Integrating MFT-qPCR techniques in constructed wetland faecal bacterial purification monitoring; a case of a typical tropical hybrid constructed wetland system

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ABSTRACT

The sanitation control of pathogens in the tropical effluents needs much more attention to ensure ecosystem health integrity and the safety of human health. The common use of chemicals in achieving this in wastewater treatment has remained unsustainable due to much health concern. Indeed, based on the numerous challenges associated with faecal pathogenic bacteria in wastewaters, the focus is now on achieving higher purification efficiencies in the elimination of the human pathogens from wastewater through eco-sustainable systems such as constructed wetlands (CWs). Hence, the need to explore the application of constructed wetlands in wastewater treatment under specific local environmental conditions for accurate understanding and improved treatment efficiency. This study therefore aimed at monitoring constructed wetlands faecal bacteria purification efficiency through integrated non-molecular membrane filtration technique and molecular quantitative polymerase chain reaction (MFT-qPCR) technique. The results showed some shortfall in the treatment system and also proved that integrating MFT-qPCR in faecal bacterial purification monitoring within a constructed wetland system provides a more accurate and reliable outcome. Additionally, the wetland purification efficiency was low (<80%) with the dissolved oxygen posing the strongest influence on faecal pathogenic bacterial purification trend across the wetland. Hence, the need to regularly carry out dredging and macrophyte harvesting as well as the use of holistic and more integrative approaches such as MFT-qPCR in managing and monitoring the performance of CWs in faecal pathogen eradication for improved CWs purification efficiency.

Key words | constructed wetland, faecal bacteria, molecular, non-molecular, purification efficiency

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INTRODUCTION

Increased water exploitation and pollution as consequences of climate change, human population growth and rise economic development have continued to impose more pressure on the quantity and quality of water as a resource

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(Zhang *et al.* 2016; Camacho *et al.* 2017). Moreover, much attention has been put on developing technologies that will ensure proper treatment, reuse and recycling of wastewater (Donde & Xiao 2017). Even though centralized and decentralized wastewater treatment systems have been developed, the overall treatment efficiency is still relatively low (Vymazal 2010). Bacterial wastewater purification is more challenging within the tropics where the existing weather and environmental conditions favor the proliferation of most bacterial pathogens (Desmarais *et al.* 2002). Wastewater related pathogenic microorganisms are the major public health concern and transferred to the surface waters when untreated or inappropriately treated wastewater is discharged into receiving water bodies (Rop *et al.* 2014, 2016; Donde *et al.* 2017) and these are used as direct source of water for domestic use and human consumption (Macharia *et al.* 2014). These facts represent a major hygienic concern, which needs to be effectively investigated and accurately prevented (Alexandros & Akratos 2016).

Due to this, the sanitation control of pathogens in the tropical effluents needs much attention to ensure ecosystem health integrity and the safety of human health (Winward *et al.* 2008; Petrie *et al.* 2015). Despite the wide range of treatment approaches, the common use of chemicals in wastewater treatment has remained unsustainable with much health concern (Toscano *et al.* 2013). This challenge has made wetland treatment as one of the preferred alternative treatment approaches (Zazouli & Kalankesh 2017). Most municipalities and institutions with high effluent generation and a complex range of biological, chemical and physical pollutants have now focused on applying wetlands in wastewater treatment (Stottmeister *et al.* 2003; Burgos *et al.* 2016). Constructed wetlands (CWs) offer a mechanism to meet increasingly stringent regulatory standards for wastewater treatment while minimizing energy inputs and polishing the treated effluents by removal of pathogens and nutrients (Smith *et al.* 2005; Cohen *et al.* 2013). Currently, there is increased adoption of CWs in wastewater purification (Donde & Xiao 2017). However, unlike the temperate zones, the operation and maintenance of CWs systems within the tropics have not been extensively studied (Yan & Xu 2014; Tianzhu *et al.* 2017).

