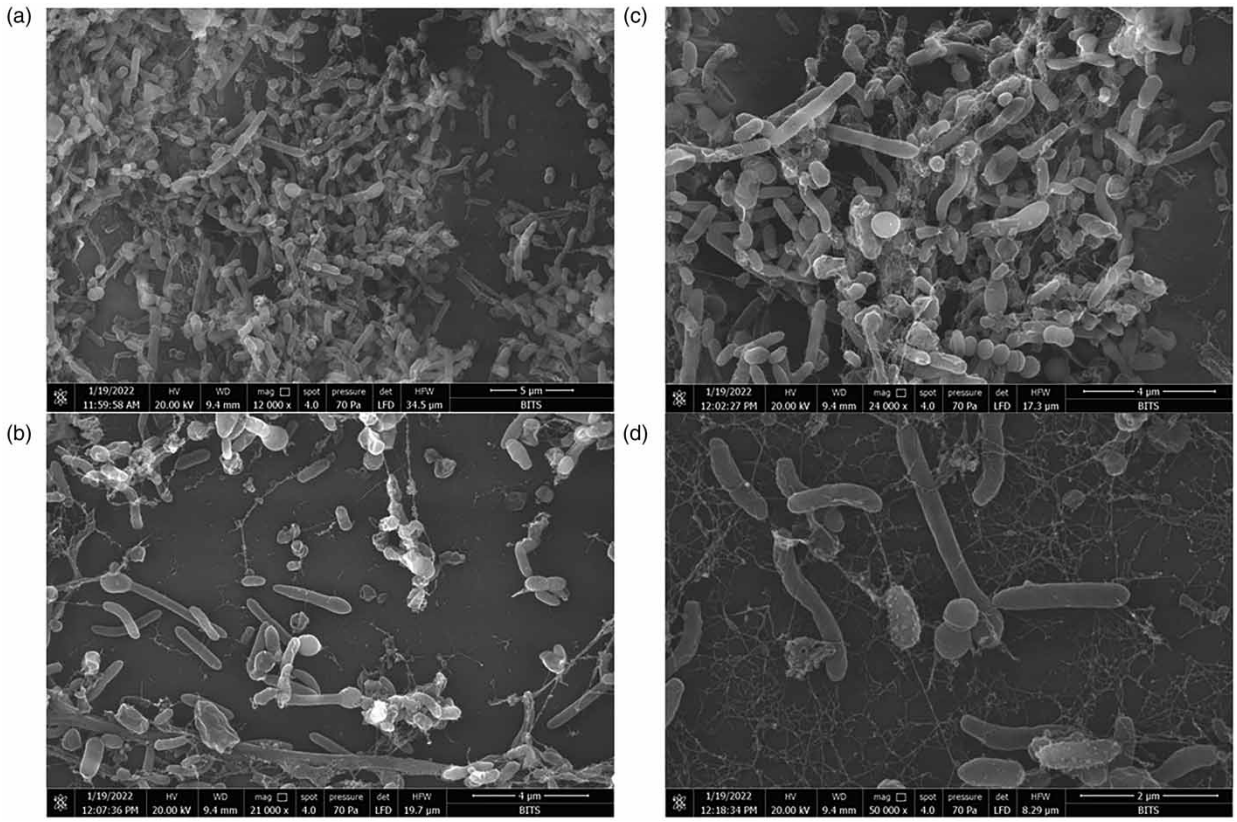


Figure 6 | SEM images of biofilm at a magnification of (a) 12,000×; (b) 21,000×; (c) 24,000×; and (d) 50,000×.

The pilot-scale UAFBR 3-day HRT was optimised based on the NO_3^- removal efficiency up to the CPCB discharge limits of <10 mg/L. The pH and temperature in each HRT zone were in a favourable range for denitrification as shown in Table 2.

