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A novel integrative performance evaluation of constructed wetland on removal of viable bacterial cells and related pathogenic, virulent and multi-drug resistant genes from wastewater systems

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ABSTRACT

Integrative performance evaluation of constructed wetland in removal different aspects of bacteria under specific local environmental conditions needs to be explored in detail to ensure selection of appropriate and highly efficient macrophytes candidates. To achieve this, integrative purification performance evaluation approach that holistically considers all the aspects of pathogenic bacterial biology (colony numbers, functional gene, species, virulent, pathogenicity and resistant genes) needs to be adopted rather than the commonly known unidimensional approaches that take into account a single bacterial aspect. This study experimentally evaluated the individual performance of three native and one exotic winter tolerant submerged macrophytes combined with a single emergent macrophyte in eradicating faecal related bacterial species and pathotypes across horizontal surface flow constructed wetland. It involved the new multi-dimensional approach that integrated the faecal bacterial colony numbers, functional gene copies, species survival, virulent and pathogenicity as well as antimicrobial resistant in constructed wetland purification evaluation. The results showed *Elodea nuttallii* and *Myriophyllum spicatum* as the best candidate partners to *Typha latifolia* for the highest Purification Efficiency ($P < 0.05$), of above 97% for removal of faecal bacteria colonies and functional genes, and more than 75% for removal of faecal bacterial strains, pathotypes, virulent and well as resistant genes. However, *M. spicatum* being a Chinese native species should be much preferred to the invasive *E. nuttallii*. Therefore, the study recommends the application of local macrophytes such as *M. spicatum* as the best candidates and the decision should emanate from such a multidimensional/intergrative purification-based evaluation approach.

1. Introduction

Constructed wastewater wetlands have shown high capability in treating different kinds of raw wastewater [1,2]. Several studies have focused on the potential of constructed wetlands in the treatment of grey and black water from domestic effluents [3] (Paule et al., 2013; Marzec et al., 2019). Domestic raw wastewater contains various pollutants that range from organic matter, nutrients to pathogenic microorganisms, and these pollutants do find their way into the major water bodies [4]. Previously, majority of studies had given much attention on organic matter and nutrients removal as opposed to pathogenic pollutants removal [3]. The macrophytes role in raw wastewater purification occurs in many ways, they stabilize the surface of the beds, provide good conditions for physical filtration, prevent vertical flow systems from clogging, insulate the surface against frost during winter and provide a huge surface area for attached microbial growth [3,5]. Constructed wetlands with mixed macrophytes that occur in high abundances have proved to provide high removal of enteric bacteria, but the treatment effect may vary among systems, nature of plants and the design of the constructed wetlands [6]. Despite hybrid and subsurface flow constructed wetlands being the most effective in raw wastewater purification, the use of surface flow constructed wetland is

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still preferred due to its low installation and cost of maintenance (Muhsin et al., 2009).

The complexities in wetland functionalities call for several alternative testing methodologies that could provide adequate information on precise sources, movements and purification efficiencies of faecal pathogenic bacteria [7]. Indeed, the presence of pathogenic microorganisms in raw wastewater pose a risk to public health when untreated or inappropriately treated raw wastewater is discharged into surface waters. These facts represent a major hygienic concern, which needs to be effectively handled [8]. Faecal pathogens in raw wastewater can cause waterborne illnesses such as diarrhoea, typhoid fever, dysentery, cholera, and ineffective hepatitis and the main exposure root to these illnesses is through faecal-oral contamination that involves the consumption of water or food containing the illnesses causing pathogens (Shakoor et al., 2012). A study by Simachew et al. (2018) identified the co-occurrence of both single and multidrug resistant bacterial isolates in raw wastewater in Ethiopia, Africa. Their finding indicated that significant number of antibiotic-resistant bacteria were removed in vegetated broken brick and gravel bed constructed wetlands than the non-vegetated gravel bed wetlands. Furthermore, Ricardo and Eloy (2013) showed that constructed wetland was better than conventional raw wastewater treatment plant in the removal of bacteria and not the drug resistant genes from raw wastewater. They recommended the identification and use of correct wetlands macrophytes for a better removal of the drug resistant genes by constructed wetlands. Wetland ecological studies have emphasized that plant species selection is the best way to further maximize pollutant removal in CWs [6,9], and that native plant species of polluted sites have proved to be good candidates [10].

According to Shah et al. [11], the application of macrophytes in municipal raw wastewater treatment under specific local environmental conditions needs to be explored in detail. This is very essential because, apart from their performance being comparable to conventional raw wastewater treatment plants, they have low operation and management costs [6] (DiMuro et al., 2014; Georgios et al., 2018; Irwin et al., 2018). Moreover, one of the tricky tasks prior to the designing of a constructed wetland treatment facility is the selection of the appropriate aquatic plant [6,12]. Additionally, the influence of weather condition mediated environmental parameters' effects on the integrity of constructed wetlands has continued to draw attention from domestic wastewater researchers, practitioners and regulators [13]. Specifically, most wetlands in China have recorded a low purification efficiency in pollutant removal during winter season due to the inability of most macrophytes to withstand the cold winter temperatures (Yan and Xu, 2014; Mo et al., 2017). Furthermore, the growth performance of most macrophytes is always hindered by the cold winter conditions, making the performance of most constructed wetlands to drop during the winter seasons [13] (Ottor and Hook, 2005; Marianna et al., 2012). In this regard, more studies are currently focusing on the utility of aquatic macrophytes for the simulated conditions, to improve raw wastewater purification efficiencies based on the existing environmental conditions or seasons [3].

The available information regarding the ideal combination of species to acquire close to 100% wastewater purification efficiencies in constructed wetlands is still inadequate in most regions [12,14]. Moreover, most study designs that have focused on evaluating the purification performance of constructed wetlands in the removal of faecal pathogenic and resistant genes have only focused on individual aspects of the bacterial biology such as colony forming units [15], gene copy numbers [16], virulence and pathogenicity [17] and resistance [18]. Faecal indicators reductions of 1.4 log that accounts to approximately 85% purification efficiency had been recorded in Echinochloa pyramidalis under horizontal surface flow constructed wetland [19]. Maria et al. recorded PE of 90% on bulrush under surface flow constructed wetland while Juncus effusus L., Scirpus validus L., and Typha latifolia L. all recorded a reduction of three order of magnitude (75% purification efficiency) [20]. Elodea nuttallii, Hydrilla verticillata, Elodea canadensis, Myriophullum spicatum and Potamogeton crispus in Wastewater Polishing Pond Enclosures gave a purification efficiency of more than 90% in the removal of bacterial colony units [6]. Therefore, this study evaluated the individual performances of three Chinese native types of winter tolerant submerged macrophytes (*Hydrilla verticillata, Myriophyllum spicatum* and *Potamogeton crispus*) and one exotic species of Japanese origin (*Elodea nuttallii*) [21], each combined with emergent macrophyte (*Typha latifolia)* in eradicating faecal related bacterial pathotypes and virulence across horizontal surface flow constructed wetlands during the winter season. It employed the new multidimensional purification performance evaluation approach that integrated the removal evaluation for faecal bacterial colony numbers, functional gene copies, species survival, virulent and pathogenicity as well as antimicrobial resistant genes.