Due to the numerous challenges associated with faecal pathogenic bacteria in wastewaters, much attention has focused in achieving higher purification efficiencies in the elimination of the human pathogens from wastewater through eco-sustainable systems such as CWs. Application of CWs in wastewater treatment under specific local environmental conditions needs to be explored in detail for accurate understanding and improved treatment efficiency (Shah *et al.* 2014; Donde *et al.* 2018). This study therefore aimed at monitoring CWs faecal bacteria purification efficiency through integrated molecular and non-molecular techniques to provide appropriate recommendation for improved wastewater treatment mechanisms.

METHODOLOGY

Study area

The studied tropical hybrid CW cell is located at Egerton University in Kenya. It receives wastewater from the students and staff residential houses, laboratories, offices and other agri-production units within the university. The wastewater first passes through two parallel series of three wastewater stabilization ponds (WSPs) prior to getting to the hybrid CW cell which was put up for further purification and polishing of the wastewater prior to its discharge into the neighbouring stream. The hybrid CW cell is located at S 00° 22.268′ and E 035° 56.441′ at an altitude of 2,223 m and it comprised of five compartments separated by 4 alternating baffles. Only the last compartment of the cell is filled with gravel. At the time of the study, the porosity of the gravel used at the bed was 0.55, with the first four compartment operating as surface flow (SF) while only the last compartment (5th) operating as sub-surface flow (SSF). The cell has a surface area of 1,051 m^2 with an approximated effective volume of 976 m^3. The depth of the cell is 0.72 m at the inlet and increases gradually to 1.2 m at a point nearest to the SSF part of the 5th compartment (Seema 2014). The first four compartments are dominated by *Pistia stratiotes* (water lettuce) while the SSF part within the 5th compartment is predominantly covered by *Typha sp.* (cattail or bulrush) and *Scirpus lacustris* (deergrass or grassweed). These macrophytes were being harvested on a yearly basis, dried and used as green manure locally. The cut macrophytes were then managed to regrow. The harvest debris was approximated at 0.5 kg/m^2/yr dry weight of macrophyte on average.

Sampling and analyses

This study was conducted between the months of July to October 2016 and later continued between the month of November 2017 to February 2018. Samples were obtained from the inlet point, baffle 2 (B2), baffle 4 (B4) and at the outlet point (Figure 1). For each sampling episode, water samples were obtained in triplicate from the inlet, baffle 2

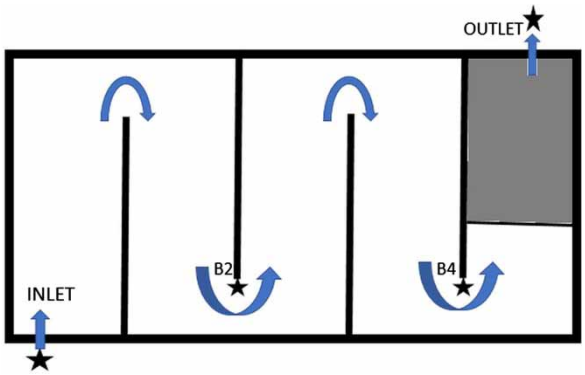

Figure 1 | A schematic representation of the tropical hybrid CW cell (shaded part is the gravel filled compartment, asterisk represent the sampling points).

(B2), baffle 4 (B4) and outlet points using sterilized bottles. Physical and chemical parameters, dissolved oxygen, temperature, pH and electrical conductivity, were measured *in situ* at each sampling episode. The water temperature, dissolved oxygen and pH were measured using a WTWÒ microprocessor pH/temperature meter. The pH meter was calibrated with pH 4 and 7 using standard buffer solutions according to manufacturer's instructions (WTW, Vienna, Austria). The electrode was rinsed with distilled water between samples. Electrical conductivity was measured using a WTWÒ microprocessor conductivity meter calibrated at 25 °C. All the samples were stored in a cool box with ice and transported to Egerton University, Department of Biological science laboratory for non-molecular analysis (membrane filtration technique (MFT)) while the molecular (quantitative polymerase chain reaction (q-PCR)) analyses were conducted at the Lake Restoration Laboratory of the Institute of Hydrobiology, Chinese Academy of Sciences.