Table 2 | Pilot-scale optimisation of UAFBR for nitrate removal

Average reduction				
Parameters	**H-VFCW (60 cm)**	**UAFBR (1-day HRT)**	**UAFBR (2-day HRT)**	**UAFBR (3-day HRT)**
COD (mg/L)	93.62 ± 6.28	64 ± 1.85	49.62 ± 0.65	44.54 ± 1.06
TC (mg/L)	189.05 ± 6.93	132.7 ± 8.01	128 ± 9.4	116.1 ± 13.06
TN (mg/L)	117.9 ± 1.97	101.6 ± 2.45	99.2 ± 2.52	97.4 ± 1.18
C:N (mg/L)	1.6 ± 0.12	1.31 ± 3.7	1.29 ± 5	1.19 ± 5.02
NO_3^- (mg/L)	23.22 ± 3.22	15.79 ± 0.8	11.51 ± 1.8	8.75 ± 1.23
TAN (mg/L)	80.39 ± 39.42	79.47 ± 5.11	76.87 ± 4.71	72.15 ± 2.67
PO_4^{3-} (mg/L)	2.49 ± 0.59	0.51 ± 0.02	0.35 ± 0.01	0.24 ± 0.03
TSS (mg/L)	35.01 ± 0.94	29.08 ± 1.72	25.58 ± 0.96	21.96 ± 1.18
pH	7.06 ± 0.09	7.18 ± 0.03	7.19 ± 0.04	7.22 ± 0.04
Temp. (°C)	-	26.28 ± 0.26	26.38 ± 0.19	26.15 ± 0.24

The highest nitrate removal efficiency was observed for 1-day HRT (32 ± 0.39%) followed by 2-day- HRT (27.11 ± 0.82%) and 3-day HRT (23.98 ± 1.53%) as shown in Table 2. The possible reason can be due to the availability of a more utilisable carbon source or high C:N ratio input for 1-day HRT (1.6 ± 0.12 mg/L) than the other two HRTs for the denitrification process (Table 2). Overall, the nitrate removal gradually decreased with a decrease in the C:N ratio, irrespective of the HRT or more treatment time (Table 2), hence the availability of a utilisable carbon source plays a critical role in nitrate removal or denitrification metabolism. Hence, the minimum HRT for the targeted NO_3^- removal (<10 mg/L) efficiency was found to be 3 days. The TAN removal was also observed by 13.16 ± 0.24%, which can be due to the ammonium assimilation process (Nelson *et al.* 2015). Hence, 3-day HRT was considered for the full-scale UAFBR implementation.

The optimised full-scale integrated UAFBR of 3-day HRT removal efficiency is shown in Figure 4. The input C:N ratio was 1.63 ± 0.09 mg/L. The final UAFBR effluent NO_3^- concentration was 7.59 ± 0.66 mg/L, which is up to the CPCB limits (Table 3). The major drawbacks of the UAFBR are; high HRT, requires a large number of carriers and prior sedimentation treatment, or else can clog the carriers (can interfere in surface biofilm formation) and the unit.

Metagenomic analysis

The UAFBR treatment is solely dependent on microbial biofilm metabolisms, hence understanding its composition and mechanism of treatment is of great importance. Thus, metagenomic sequencing unravelled the biofilm population and functional

Table 3 | Results of an optimised SHH toilet integrated treatment system

	Average reduction observed				
Parameters	**Septic tank**	**H-VFCW**	**UAFBR**	**In situ ECl_2**	**GAC**
COD (mg/L)	675.59 ± 34.32	78.82 ± 5.05	43.06 ± 0.71	29.69 ± 1.7	9.23 ± 0.86
TN (mg/L)	213.07 ± 92.67	125.04 ± 4.65	91.9 ± 2.17	87.1 ± 1.91	6.2 ± 0.51
NO_3^- (mg/L)	117.48 ± 51.67	19.5 ± 0.51	7.59 ± 0.66	7.01 ± 0.49	7.1 ± 0.36
TAN (mg/L)	239.43 ± 16.88	118.14 ± 1.78	102.6 ± 1.71	86.58 ± 1.67	4.97 ± 0.5
PO_4^{3-} (mg/L)	20.93 ± 1.00	0.95 ± 0.16	0.33 ± 0.2	0.08 ± 0.04	NA
TSS (mg/L)	111.52 ± 0.45	27.2 ± 1.24	22.03 ± 0.73	16.73 ± 0.37	13.95 ± 0.39
***Coliform* (cfu/mL)**	44.75×10^4 ± 19.9	3.08×10^4 ± 0.98	2×10^4 ± 0.68	0	NA
pH	7.12 ± 0.08	7.19 ± 0.03	7.31 ± 0.04	7.46 ± 0.09	8.4 ± 0.08
Residual chlorine (mg/L)	NA	NA	NA	1.64 ± 0.68	0.23 ± 0.06

NA, not analysed.

diversity (focused on nitrogen, phosphorous, and carbohydrates (carbon) pollutants removal). The decoded raw metagenomic data for taxonomy and functional annotation classification by MG-RAST are as follows.