2. Methods

2.1. Experimental set-up

The experimental set-up consisted of a physical microcosm model of raw wastewater treatment plant that was constructed within the domestic wastewater experimental field station at Lake Dianchi (between $102^0 35'$ to $102^0 50'$ E and $24^0 40'$ to $25^0 00'$ N) in Kunming City of Yunnan Province of the Peoples' republic of China. The field station was run by the Institute of Hydrobiology of the Chinese Academy of Sciences [6]. This study was conducted through 6 batch experimental runs with every experimental run lasting for 18 days between the winter months of October 2016 to December 2017. The experiment was set-up as illustrated in Fig. 1 Part A. It involved four pairs of experimental tanks plus additional one pair as control. Each pair was made of two interconnected plastic tanks, with each tank measuring 1.2 m in length × 0.6 m in width × 0.6 m in depth. The first tanks were marked A1, A2, A3, A4 and A5, while the second tanks were marked B1, B2, B3, B4 and B5 respectively for pairs 1, 2, 3, 4 and 5. In pairs 1 to 4, the A tanks were filled with substrate of mixed soil sediment (silt, sand and clay) of fine particles (0.25–0.5 mm diameter) from lake bottom with porosity values of 0.17–0.27 to 20 cm wet depth and emergent macrophyte *Typha latifolia* (TL) planted into them. The B tanks were filled with the same substrate to 10 cm depth and different submerged macrophytes planted into them; *Hydrilla verticillate* (HV) in B1*, Elodea nuttallii* (EN) in B2*, Myriophyllum spicatum* (MS) in B3 and *Potamogeton crispus* (PC) in B4. The 5^{th} pair of tanks was used as control (C), with the same setting but no macrophytes planted in them (Fig. 1 Part B). The plant density was 20 individuals per tank and 30 individuals per tanks respectively for submerged and emergent macrophytes. The root depth was within the range of 5 to 10 cm and 10 to 15 cm respectively for submerged and emergent macrophytes. The experiment was carried out after one month from the macrophyte planting time and ended before the flowering stage. All the pairs had a common reservoir that supplied domestic raw wastewater into the system through A tanks to B tanks up to the 55 cm height. The raw wastewater had the initial characteristics of 60 mg/L, 6 mg/L, 0.2 mg/L, 1 ~ 2.0 mg/L, 200 mg/L and 6–8 mg/L respectively for Chemical Oxygen Demand (COD), Nitrate, Nitrite, Ammonium, total suspended solids and total Nitrogen. The flow rate of water into the A tanks was 400 mL/min (3.45×10^7 mm/day) while the hydraulic retention time between the inflow points at As and out flow points at Bs was approximately 2 days on average for each set of tanks. The lower hydraulic retention time of 2 days was chosen based on the recommendation by Maria et al. (2015), which showed that the lower hydraulic retention time enables constructed wetland to exhibit the highest pollutant removal efficiency. All the experimental tanks were acclimatized to raw domestic wastewater condition through flashing by the raw wastewater prior to the study.

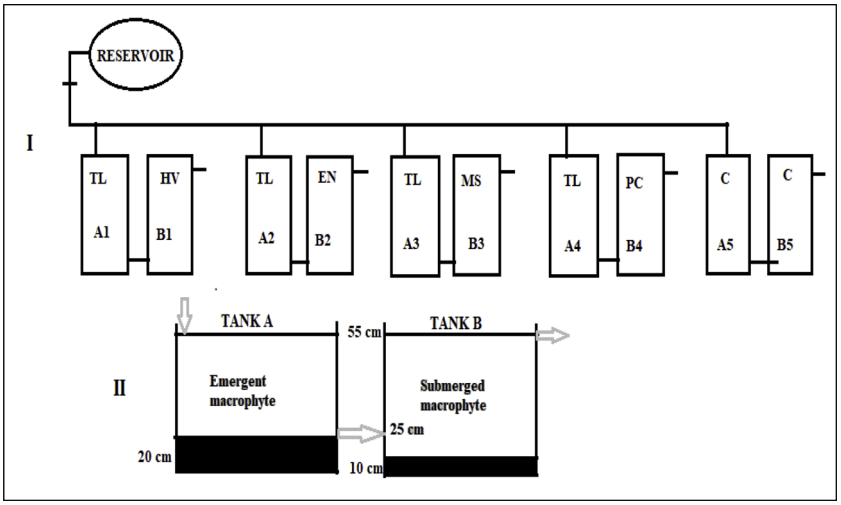


Fig. 1. Experimental set-up showing the arrangement the pairs of tanks (I) and the depth of the wet substrate, the macrophyte and movement of water between Tanks A and B (II). (*Typha latifolia* (TL), *Hydrilla verticillate* (HV), Elodea nuttallii (EN), *Myriophyllum spicatum* (MS), *Potamogeton crispus* (PC), control (C)).

2.2. Sampling and analyses

For each of the experimental run, sampling was done in triplicate after every six days, giving a total of 18 sampling episodes. During every sampling episode, measurement of Physico-chemical parameters; temperature, Dissolved Oxygen (DO), conductivity, Total Dissolved Solids (TDS), salinity and pH was done *in situ* using YSI ProODO™ handheld multi parameter meter (USA). This was carried out in triplicate in influent and in all the A and B tanks. The samples for bacterial analysis were obtained from the inflowing raw wastewater prior to its introduction into the A tanks, the raw wastewater that leaves A tanks (enter B tanks) and raw wastewater that leaves B tanks. In addition, samples for bacterial analysis were also obtained from the sediment and biofilm in all the tanks. The bacterial analyses involved Membrane Filtration Technique (MFT), Polymerase Chain Reaction (PCR), real time quantitative-PCR (qPCR) and DNA sequencing to determine the abundances of total coliforms, *E. coli* and faecal streptococci, strains and virulence/pathotypes of dominant persistent members of *Enterobacteriaceae* and *Enterococcaceae* at different treatment points.

2.3. Determination of bacterial abundances through MFT

Bacterial abundances across the treatment tanks was measured using Membrane Filtration Techniques (MFT) as outlined in American Public Health Association (APHA) [22], Public Health England (PHE) [23] and as summarised in Donde et al. [24]. In summary, the laboratory analysis was done within 24 hours after sampling to eliminate errors in bacterial counts as a result of bacterial growth or die off. There was strict adherence to aseptic techniques. The abundance of coliforms and intestinal enterococci were determined using selective Chromocult Coliform Agar (Merck) and Enterococcus selective agar (Sigma-Aldrich) respectively. Incubation conditions were 37 °C for 24 hours for coliform bacteria and 44 °C for 36 hours for enterococcus bacteria.

2.4. Determination of bacterial abundances through PCR

Bacterial abundances across the treatment tanks was also achived by quantifying the functional gene copies through realtime QPCR following the procedure under Quirós et al. (2015) and as previously summarized in Donde et al. [24]. This was carried out in all the samples from inflowing raw wastewater prior to its introduction into the A tanks, the raw wastewater that leaves A tanks (enters B tanks) and raw wastewater that leaves B tanks. In addition, realtime QPCR was also carried out for samples obtained from the sediment and biofilm in all the tanks.