Bacterial abundance through MFT

MFT was performed to quantify total coliforms and *Escherichia coli* counts using selective Chromocult Coliform Agar (Merck). Aseptic filtration was done separately for each dilution by passing the sample through a membrane filter (47 mm diameter, 0.45 μm pore size) on a filtration unit. The filter was taken off using a pair of forceps and placed onto the plate containing culture media and incubated at 37 °C for 24 h. This procedure was accomplished within 6–24 h from sampling time to ensure that there was no post-sampling bacterial growth or die off. Typical colonies appearing pink and blue were counted as total coliforms while those blue colonies alone were counted as *E. coli*. Numbers of cells were expressed as CFUs/100 mL (Public Health Association (APHA) 2005; Public Health England (PHE) 2014).

Gene copy numbers through q-PCR

For this molecular technique, filters with bacterial colonies were stored under −20°C until the analyses time. EZNA water DNA kits (Omega, America) was used in the DNA extraction for each water sample. Primers targeting the functional genes, *LacZ* which encodes *β*-galactosidase, a characteristic specific for all total coliforms, *uidA* which encodes for *β*-glucuronidase, a characteristic specific for *E. coli* plus other faecal coliforms and *cyd* that codes for cytochrome d complex, a characteristic specific for *E. coli* were used to quantify the total coliforms, faecal coliforms and *E. coli* (Quirós *et al.* 2015). The specific primer details are provided in Donde *et al.* (2017). The signal dye SYBR Green I (Bio-Rad, USA) was used as the real time qPCR detector in the qPCR products amplification. The amplification was carried out under the following conditions; 94°C for 3 min for initial stage, 34 cycles with 94°C for 30 s denaturation, 58°C for 30 s annealing and an extension at 72°C for 30 s for all the genes (*lacZ*, *uidA* and *cyd*). The amplification was performed in 20 μL reaction volumes that contained 10 μL iTaq universal SYBR Green Supermix (Bio-Rad, United States of America), 7 μL of double distilled water, 1 μL of both the forward and reverse primers and 1 μL of the template (sample). All the samples were analyzed in triplicates. The amplification reaction specificity was checked through a final melting curve analysis. The qPCR standard was produced using the appropriate primers. The analysis of PCR amplification products was accomplished by electrophoresis in an agarose gel within a 0.5 × tris-borate-EDTA(TBE) buffer and followed by the purification of the PCR products to check for the specificity. The ligation of the PCR products was made into pMD18-T vector (Takara, Japan) after the agarose confirmatory test. The primer specificity to the target sequences was then checked through Basic Local Alignment Search Tool analysis and positive clones were separated. The positive clones were then subjected to plasmid DNA extraction using Axy Prep plasmid mini pre-kits (Axygen, China). Circular plasmids digestion was done through ingestion with Barma HI (Takara, Japan) and qPCR standard curve generated. The measurement of the standards was done in triplicates and the r^2 value and amplification efficiency were used in the confirmation of their validity. The target bacterial population/concentration numbers were

normalized as the DNA concentration quantified gene copies/100 mL water (Changsoo *et al.* 2006).

Purification efficiencies

Purification efficiencies (PE) was determined at different stages of the system (inlet to B2, B2 to B4 and B4 to outlet) and between the inlet and outlet points of the cell. This was accomplished by working out the percentage change in the quantity of water (MFT and qPCR values) that enters and leaves the points. The PE at inlet-B2 was calculated as [(mean values at inlet-mean values at B2/mean values at inlet) × 100], PE at B2–B4 was calculated as [(mean values at B2- mean values at B4/mean values at B2) × 100], PE at B4-outlet was calculated as [(mean values at B4- mean values at outlet/mean values at B4) × 100] and PE at inlet-outlet was calculated as [(mean values at inlet-mean values at outlet/mean values at inlet) × 100.

Data presentation and statistical analyses

The presentation of data was done through tables, figures and graphs drawn on Microsoft Excel and Minitab statistical package version 14. Statistical analyses were performed with the Minitab statistical package. Mean and median values were respectively calculated for physico-chemical parameters and bacterial colonies and gene copies. Median comparison was performed using One Way Analysis of Variance (ANOVA) on ranks at 95% confidence level. In case of significant differences in ANOVA on ranks, Tukey tests were performed as post hoc test to determine the points of variation. Principal component analysis (PCA) and Pearson's correlation was performed between the physico-chemical parameters, bacterial colony forming units and bacterial gene copy numbers.