Microbial taxonomy

The microbial population diversity proportions are 98.49 ± 0.2% bacteria, 0.636 ± 0.17% archaea, 0.594 ± 0.001% eukaryote, 0.278 ± 0.03% viruses, and 0.002 ± 0.001% other sequences. At the phylum hierarchy level bacteria and archaea showed 27 and five groups, respectively, as shown in Figure 7. The higher level of the hierarchy was centered around bacteria, particularly those that are capable of removing nitrogen and phosphorus. The dominated *Proteobacteria*, broadly plays an important role both in natural and artificial (CW) environments, like nitrification, denitrification, and PAOs (majorly by *Betaproteobacteria* and *Gammaproteobacteria class)* (Zeng *et al.* 2016). The other dominant phylum were *Firmicutes, Bacteroidetes and Actinobacteria*, which are also popularly known to be the diverse denitrifying phylum.

The *Proteobacteria* was dominated by *Betaproteobacteria* (68.74 ± 11.66%) and *Gammaproteobacteria* (18.01 ± 4.9%) (Supplementary material, Table S1). The revealed prominent denitrifying genera of *Betaproteobacteria* are *Achromobacter*, *Janthinobacterium*, *Thiobacillus,* etc. (Supplementary material, Table S2). The prominent denitrifying genera of *Gammaproteobacteria* are *Pseudomonas*, *Xanthomonas*, *Acinetobacter,* etc. (Supplementary material, Table S3).

The study also revealed some of the prominent PAO genera of *Betaproteobacteria*, i.e., *Candidatus* Accumulibacter, and also of *Gammaproteobacteria* genus, i.e., *Acinetobacter* and *Pseudomonas*.

Microbial function

The study unravelled the abundance of 28 types of metabolisms (Supplementary material, Table S4). Furthermore, the metabolisms of interest related to nitrogen, phosphorous, and carbohydrates (C source) were analysed (Figure 8), and the enzymes associated with nitrogen metabolism were investigated (Supplementary material, Figure S1(a–d)).