2.5. Macrophytes purification efficiencies

Purification efficiencies was determined by comparing the quality of water that enters and leaves A tanks (A inlet against A outlet), quality of water that enters and leaves B tanks (B inlet against B outlet) as well as comparing the quality of water that enter A tanks verses what leaves B tanks (A inlet against b outlet).

2.6. Variation in Escherichia and Enterococcus strains across the treatment tanks

The variation in the species of *Escherichia* and *streptococci* across the constructed wetland treatment system was evaluated through sequencing of 16S rRNA gene using Primers (27F: 5-GAGTTTGATCCTGGCT CAG-3 and 1492R: 5- GGTTACCTTACGACTT-3). The detailed procedure is previously outlined in Donde et al. [6].

2.7. Detection of Escherichia and Streptococci pathotypes and resistant genes

Detection of genes that are associated with five types of Diarrheagenic *E. coli* (DEC); Entero-pathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), Enterotoxigenic *E. coli* (ETEC), Entero-aggregative *E. coli* (EAEC) and Entero-invasive *E. coli* (EIEC) was done. For the detection of *E. coli* Diarrheagenic genes, two multiplex PCR assays were used together with appropriate primers. For detection of *Enterococcus* pathotypes, genes associated with *Eterococcus faecalis* and *E. faecasum* were targeted and detected using appropriate primers. All the analysis procedure used in this section are described in detail in Donde et al. [6]. Detection of antimicrobial resistant genes was performed according to the protocol described in Momtaz et al. [25].

3. Results

3.1. Physico-chemical parameters across the tanks

The values of physico-chemical parameters (Temperature, DO, Conductivity, TDS, Salinity and pH) were as provided in Table 1. There was statistical difference in temperature, DO and pH values between and within most of the A tanks and B tanks in pairs 1 to 4. Conductivity, TDS and salinity did not show statistical difference between tanks 2 to 5. However, temperature, DO and pH showed significant difference across all the tanks.

Table 1
Mean ± standard error values of Physico-chemical parameters

Tanks	TEMP (°C)	DO (mg/L)	COND (µs/cm)	TDS (mg/L)	SAL (ppt)	pH
Influent	14.12 ± 0.64 A	11.19 ± 0.65 A	731.39 ± 7.14 A	514.49 ± 6.75 A	0.31 ± 0.01A	9.86 ± 0.05 A
1A	13.7 ± 0.48 B	5.73 ± 0.58 B	654.3 ± 9.93 B	437.72 ± 13.38 B	0.37 ± 0.01 A	8.19 ± 0.06 B
1B	14.68 ± 0.67 C	11.20 ± 0.52 A	646.05 ± 5.64 B	418.254.13 B	0.32 ± 0.01 A	9.65 ± 0.04 A
2A	13.77 ± 0.64 B	11.21 ± 0.87 A	642.82 ± 11.70 B	406.33 ± 7.02 B	0.31 ± 0.01 A	9.38 ± 0.06 A
2B	14.49 ± 0.71 C	10.15 ± 0.43 C	655.66 ± 10.78 B	430.64 ± 6.68 B	0.32 ± 0.01 A	10.30 ± 0.06 C
3A	13.91 ± 0.57 B	9.31 ± 0.73 C	613.69 ± 7.77 B	399.09 ± 5.56 B	0.30 ± 0.01 A	9.86 ± 0.07 A
3B	15.08 ± 0.65 C	10.57 ± 0.71 AC	687.08 ± 11.81 B	439.61 ± 9.69 B	0.34 ± 0.01 A	10.56 ± 0.06 C
4A	13.91 ± 0.58 B	9.32 ± 0.76 C	693.69 ± 16.64 B	449.85 ± 10.84 B	0.34 ± 0.01 A	9.32 ± 0.06 A
4B	14.78 ± 0.66 C	12.47 ± 0.61 D	686.91 ± 12.15 B	459.20 ± 13.93 B	0.34 ± 0.01 A	10.31 ± 0.04 C
5A	14.11 ± 0.64 A	11.20 ± 0.65 A	730.39 ± 7.14 A	515.49 ± 6.75 A	0.31 ± 0.01 A	9.85 ± 0.05 A
5B	14.55 ± 0.64A	10.71 ± 0.38 AC	747.32 ± 33.03 A	533.71 ± 7.21 A	0.33 ± 0.01 A	9.81 ± 0.08 A
P-value	0.0205	0.0344	0.0425	0.0435	0.0725	0.0352

Values with different letters within the rows are significantly different at P = 0.05, TEMP = Temperature, DO = Dissolved oxygen, COND = Conductivity, TDS = Total Dissolved Solids and SAL = salinity

3.2. Bacterial abundances through MFT

The reduction trend in total coliform, *E. coli* and faecal streptococci values through MFT were as provided in Figs. 2A, 2B and 2C. The raw water (prior to treatment) had higher values than that at the semi treatment stage (B inlets) and the one after the full treatment (B water). There were statistical differences between the semi treated wastewater and the one after full treatment across all the pairs. Tanks 1 to 4 showed higher purification efficiencies for total coliform, *E. coli* and faecal streptococci as compared to tank 5 (control).

3.3. Variation in bacterial abundances through qPCR across the tanks

The reduction trend in median values for gene copy numbers for *lacZ*, *uidA* and *cyd* through QPCR were as provided in Figs. 3A, 3B and 3C. The median values for gene copies numbers in raw water were higher than what was in the semi treatment stage (B inlets) and the one after the full treatment (B water) in pairs 1 to 4. However, the values for control (tank 5) were significantly higher at the semi treatment stage (5B-inlet), with slightly lower values than the one in raw water (*lacZ* and *cyd*) or higher values than the one in raw water (*uid*A). The values at the semi treatment stage of tank 5 (5B-inlet) recorded a wider range in median 25th and 75th percentile values than in all other tanks.

3.4. Variation in colony forming units and functional genes across the treatment tanks

The Purification Efficiencies (PE) values were calculated based on the reduction trends in total coliforms, *E. coli* and faecal streptococci as well as for *lacZ, uidA* and *cyd* genes across the treatment tanks. The PE values are provided in Tables 2 and 3. PE were calculated using the formula; PE= (Inlet-Outlet)/Inlet×100 are provided in. The PE were calculated for A tanks [(A nlet - A Outlet)/A inlet×100], B tanks [(B Inlet – B Outlet)/B Inlet × 100] as well as for fully treated wastewater from A inlet to B outlet [(A Inlet-B Outlet)/A Inlet×100]. For fully treated wastewater (from A inlet to B outlet), the PE for total coliform was highest at tank 2 and lowest at tank 5, for *E. coli* was highest at tank 2 and lowest at tank 5, for faecal streptococci was highest at tank 3 and lowest at tank 5, for *lacZ* genes was highest at tank 4 and lowest at tank 3, for *uidA* gene was highest at tank 1 and lowest at tank 3 while for *cyd* was highest at tank 4 and lowest at tank 5. The average PE for the removal of viable indicator bacteria and faecal pathogenic bacterial functional genes is provided in (Fig. 4).