RESULTS

Physico-chemical parameters

Results on physico-chemical parameters are provided in Table 1. There was a significant difference between the sampling sites for all the parameters except the pH. In most instances, parameters showed lack of significant difference between B2, B4 and outlet sites based on Tukey test.

Bacterial abundance through MFT

The bacterial abundance through MFT are provided in Figure 2 for total coliforms and Figure 3 for *E. coli*. There was lack of statistical differences between the total coliforms' colony forming units in inlet and outlet points and the median values were highest at B2 sampling point. B4 showed the lowest median values. For *E. coli* abundance, inlet and B2 points showed no significant difference. The highest median values occurred at B2 while the lowest values were found at B4.

Gene copy numbers through q-PCR

The result on gene copy numbers are provided in Figure 4 for numbers of *lacZ* gene and Figure 5 for *uidA* and *cyd* genes. For *lacZ* gene, there was no statistical difference between B2 and B4 and, as expected, inlet point accounted for the highest gene copy numbers. For *uidA* gene, there was no significant difference in gene copy number in B2 and outlet points, with the highest median values being recorded at inlet point. For *cyd* gene, there was also no significant difference in gene copy numbers at B2 and outlet points,

Table 1 | Values for physico-chemical parameters

	INLET	BAFFLE 2	BAFFLE 4	OUTLET	*P*-values
DO (mg/L)	1.42 ± 0.07 (1.22–1.52) A	1.22 ± 0.13 (0.94–1.57) BC	0.95 ± 0.07 (0.86–1.25) B	1.52 ± 0.05 (1.38–1.62) D	0.025
Temp (°C)	20.24 ± 0.84 (18.80–23.20) A	19.51 ± 0.52 (18.40–20.50) C	19.62 ± 0.57 (18.80–21.30) C	19.76 ± 0.76 (17.90–22.10) C	0.034
pH	7.72 ± 0.29 (6.87–8.10) A	7.76 ± 0.24 (6.95–8.24) A	7.84 ± 0.29 (7.52–9.05) A	7.81 ± 0.10 (7.61–8.10) A	0.028
EC (μS/cm)	1,123.94 ± 74.44 (91,006.80–1,417.20) A	1,130.80 ± 162.51 (1,082.60–1,992.30) A	1,090.72 ± 4.24 (1,078.40–1,102.40) B	1,127.40 ± 27.19 (1,095.60–1,209.50) A	0.045

Mean values ± standard deviation with the minimum and maximum values in bracket for dissolved oxygen (DO), temperature (temp), pH and electrical conductivity (EC). Means with the different letters within each raw are significantly different at $p = 0.05$, $n = 30$.

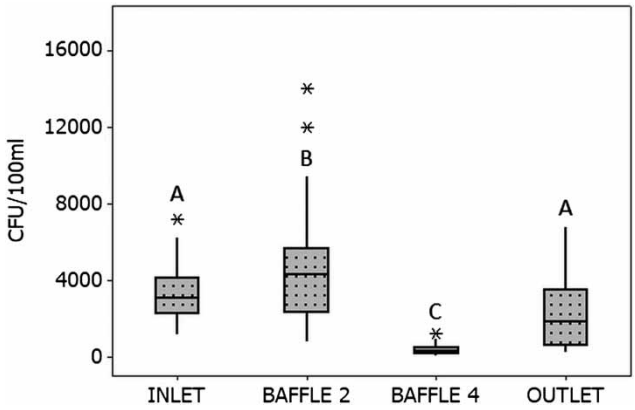

Figure 2 | Box and whisker plots of median × 10^3 (25%, 75% intervals) values for abundances of total coliform across the CW. Box range is the 25th–75th percentile. Asterisk represent the outliers and whisker range is the maximum and minimum values. The median is represented by solid horizontal lines in each box. Where ANOVA on ranks was significant ($p < 0.05$, $n = 30$), Tukey tests were performed to determine sites that were significantly different for each parameter (indicated with different letters).