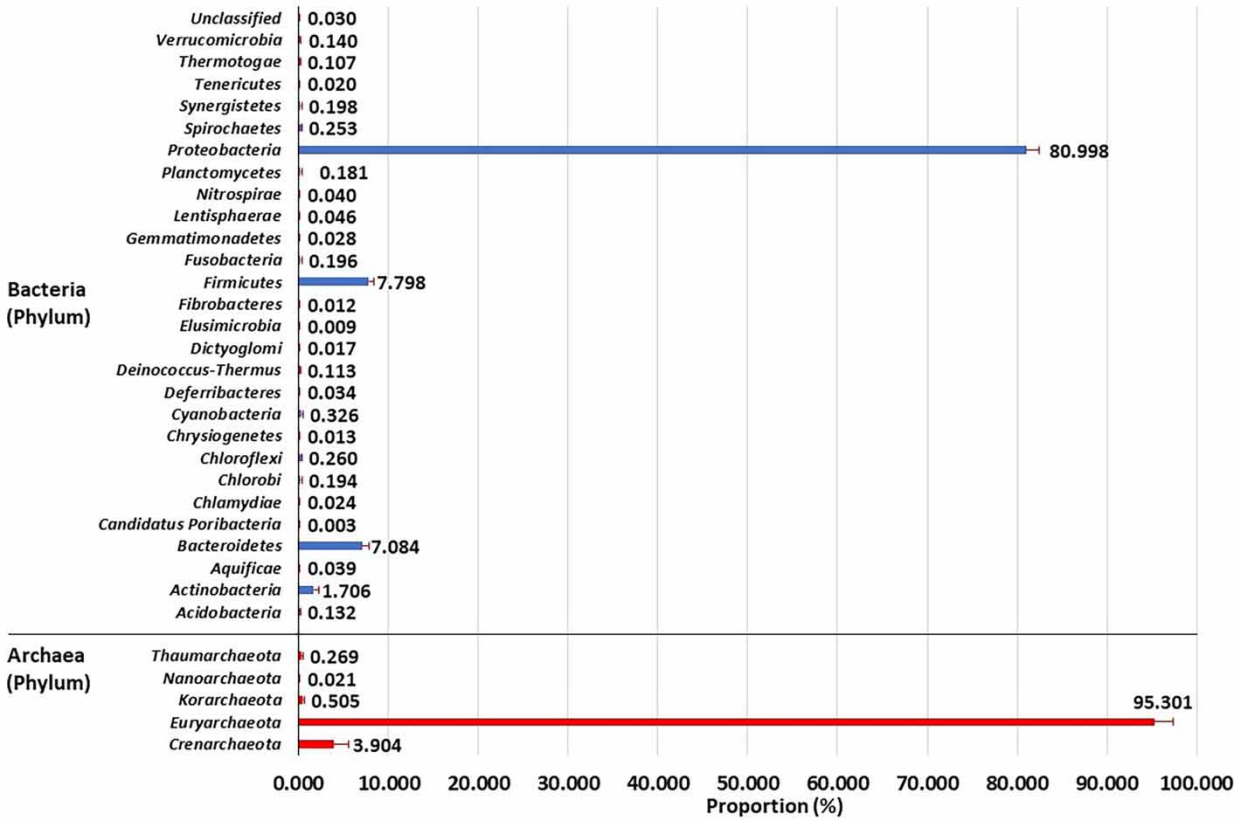


Figure 7 | Diversity of microbial community against Ref Seq database of biofilm metagenomes depicting hierarchical levels of phylum.

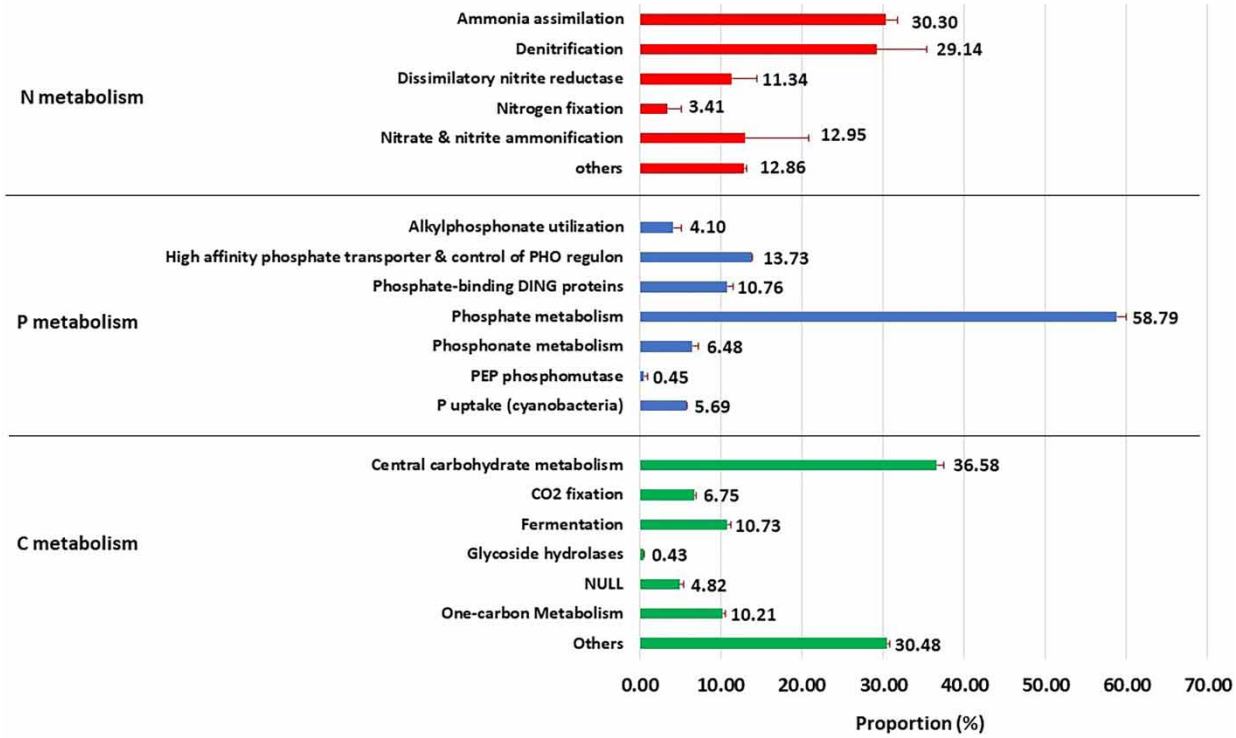


Figure 8 | Functional annotation of biofilm metagenomes classified against metabolism of nitrogen (N), phosphorous (P), and carbohydrates (C) in SEED subsystem level III.