3.5. Variation in Escherichia and Enterococcus strains across the treatment tanks

The detection of various species of *Escherichia* and *Streptococci* are recorded in Table 4. A total of 13 *Escherichia* strains were detected in raw water from the source reservoir. The following were the consequent total number of *Escherichia* strains recoded at the outflows from A tanks and out flows from B tanks; 7 and 3, 6 and 1, 5 and 1, 7 and 5, 12 and 9 respectively for tanks 1, 2, 3, 4, and 5. Generally, low *E. coli* species were recorded in tanks 2 and 3 than in tanks 1, 4 and 5. *E. coli* O157:H7 and its closest relative *E. coli* strain TYN 130606 [6] showed the highest resistant to the treatment process as compared to the other species across all the tanks. A total of 7 *Enterococcus* strains were detected in raw water from the source reservoir. The following were the consequent total number of *Enterococcus* strains recoded at the outflows from A tanks and outflows from B tanks; 5 and 3, 4 and 2, 3 and 1, 5 and 4, 7 and 6, respectively for tanks 1, 2, 3, 4, and 5. Lowest number of *Enterococcus* strains were recorded in tank 3. The average PE based based on the removal of *Escherichia* and *Enterococcus* strains across the treatment tanks is provided in Fig. 5.

3.6. Detection of Escherichia Pathotypes and its and antimicrobial resistant genes across the treatment tanks

As highlighted in the methodology section, multiplex PCR technique [6,26,27] was run to detect the presence of virulence genes associated with various *E. coli* pathotypes and those associated with two Enterococcus species. There were total of 18 Multiplex PCR runs that corresponded to each sampling episode as described in the sampling and analyses section. The detection of gene markers for Enterotoxigenic *E. coli* (ETEC), Entero-aggregative *E. coli* (EAEC), typical and atypical Enterohemorrhagic *E. coli* (EHEC), typical and atypical Entero-pathogenic *E. coli* (EPEC) and Entero-invasive *E. coli* (EIEC) as well as those for enterococcus species are provided in Table 5 and Fig. 6. Tanks 2 and 3 recorded the lowest values of both the *E. coli* pathotypes and Enterococcus related genes. The results on the purification efficiency based on the detection of resistant genes across the treatment tanks are provided in Table 6. Tanks 2 and 3 proved to be the most effective in the eradication of *E. coli* related resistant genes. The average PE based on the removal of *E. coli* pathotypes, *Enterococcus* related genes and antimicrobial resistant genes across the treatment tanks is provided in Figs. 6 and 7.

Considering the Purification Efficiency (PE) in the removal of all the faecal bacterial characteristics, tanks 2 and 3 further showed higher PEs values which were of more than 75% for each characteristic (*E. coli* colony CFU, total coliforms CFUs, Faecal streptococci CFUs, *E. coli* Resistant genes, *Escherichia and streptococci* strains and *E. coli* pathotypes (Fig. 4.12) and the average PE across the tanks based on all the studied parameters is provided in Fig. 8A and 8B.

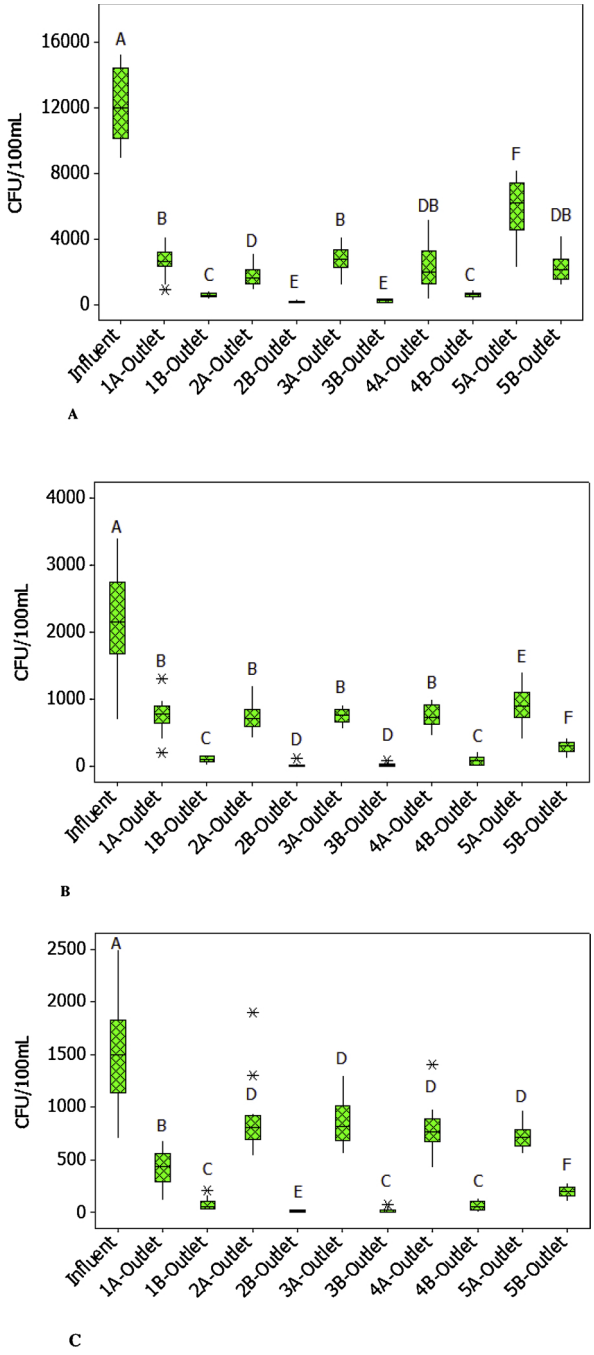


Fig. 2. A: Box and whisker plots of median (25%, 75% interval) Values of total coliforms in influent, outlet of A tanks and outlets of B tanks. Box range is the 25th-75th percentile. Whisker range is the maximum and minimum values. The median is represented by solid horizontal lines in each box. Where analysis of variance (ANOVA) on ranks was significant ($P < 0.05$, $n = 15$), Tukey tests was performed to determine sites that were significantly different for each parameter (indicated with different letters). **B:** Box and whisker plots of median (25%, 75% interval) Values of *E. coli* in influent, outlet of A tanks and outlets of B tanks. Box range is the 25th-75th percentile. Whisker range is the maximum and minimum values. The median is represented by solid horizontal lines in each box. Where analysis of variance (ANOVA) on ranks was significant ($P < 0.05$, $n = 15$), Tukey tests was performed to determine sites that were significantly different for each parameter (indicated with different letters). **C:** Box and whisker plots of median (25%, 75% interval) Values of faecal streptococci in influent, outlet of A tanks and outlets of B tanks. Box range is the 25th-75th percentile. Whisker range is the maximum and minimum values. The median is represented by solid horizontal lines in each box. Where analysis of variance (ANOVA) on ranks was significant ($P < 0.05$, $n = 15$), Tukey tests was performed to determine sites that were significantly different for each parameter (indicated with different letters).