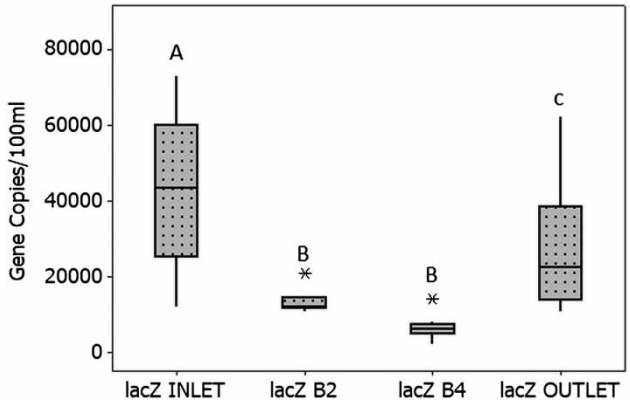

Figure 4 | Box and whisker plots of median × 10^4 (25%, 75% intervals) values for *lacZ* gene copies across the CW. Box range is the 25th–75th percentile. Asterisk represent the outliers and whisker range is the maximum and minimum values. The median is represented by solid horizontal lines in each box. Where ANOVA on ranks was significant ($p < 0.05$, $n = 10$), Tukey tests were performed to determine sites that were significantly different for each parameter (indicated with different letters).

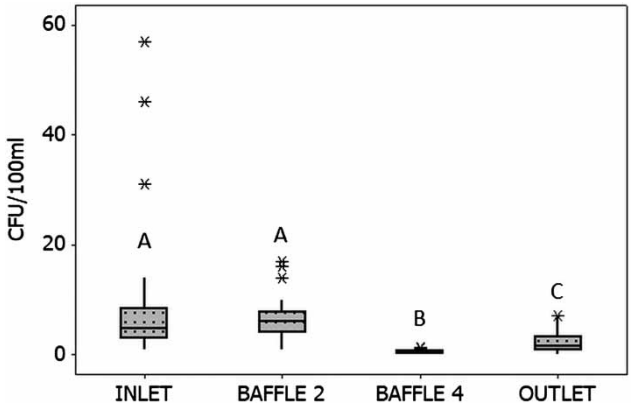

Figure 3 | Box and whisker plots of median × 10^3 (25%, 75% intervals) values for abundances of *E. coli* across the CW. Box range is the 25th–75th percentile. Asterisk represent the outliers and whisker range is the maximum and minimum values. The median is represented by solid horizontal lines in each box. Where ANOVA on ranks was significant ($p < 0.05$, $n = 30$), Tukey tests were performed to determine sites that were significantly different for each parameter (indicated with different letters).

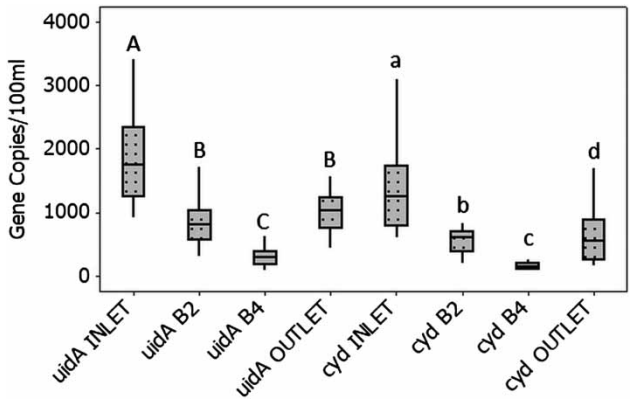

Figure 5 | Box and whisker plots of median × 10^4 (25%, 75% intervals) values for for *uidA* and *cyd* genes copies across the CW. Box range is the 25th–75th percentile. Asterisk represent the outliers and whisker range is the maximum and minimum values. The median is represented by solid horizontal lines in each box. Where ANOVA on ranks was significant ($p < 0.05$, $n = 10$), Tukey tests were performed to determine sites that were significantly different for each parameter (indicated with different capital letters (*uidA*) and small letter (*cyd*)).

with the highest median values being recorded also at the inlet point.