Nitrogen metabolism

The major nitrogen metabolism abundance and dominance are shown in Figure 8. Broadly, the assimilatory pathways (ammonia assimilation, DNRA, and nitrogen fixation) dominated the dissimilatory pathway (denitrification and dissimilatory nitrite reductase) (Figure 8). The major reason can be due to the nitrate or nitrogen-limited conditions in the UAFBR system, directing the metabolic adaptation to conserve (assimilatory pathway) the nitrogen sources.

Ammonia assimilation is an adaptation to conserve inorganic nitrogen to an organic-nitrogen form intracellularly for various cellular activity requirements in a less nitrogen environment (Nelson *et al.* 2015). Hence, the anaerobic UAFBR treatment showed a reduction in ammonium concentration and that too more on 3-day HRT (least nitrate availability stage) (Table 2). The 19 variety of metabolism-associated enzymes were confirmed and were dominated by *Glutamate-ammonia-ligase adenylyl transferase (EC 2.7.7.42)*, *Glutamate synthase [NADPH] large chain (EC 1.4.1.13)* (Supplementary material, Figure S1(a)).

The major nitrate removal in UAFBR treatment was due to the *denitrification* metabolism (dissimilatory pathway) (Figure 8). The 20 variety of metabolism-associated enzymes were dominated by *Nitrous-oxide reductase (EC 1.7.99.6)*, *Nitrous-oxide reductase maturation protein NosD)* (Supplementary material, Figure S1(b)).

The second major nitrate removal was due to **nitrate and nitrite ammonification metabolism or DNRA**, which directly reduces nitrate or nitrite to ammonium (van den Berg *et al.* 2016). The DNRA metabolism is dominated under nitrate-limiting anaerobic conditions, with a mechanism to conserve the nitrogen source as ammonium in the habitat. The microorganisms with the *Nrf-gene can perform DNRA*, e.g., obligate anaerobes: *Clostridium, Desulfovibrio and* facultative anaerobes: *Enterobacter, Escherichia, Klebsiella, Pseudomonas* (Kuypers *et al.* 2018). The seven varieties of *Nrf*-gene presence were confirmed (Supplementary material, Figure S1(c)).

The **dissimilatory nitrite reductase** is in conjunction with the denitrification process, where the nitrite is reduced to nitric oxide rather than to ammonia (Punyapwar & Mutnuri 2020). Hence, it also contributes to the nitrate or nitrogen removal process. The 10 variety of metabolism-associated enzymes were confirmed and were dominated by *Nitrite reductase-associated c-type cytochrome NirN*, *Heme d1 biosynthesis protein NirF* (Supplementary material, Figure S1(d)).

Phosphorus and carbohydrates metabolism

The metagenomic study revealed seven types of microbial phosphorus removal mechanisms and their dominance is shown in Figure 8. The functional annotation of carbohydrates revealed the presence of heterotrophic and autotrophic modes of carbon utilisation. The autotrophic mode was confirmed by the CO_2 fixation metabolism with an abundance of 6.75% and the remaining percentage represented the heterotrophic metabolic type (Figure 8).

Both heterotrophic and autotrophic denitrification modes are present based on the denitrifying genera revealed through metagenomics and the co-occurrence of carbon metabolism modes. The revealed denitrifying genera with heterotrophic modes are *Pseudomonas, Xanthomonas, Acinetobacter, Serratia, Aeromonas, Halomonas, Achromobacter, Janthinobacterium, Kingella, Variovorax, Neisseria, Vibrio* and the denitrifying genera with autotrophic modes are *Paracoccus, Thiobacillus, Gallionella, Leptothrix, Thiomicrospira.* Hence, the autotrophic denitrification can be the reason for the nitrate removal even in the less C:N ratio on third day HRT of the UAFBR treatment system.