4. Discussion

Several studies have shown significant reduction of *E. coli* between constructed wetland influent and effluent, and suggested to be due to the dynamics in the nature and quantity of nutrients, competition/predation by other microorganisms, release of bactericidal exudates by plants as well as bacterial die off that occurs across the treatment system [3,8]. The reduction of *E. coli* was an evident of the significant removal of pathogens that may enter into the surface water from different forms and sources of raw wastewater. The removal of the main pollutants from the raw wastewater would ensure clean and safe environment and reduced outbreak of water related illnesses [17]. The effects of physico-chemical parameters on the survival of bacteria have been extensively studied [28]. Changes in the physico-chemical parameter values between the A tanks and B tanks for each set was more noticeable for dissolved oxygen than for all the other parameters. The small general decline in DO values between the A tanks and the B tanks could be due to the increase in temperatures between the A tanks and B tanks as a result of the increased ability of the sun's radiation to penetrate through the floating macrophytes as opposed to the emergent macrophyte (cattail). The statistical differences in temperature, DO and pH values between most of the A tanks and B tanks in pairs 1 to 4 could be attributed to the consequential variation in bacterial counts as earlier reported in Salem et al. [29] and Rop et al. [4]. The relatively higher values of DO could be as a result of aeration that was caused by the supplying water pump as well as the strong wind.

Previous studies have shown a reduction trend in the bacterial colony counts across the constructed wetland systems. A study by Ibekwe and Murinda had showed significant reduction in *E. coli* populations from wetland influent to the final effluent and recommended the use of continuous flow-constructed wetlands in reducing contaminants from different waste water sources [17]. Generally constructed wetlands were found to be more efficient than algae-based systems in bacterial removal efficiencies [30]. Under stable environmental conditions, the percentage removal efficiencies have been over 90%, but no integrative efficiency evaluation has been done during the harsh season when the environmental conditions are unfavorable to the survival of most macrophytes [8]. The present study showed that raw water had the highest bacterial counts for total coliforms, *E. coli* and faecal streptococci than the water that was leaving all the A and B tanks. The bacterial colony forming units at the outlets of B tanks for sets 1 to 4 were all lower than that at the out let of the control set (5B). The trend was replicated in the functional gene copy numbers of *lacZ*, *uidA* and *cyd* through QPCR. This was an evidence that macrophytes play significant role in faecal pathogenic bacteria removal and combining both the two sets of macrophytes (emergent and submerged) was key to achieving that. However, of interest to note was the lower MFT values for total coliforms, *E. coli* and faecal streptococci as well as the lower QPCR values for *lacZ*, *uidA* and *cyd* copies in tank 5B than the A tanks in sets 1 to 4. This raises an interesting argument, where the control (5B) with no macrophyte depicting some sort of faecal bacterial removal. This unexpected finding could hypothetically be attributed to the role of ultraviolet radiation from the short sunny periods that occurred during the experiment and contributed in eradicating faecal bacterial pathogen. The control set of tanks were open with no macrophytes, hence the microbes could have been directly exposed to the ultraviolet radiation that caused their reduction in counts due to die-off as had been showed in studies by Donde et al. [31] and Alvarez et al. [32]. This finding leads to a suggestion that integrating UV radiation into constructed wetland technology may be fundamental in achieving the highest raw wastewater purification efficiencies in a much more sustainable manner.

The *T. latifolia* has been recorded to be a better macrophyte for removal of bacterial cells from domestic wastewater with a purification efficiency of greater than 90% [33]. The have been high removal of epiphytic bacterial community from raw wastewater flowing across *H.*

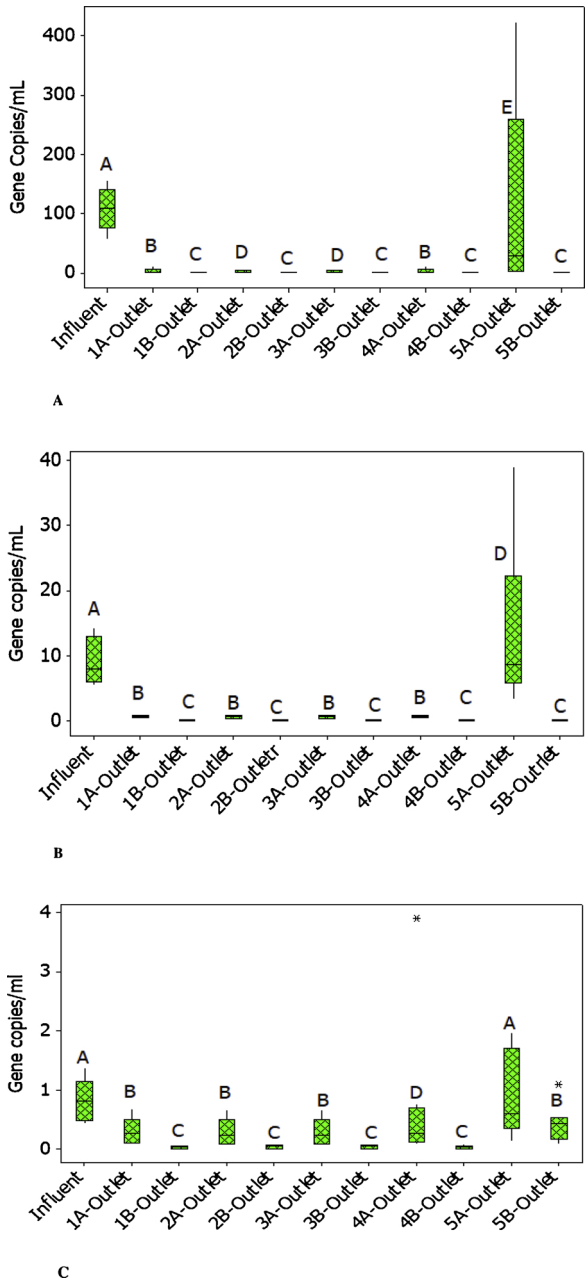


Fig. 3. A: Box and whisker plots of median (25%, 75% interval) Values $\times 10^6$ copy numbers of *lacZ* gene in influent, outlet of A tanks and outlets of B tanks. Box range is the 25th-75th percentile. Whisker range is the maximum and minimum values. The median is represented by solid horizontal lines in each box. Where analysis of variance (ANOVA) on ranks was significant ($P < 0.05$, $n = 15$), Tukey tests was performed to determine sites that were significantly different for each parameter (indicated with different letters). **B:** Box and whisker plots of median (25%, 75% interval) Values $\times 10^6$ copy numbers of *uidA* gene in influent, outlet of A tanks and outlets of B tanks. Box range is the 25th–75th percentile. Whisker range is the maximum and minimum values. The median is represented by solid horizontal lines in each box. Where analysis of variance (ANOVA) on ranks was significant ($P < 0.05$, $n = 15$), Tukey tests was performed to determine sites that were significantly different for each parameter (indicated with different letters). **C:** Box and whisker plots of median (25%, 75% interval) Values $\times 10^6$ copy numbers for *cyd* gene in influent, outlet of A tanks and outlets of B tanks. Box range is the 25th–75th percentile. Whisker range is the maximum and minimum values. The median is represented by solid horizontal lines in each box. Where analysis of variance (ANOVA) on ranks was significant ($P < 0.05$, $n = 15$), Tukey tests was performed to determine sites that were significantly different for each parameter (indicated with different letters).