Purification efficiencies

The values on purification efficiencies (PE) at different points within the CW were calculated as described in methodology section and are provided in Table 2. Generally, there were higher PE values for all the parameters between B2 and B4 points. Point B4 to outlet recorded negative PE

Table 2 | Purification efficiencies

Parameters	INLET-B2	B2-B4	B4-OUTLET	INLET-OUTLET
E. Coli	30.63	92.00	**− 322.78**	76.53
Total coliforms	**− 37.28**	91.38	**− 472.21**	32.30
lacZ	69.59	49.45	**− 302.13**	38.18
uidA	55.24	65.02	**− 243.03**	46.29
Cyd	60.66	73.43	**− 329.89**	55.07

values (increased colony and gene counts) for all the parameters, additionally, only total coliform recorded a negative PE at inlet to B2 points. The overall PE (inlet to outlet) was below 80% through both the molecular and non-molecular techniques.

Association between variables through PCA and correlation analysis

The association between physico-chemical parameters and MFT values across the entire CW system through PCA are provided in Table 3 and Figure 6. The high coefficient values (> ± 0.5 threshold) marked in bold within the table shows a higher correlation between the principal components and the variables. The PCA of the wetland purification process showed that the 3rd to the 6th principal components (PC3, PC4, PC5 and PC6) explained 48.97 variance ([eigenvalue/number of factors] ×100) within the CW system, with PC3 (dissolved oxygen) and PC4 (temperature) explaining the highest variance as compared to other physico-chemical parameters. The loadings of the two principal components for the wetland purification clustered together with the total coliforms (TC), *E. coli* and dissolved oxygen, indicating that the effect of dissolved oxygen was greater than the other physico-chemical parameters. There was significant Pearson's correlation between bacterial colonies and dissolved oxygen (Table 4).

DISCUSSION

Physico-chemical parameters play important roles in faecal bacterial growth and die off. In most CW systems, the purification efficiencies are dependent on the physico-chemical variables, indeed the survival and death of faecal pathogenic parameters is greatly controlled by factors such as temperatures, dissolved oxygen and pH. Dissolved oxygen concentration and pH increases attributed to algae in algal-based treatment ponds results in the inactivation of faecal coliforms and this would also be expected in vegetated CWs (Alexandros & Akratos 2016). The significant differences in most of the physical–chemical parameters between the sampling sites could be one of the crucial factors that shaped both the colonies as well as genes count within the various baffles.

The lack of statistical difference in the total coliforms' colony forming units between the inlet and outlet points may be an indication that the CW system was not efficient in the elimination of total coliforms from the wastewater or may be because the total coliforms have the ability to thrive and multiply in such an environment. In fact, it resulted in the proliferation of the total coliforms as evident by the highest median values at B2. A similar scenario also occurred with respect to *E. coli* purification where inlet and B2 points showed no significant difference with the highest and lowest median values being recorded at B2 and B4, respectively. Such an expected finding pointed out to the existence of certain environmental conditions within B2 outlet that promoted the proliferation of the bacteria or indicate the role of other animals such as birds in reintroducing the faecal related bacteria into the wetland systems. This finding was in support of the findings in a study by Seema (2014) that the wetland system was not effective in nutrient purification. Indeed, the documented positive correlation between nutrients quantities and bacterial abundances further supports this argument (Kora *et al.* 2017). However, it was inconsistent with the studies by Smith *et al.* (2005) for pathogen indicator bacteria removal in agricultural CWs.