Optimised in situ ECl_2 disinfection

The in situ ECl_2 was integrated to resolve the incomplete disinfection by the nature-based treatment units (H-VFCW and UAFBR). The complete disinfection was achieved with the optimum parameters of 44.48 Am^{-2} of current density and 20 min of electrolysis time and with residual chlorine of 1.64 ± 0.68 mg/L. The achieved WW treatment volume was 154 L/day. The power consumption was 1.81 ± 0.14 $KWhm^{-3}$, which is significantly lower than any treatment scheme that employs in situ electrochlorination disinfection. The present optimised EC power consumption cost was only 6.15 INR m^{-3}. In contrast, the power consumption cost of a previously studied in situ ECl_2 disinfection unit by Gogoi *et al.* (2022) and Talekar *et al.* (2018) was 21.22 INR m^{-3} (6.24 ± 0.12 KWh/m^3) and 54.4 INR m^{-3} (16 ± 3 kWh/m^3), respectively. The reason can be because of an increase in current density by 4.45 Am^{-2}, less electrolysis time, and a low organic input load (due to pre-integrated H-VFCW and UAFBR than the previously studied EC units which had only U-VFCW pre-integrated).

Optimised GACC for TAN removal

The EC effluent concentration of TN and TAN was above the CPCB discharge limits (Table 3); hence, the GACC was integrated to remove the residual TAN based on the ammonia volatilisation method. GACC was set up in an open environment for ammonia volatilisation enhancement.

The laboratory-scale GACC input flow rate was optimised at 14 mL/min and was later scaled up to an optimum input flow rate of 200 mL/min for the field-scale GACC treatment (153 L/day). The GACC showed the highest removal efficiency for TAN and TN by 94.26 ± 0.46% and 92.88 ± 0.45%, respectively, than the other treatment units (Figure 4). The removal efficiency of other parameters is shown in Figure 4. However, the GACC ammonium removal suddenly decreased after 38 days of use (*breakpoint*), with an effluent TAN concentration above CPCB limits of 5 mg/L. The major reason might be due to the drop in pH to 7.3, which is below the ammonia volatilisation pH ($\geq$7.8). Hence, preventing ammonium conversion to volatile ammonia rather it was eluted in the effluent. Another reason might be due to GAC's active surface being gradually occupied by organic matter, Cl_2, etc. The present GACC study was to investigate the AVM application and remove TAN up to CPCB limits. Therefore, it was studied only till the breakpoint of the GACC. The cost of GAC used in the field-scale column was 149.82 GBP.

Furthermore, the changes in physio-chemical properties of GACC during the ammonia volatilisation process can be studied. The saturated GAC can be reactivated for use by either chemical or physical methods by dissolving or removing the adsorbate (Nwankwo 2018). However, the possibility of increasing the shelf life of the GACC specifically for TAN removal can be increased (as the ammonia formed in the column volatilises readily), by identifying and resolving the interfering pollutants in the WW input, like COD, TSS, Cl_2, etc.

CONCLUSION

Thus, the optimised decentralised integrated treatment systems achieved the CPCB effluent discharged standards. The H-VFCW design effectively resolved the VFCW issue of a hike in nitrate and also enhanced the overall BW treatment potential. The metagenomic study of UAFBR unravelled the potential of the heterotrophic and autotrophic modes of denitrification. Thus, the study of an autotrophic denitrification potential and its feasibility for WW treatment could be of

significant solution for the carbon crisis or the expense of dosing external carbon sources for denitrification. The aim of TAN removal by GACC and power consumption reduction of in situ ECl_2 was efficiently achieved.

The integrated H-VFCW and UAFBR treatment was achieved with zero energy operation and maintenance costs. This promises great sustainability for developing countries or future climate-resilient WW treatment technology. Furthermore, the UAFBR potential for co-integrated biogas production while treating BW can be studied.

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AUTHOR CONTRIBUTIONS

S.M. and J.K.G. conceived and designed the integrated treatment system. J.K.G. completed all the experiments and manuscript writing. S.P. guided the metagenomic analysis.

DATA AVAILABILITY STATEMENT

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

CONFLICT OF INTEREST

The authors declare there is no conflict.

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