verticillata with a removal efficiency of greater than 95 % [34,35]. Sun et al. [36] showed the good ability of *Myriophyllum* sp. in the removal of nitrite-oxidizing bacteria from swine raw wastewater. The over 88% performance of *P. crispus* in the removal epiphytic and other bacteria communities have also been documented [37,38]. The allelopathy of *E. nuttallii* is one of its characteristics that has made it to be greatly considered in constructed wetlands for treatment of wastewater containing pathogenic bacteria species, especially cyanobacteria in epiphytic biofilms [39,40]. Based on this characteristic, *E. nuttallii* has achieve an efficiency of above 94% in the removal of most pathogenic microorganisms from both raw industrial and domestic wastewater [6,41]. The present study considered the reduction trends for both the Colony Forming Units (CFU) of total coliforms, *E. coli* and faecal streptococci through MFT and the gene copy numbers for *lacZ, uidA* and *cyd* through qPCR in calculating the system's PEs. Emphasis was given to the PE values for the entire treatment process (from A inlet to B outlet) as it covered the reduction trends across each of entire set (A and B combined). Based on this, the PE for total coliform was highest at tank 2 and lowest at tank 5, for *E. coli* it was highest at tank 2 and lowest at tank 5, for faecal streptococci it was highest at tank 3 and lowest at tank 5, for *lacZ* genes it was highest at tank 4 and lowest at tank 3, for *uidA* gene it was highest at tank 1 and lowest at tank *3* while for *cyd* was it highest at tank 4 and lowest at tank 5. By considering the PE values, tank 2 was the best set, and the control (tank 5) was the poorest set. This finding not only validated the role of macrophyte in raw wastewater treatment but also proved that integrating both emergent and submerged macrophyte makes the process more efficient. Indeed, out of the known submerged macrophyte, *E. nuttallii* and *M. spicatum* proved to be the best candidate partners to *T. latifolia* in achieving the highest PE in removing faecal pathogenic bacteria. Moreover, Shah et al. [11] and Phillippe et al. [10] had recommended the use of locally adopted and available plants as the best candidates in constructed wetland technology.

Faecal pathogenic bacterial strains exhibit different resistance to treatment efforts [42,43]. The 13 strains of *Escherichia* and 7 *Enterococcus* strains detected in raw water/untreated wastewater drastically declined in all the subsequent treatment sets. Indeed, B tanks recorded lower numbers than A tanks in all the sets. The control set (tank 5) recorded higher number of both *Escherichia* and *Enterococcus* strains than other sets/tanks. *E. coli* O157:H7 and its closest relative *E. coli* strain TYN 130606 exhibited higher resistance to more treatment alternatives/sets than all the other strains. Tanks 2 and 3 recorded the lowest number of *Escherichia* strains. The decline in detection of *Escherichia* and *Enterococcus* strains in all the treatment sets as compared to raw water and the water within the control unit (Tanks 5) was a prove that macrophytes additionally play a significant role in the elimination of faecal pathogenic bacterial strains from raw wastewater. The faecal bacteria removal mechanisms within a wetland system inhabited by macrophytes have been documented to include natural die-off owing to starvation or predation, sedimentation, filtration, and adsorption [3,6]. In most systems, the removal potential by these mechanisms depend on the physical, chemical and biological nature of the wetlands [5]. A reduction values of *E. coli* has been recorded at 1.7 log10 CFU/100 ml in fall, spring, and summer, as compared to 1.0 log10 CFU/100 ml recorded in winter [44]. Additionally, higher purification efficiencies have been recorded in horizontal subsurface flow CWs than in surface flow CWs, and emphasis have been laid on hybrid CWs that have further improved the removal of *E. coli*, total coliforms and faecal coliforms by 1.5, 1.2, 0.3 log10 CFU/100 ml, respectively [44]. Indeed, hybrid wetlands systems that incorporate different macrophytes, varied flow regimes as well as UV radiation may be the best alternative for dealing with some of the deadliest and highly persistent forms of faecal pathogenic bacterial strains such as the *E. coli* O157:H7 and its closest relative *E. coli* strain TYN 130606.

Studies have demonstrated varying survival capabilities of different faecal bacterial pathotypes and the abundance in their respective

Table 2
Percentage Purification efficiencies of winter tolerant macrophytes in f faecal pathogenic bacteria removal.

	TOTAL COLIFORMS			*E. Coli*			FAECAL STREPTOCOCCI		
	A(inlet)-A(outlet)	B(inlet)-B (outlet)	A(inlet)-B (outlet)	A(inlet)-A(outlet)	B(inlet)-B (outlet)	A(inlet)-B (outlet)	A(inlet)-A(outlet)	B(inlet)-B (outlet)	A(inlet)-B (outlet)
TANK 1	77.92	78.87	95.33	63.72	87.82	95.58	71.00	86.21	96.00
TANK 2	86.67	90.00	98.67	66.98	98.59	99.53	46.33	98.76	99.33
TANK 3	76.67	91.07	97.92	62.93	98.68	99.51	45.33	99.39	99.67
TANK 4	83.33	70.50	95.08	66.05	87.67	95.81	48.67	92.21	96.00
TANK 5	48.33	66.13	82.50	56.10	65.56	84.88	52.67	71.83	86.67

Table 3
Percentage Purification efficiencies of winter tolerant macrophytes in removal of faecal related pathogenic bacterial genes.

	lacZ gene			*uidA* gene			*cyd* gene		
	A(inlet)-A(outlet)	B(inlet)-B (outlet)	A(inlet)-B (outlet)	A(inlet)-A(outlet)	B(inlet)-B (outlet)	A(inlet)-B (outlet)	A(inlet)-A(outlet)	B(inlet)-(Boutlet)	A(inlet)-B (outlet)
TANK 1	98.54	94.65	99.92	91.64	93.76	99.48	66.46	84.55	94.81
TANK 2	98.83	93.19	99.92	91.64	92.78	99.40	70.12	78.37	93.54
TANK 3	98.83	90.67	99.89	91.63	90.45	99.20	70.12	77.35	93.23
TANK 4	98.50	95.03	99.93	91.32	93.26	99.42	66.46	84.55	94.82
TANK 5	72.94	99.70	99.92	−7.55	99.36	99.31	27.44	26.30	46.53