Based on *lacZ* gene copy numbers, the lack of statistical difference between points B2 and B4 also points to the inefficiency in the purification process for total coliforms. In contrast, values for colony forming units of both the total coliforms and *E. coli* through MFT, there were higher median values for *lacZ* gene at the inlet

Table 3 | Principal component coefficients and percentage variances

Variables	PC1	PC2	PC3	PC4	PC5	PC6
E. coli	**−0.519**	0.042	0.02	**0.635**	−0.151	**−0.55**
Total coliforms	**−0.596**	0.16	0.218	0.15	0.061	**0.739**
Dissolved oxygen	−0.448	0.038	−0.243	**−0.61**	**−0.592**	−0.126
Temperature	−0.374	**−0.505**	0.304	−0.368	**0.56**	−0.253
pH	0.1	**−0.828**	0.036	0.235	−0.437	0.238
Conductivity	−0.158	−0.175	**−0.894**	0.101	0.344	0.125
% Variance	31.94	19.09	17.19	14.26	10.80	6.69

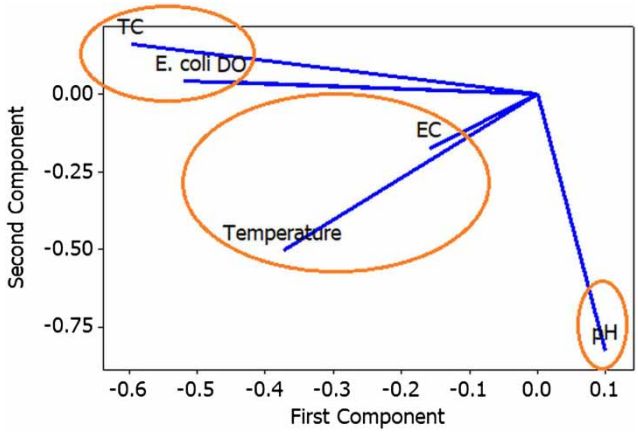

Figure 6 | Loading plots indicating the association between the physico-chemical variables (dissolved oxygen (DO), temp (temperature) and EC (electrical conductivity (pH)) and the biological parameters (total coliforms (TC)) and *E. coli* from pooled data.

points than at all the other points. Indeed, this showed the need of integrating both the MFT and qPCR techniques in any CW purification studies for a more accurate and reliable conclusion as earlier recommended in Donde & Xiao (2017). Based on *uidA* gene copies, the lack of significant difference between B2 and outlet points indicated similarity in the proliferation of *E. coli* and/or other faecal bacteria between those sites (Donde *et al.* 2017). Indeed, the highest median values for *uidA* genes were recorded at inlet point. The trend in gene copy numbers for *cyd* was similar to that of *uidA*. Since *cyd* gene is a functional gene that codes for cytochrome d complex, a characteristic specific for *E. coli,* such result revealed that the wetland had a similar purification trend for both the *E. coli* as well as other faecal coliforms. In fact, this finding supports the use of *E. coli* as an indicator organism in faecal pollution monitoring programs (APHA, 2005).

The higher PE values recorded between B2 and B4 showed that as the faecal bacterial pathogens leaves the inlet point, the purification was perfect and hence greater reduction of both the colony forming units and gene copies. However, the negative PEs recorded between point B4 and outlet for both the colony units and gene copies pinpointed the actual stage where CW system's purification process went wrong. This could be because of low dredging and plant harvesting frequency (once per year). Based on this finding, there was a process hitch-up at the 5th compartment of the CW system. This may also be arising from birds and other animals at the macrophytes in B4 which could

Table 4 | Pearson's correlation between physico-chemical and bacterial parameters

	E. coli	TC	*lazZ*	*uidA*	*cyd*	DO	Temperature	pH	EC
E. coli	1	0.517**	−0.034	−0.033	0.026	0.196*	0.155	−0.019	0.124
		0.000	0.835	0.837	0.874	0.032	0.091	0.837	0.178
TC		1	−0.267	−0.217	−0.109	0.326**	0.302**	−0.174	0.011
			0.095	0.178	0.504	0.000	0.001	0.057	0.902
lazZ			1	0.745**	0.536**	−0.085	−0.159	0.304	−0.120
				0.000	0.000	0.602	0.328	0.057	0.459
uidA				1	0.573**	−0.041	−0.086	0.365*	−0.005
					0.000	0.803	0.597	0.021	0.973
Cyd					1	0.149	−0.026	0.308	0.310
						0.360	0.873	0.053	0.052
Dissolved oxygen						1	0.214*	−0.098	0.161
							0.019	0.287	0.079
Temperature							1	0.162	0.015
								0.077	0.872
pH								1	0.037
									0.685
EC									1