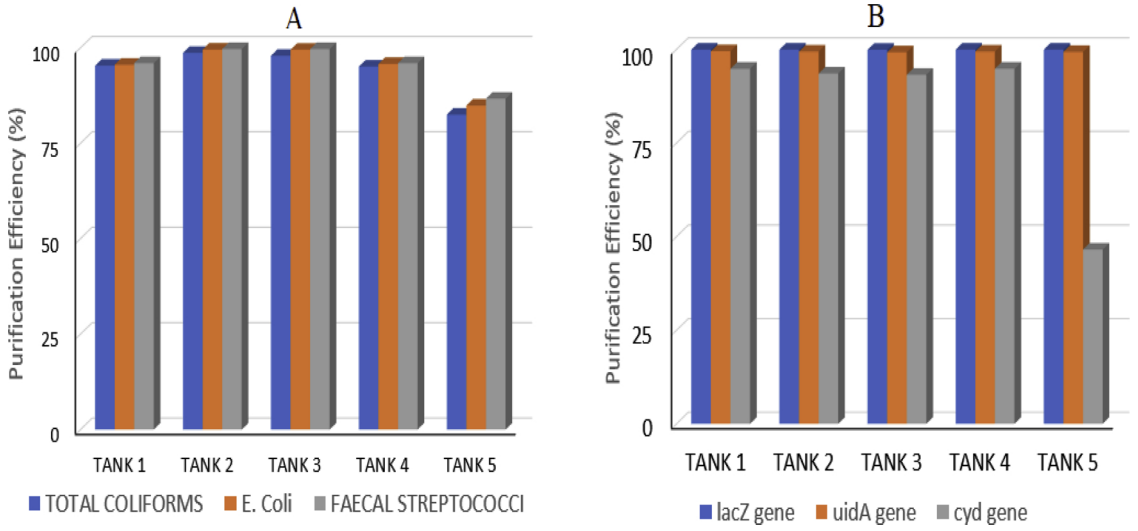


Fig. 4. Average purification efficiencies across the tanks based on the removal of viable indicator bacteria (A) and faecal pathogenic bacterial functional genes (B).

virulence genes across a wetland ecosystem [6,26,45] (Ibekwe et al., 2016). More markers for various *E. coli* pathotypes and those for *E. faecalis* and *E. faecium* detected in raw water than in treated wastewater was indicative of potential role of macrophyte dominated wetland system in eradicating faecal pathogenic bacteria pathotypes. Indeed, A tanks within sets 1 to 4 recorded the higher detection levels of DNA markers for various *E. coli* pathotypes and those for *E. faecalis* and *E. faecium* than the B tanks. This was a further prove that having a system that combines emergent and submerged macrophytes ensures a proper raw wastewater purification through elimination of more virulence genes. The recorded lower detection values within tanks 2 and 3 can support the decision on the most appropriate submerged macrophyte that can provide the best partner to *T. latifolia* for higher efficiency in raw wastewater purification. Additionally, the high purification efficiency for tanks 2 and 3 in the removal of *E. coli* multi-drug resistant genes, further showed *Elodea nuttallii* and *Myriophylum spicatum* as the best candidates in faecal bacterial eradication based on the prevailing conditions.

Comparing the performance of all the tanks based on their respective combined potential in the removal of faecal bacterial colony numbers, functional gene copies, species survival, virulent and pathogenicity as well as antimicrobial resistant genes, provide a prudent decision on the best macrophyte combination for efficient domestic raw wastewater treatment. This integrated approach increases the reliability and accuracy in making decision for putting up and modifying any constructed wetland system because it is multi-parameter based. It is therefore an improvement on the single parameter based constructed wetland studies such as study on coliforms and enteric bacteria abundances by Domingo and Lowe [16], on resistant genes by Chen et al. [18], on bacterial community by Wang et al. [15], and on *E. coli* abundances by Ibekwe and Murinda [17]. In each of these studies, only a single parameter aspect was considered in making the conclusion on

Table 4
Purification based on the detection of *Escherichia* and *Enterococcus* strains across the treatment tanks.

Escherichia and *Enterococcuss* trains	Tanks										
	Raw water	1A	1B	2A	2B	3A	3B	4A	4B	5A	5B
Escherichia coli strain 42 L	D	*	*	*	*	D	*	D	*	D	*
Escherichia coli strain E191-4	D	D	*	*	*	*	*	D	D	D	D
Escherichia coli strainCCFM8340	D	*	*	D	D	*	*	*	*	D	D
Escherichia coli strain TYN 130606	D	D	D	D	*	D	*	D	D	D	D
Escherichia coli strain S10	D	D	*	D	*	*	*	D	*	D	D
Escherichia coli strain CCFM8332	D	*	*	*	*	D	*	*	*	D	D
Escherichia coli strain C-X5B	D	*	*	*	*	*	*	D	D	*	*
Escherichia coli strain CCFM8331	D	*	*	*	*	*	*	*	*	D	D
Escherichia coli O157:H7	D	D	D	D	*	D	*	D	D	D	D
Escherichia coli strain D5	D	D	*	*	*	*	*	*	*	D	D
Escherichia fergusonii strain ATCC 35469	D	D	*	D	*	*	*	D	D	D	D
Escherichia hermannii strain CIP 103176	D	*	*	*	*	D	D	*	*	D	*
Escherichia marmotae strain HT073016	D	D	D	D	*	*	*	*	*	D	D
Enterococcus faecalis strain LMG 7937	D	D	*	*	*	D	*	*	D	D	D
Enterococcus faecalis strain JCM 5803	D	D	*	D	D	*	*	D	D	D	D
Enterococcus faecalis strain ATCC 19433	D	*	*	D	*	D	*	D	*	D	D
Enterococcus faecalis strain NBRC	D	D	D	*	*	*	*	D	*	D	D
Enterococcus hirae strain ATCC 9790	D	*	*	D	*	*	*	D	D	D	*
Enterococcus faecium strain NBRC 100486	D	D	D	*	D	D	D	*	D	D	D
Enterococcus faecium strain DSM 20477	D	D	D	D	*	*	*	D	*	D	D
Total Detection (td)	**20**	**12**	**6**	**10**	**3**	**8**	**2**	**12**	**9**	**19**	**17**
% Purification Efficiency (PE)	**0**	**40**	**70**	**50**	**85**	**60**	**90**	**40**	**55**	**5**	**20**

D Indicates detection of the strain; * Indicates absence of the strain; Maximum detectable strains (mds) = 20, % PE = (mds-td/mds) ×100.

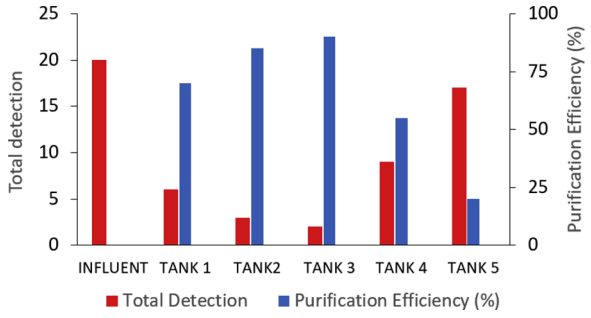


Fig. 5. Average purification efficiencies across the tanks based on the detection of *Escherichia* and *Enterococcus* strains across the treatment tanks.

Table 5
Purification based on the detection of *E. coli* pathotypes virulence and Enterococcus related genes.

E. coli pathotypes and Enterococcus species	Tanks										
	Raw water	1A	1B	2A	2B	3A	3B	4A	4B	5A	5B
ETEC	16	8	3	4	0	0	0	5	2	12	10
EAEC	14	5	2	4	1	0	0	3	2	14	13
EHEC (Typical)	10	4	1	0	0	4	2	2	1	10	10
EHEC (Atypical)	8	6	2	1	0	2	0	0	0	6	6
EPEC (Typical)	12	8	2	0	0	0	0	2	0	10	9
EPEC (Atypical)	15	0	0	0	0	3	0	5	2	11	7
EIEC	9	2	0	4	2	2	2	0	0	8	8
Enterococcus faecalis	13	4	0	0	0	2	0	2	0	9	6
Enterococcus faecium	16	5	1	2	0	0	0	2	1	13	12
Total Detection (td)	**113**	**42**	**11**	**15**	**3**	**13**	**4**	**21**	**8**	**93**	**81**
% Purification efficiency	**30**	**74**	**93**	**90**	**98**	**92**	**98**	**87**	**95**	**43**	**50**

Maximum detection limit (mdl) per sample = 162 (18 × 9); % Purification efficiency = (mdl-td/mdl) × 100

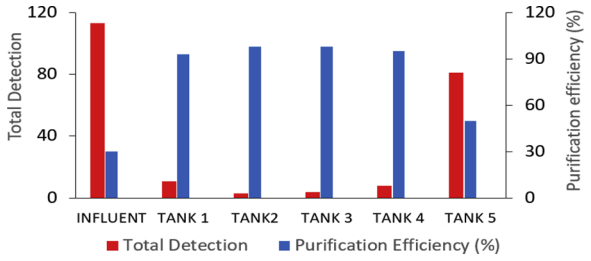


Fig. 6. Average purification efficiencies across the tanks based on removal *E. coli* pathotypes and Enterococcus related genes.

their respective constructed wetland purification efficiencies. However, the present study employed the new multi-parameter/multi-aspect purification performance evaluation approach that integrated the removal evaluation for; faecal bacterial colony numbers, functional gene copies, species survival, virulent and pathogenicity as well as antimicrobial resistant genes in determining the best winter tolerant macrophytes combination of the highest purification efficiency based on the prevailing winter condition.

5. Conclusion and recommendations

Macrophyte play a role in faecal pathogenic bacteria eradication and combination of the local emergent and submerged macrophytes species is key in achieving high PEs. Indeed, multi-parameter/multi-aspect purification-based evaluation approach provides a more reliable and conclusive decision on the best macrophyte combination in faecal bacterial elimination. According to this study, *E. nuttallii* and *M. spicatum* were the best candidate partners to *T. latifolia* for the highest Purification Efficiency ($P < 0.05$), of above 97% for removal of faecal bacteria colonies and functional genes, and more than 75% for removal of faecal bacterial strains, pathotypes, virulent as well as resistant

Table 6
Purification efficiencies based on the detection of antimicrobial resistant genes in *E. coli* isolates

Antimicrobial agent	Resistant gene	Size (bp)	Tanks									
			Raw water	1A	1B	2A	2B	3A	3B	4A	4B	5A	5B
Streptomycin and gentamicin	aadA1	447	D	D	*	*	*	D	*	D	*	D	D
	aac(3)-IV	286	D	D	D	*	*	*	*	D	D	D	D
Sulfonamide	sul1	822	D	*	*	D	D	*	*	*	*	D	D
Beta-lactams	blaSHV	768	D	D	D	D	D	D	*	D	D	D	D
	blaCMY	462	D	D	D	D	*	D	D	D	*	*	D
Erythromycin	ere(A)	419	D	*	*	*	*	D	*	*	*	D	D
Chloramphenicol	catA1	547	D	D	*	D	*	*	*	D	D	D	*
	cmlA	698	D	*	*	*	*	D	*	*	*	D	D
Tetracycline	tet(A)	577	D	D	D	D	D	D	D	D	D	*	D
	tet(B)	773	D	D	*	*	*	*	*	*	*	D	D
Trimethoprim	dfrA1	367	D	D	D	D	*	*	*	D	D	D	*
Quinolones	qnrA	670	D	*	*	*	*	D	D	*	D	D	*
Total detection (td)			**12**	**8**	**5**	**6**	**3**	**7**	**3**	**7**	**6**	**10**	**9**
% Purification efficiency (PE)			**0**	**33**	**58**	**50**	**75**	**25**	**75**	**25**	**50**	**17**	**25**

D Indicates detection of the resttant gene; * Indicates absence of the resistant gene; Maximum detectable genes (mdg) = 12; % PE = (mdg-td/mdg) ×100.

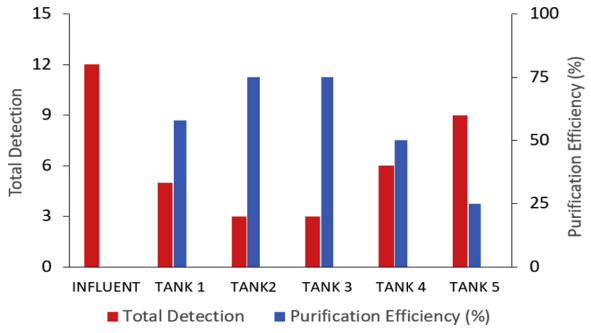
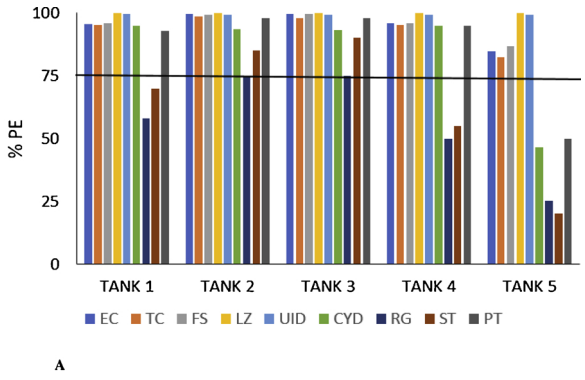
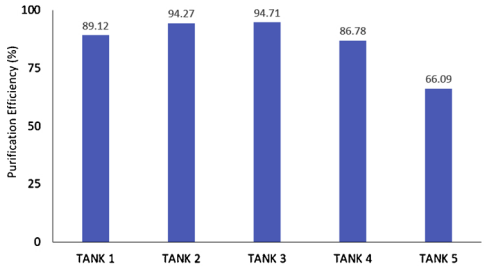


Fig. 7. Average detection levels and purification efficiencies across the tanks based on the detection of antimicrobial resistant genes in *E. coli* isolates.



A



B

Fig. 8. A: Percentage Purification Efficiencies (PE) in the removal of *E. coli* (EC), total coliforms (TC), Faecal streptococci (FC), *lacZ* genes (LZ), *uidA* genes (UID) and *cyd* genes (CYD), *E. coli* resistant gene (RG), *E. coli* strains (ST) and *E. coli* pathotypes (PT) across the tanks. **B:** Average purification efficiencies across the tanks.

genes. However, more preference should be given to the native *E. nuttallii* as opposed to invasive *M. spicatum*. Therefore, the study confirmed that macrophytes play significant role in the elimination of faecal pathogenic bacterial strains and pathotypes from raw wastewater. Additionally, ultraviolet radiation from the sun light may have played a significant role in the removal of faecal bacterial pathogens. In this regard, for efficient domestic raw wastewater treatment, local macrophytes that are well adapted to the existing environmental and seasonal conditions should be the best candidates and the decision should emanate from a multi-parameter/multi-aspect purification evaluation approach. We also recommend further research that will investigate the possibility of integrating UV radiation into various types of constructed wastewater treatment system as well as studies to unlock the resistant mechanisms by various forms of faecal pathogenic bacteria for better control of their health-related implications.

Declaration of Competing Interest

There is no conflict of interest between the Authors and the Funder.

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