Total coliforms (TC), dissolved oxygen (DO), electrical conductivity (EC).
**Correlation is significant at the 0.01 level (2 tailed).
*Correlation is significant at the 0.05 level (2 tailed).

have contributed to reintroduction of faecal bacterial pathogens. Additionally, biofilm development on the gravels here could be scrubbed into effluents at the outlet, hence higher bacterial parameters at effluent than at B4 resulting in negative removal performances between B4 and outlet. Based on this, there is need for consistent periodic cleaning or dredging the gravel.

The overall PEs (from inlet to outlet point) were all too low (below 80%). Despite *E. coli* recording a relatively higher PE based on both the colony counts and *cyd* gene copies, other parameters (total coliforms, *lacZ* gene and *uidA* gene) recorded PEs of below 60%. Indeed, total coliforms and *lacZ* gene recorded the lowest PEs. Such findings have therefore strengthened the need to employ integrating MFT-qPCR techniques in faecal bacterial pathogens purification monitoring within a CW system. Indeed, studies by Donde *et al.* (2017) had laid emphasis on integrated monitoring tools as the best pollution monitoring and control strategies and efficient ways in managing and conserving the aquatic ecosystem health.

The high coefficient values recorded through PCA indicated a higher correlation between the principal components and the variables. Both the total coliforms and *E. coli* colony forming units has a stronger correlation with the first principal component. Total coliforms and *E. coli* colony forming units also had a significant Pearson's correlation with dissolved oxygen. Among the physico-chemical parameters, dissolved oxygen and temperature explained the highest variance within the CW system as compared to pH and electrical conductivity. Indeed, the loadings of the two principal components for the wetland purification clustered together the total coliforms (TC), *E. coli* and DO. This was an indication of the stronger influence dissolved oxygen played on the bacterial colony counts and genes copies across the CW system as compared to other physico-chemical parameters. Additionally, it was an evidence that the bacteriological purification trend was highly dependent on DO more than other measured physico-chemical parameters. This finding could be beneficial in placing corrective measures to ensure that the wetland purification integrity is accurately restored. This can be achieved by ensuring that specific environmental variables that play a higher role in faecal pathogenic bacterial growth are targeted and corrected. In fact, Kora *et al.* (2017), highlighted the level of organic greatly influence both that bacterial community structures directly or indirectly by controlling other environmental variables.

Removal of microbes from wastewater has been a major concern (Solano *et al.* 2004). The microbe's removal mechanisms from CWs include natural die-off owing to starvation or predation, sedimentation, filtration, and adsorption (Jasper *et al.* 2013). The main process involved in pathogen removal is sedimentation where there is vast accumulation of coliforms and other bacteria (Quiñónez-Díaz *et al.* 2001). The sediments provide microbes with longer survival ability (Solano *et al.* 2004). Other microbes tend to attach to colloidal material, which takes longer to settle out, and eventually settle out in a loose layer above sediment which can be disrupted by human activity or natural storms and cause reintroduction of the pathogens back to the treatment system (Jasper *et al.* 2013), as was noted between baffle 4 and outlet point. The sediment grain size may also determine the CW removal efficacy, as a smaller grain size has a larger specific surface area for interactions. Although bacteria removal mechanisms by CWs is still debatable, studies have compared processes such as filtration, adsorption, natural death, and predation by other microorganisms (Wu *et al.* 2016). Studies have also proved that the presence of an active microbial community tremendously accelerates the inactivation and decay of pathogens in aquatic systems (Flemming *et al.* 2016). Additional pathogen filtration approach is through the root system of wastewater wetland plants. Attachment to the root system plays a role in pathogen elimination from wastewater (Karim *et al.* 2004).

Generally, this study highlighted the inefficiency of the studied tropical hybrid CW system, especially in producing effluent with recommended quality for drinking water. The effluent from the outlet point had CFU of 2.0×10^7 and 1.0×10^7, respectively, for total coliform and *E. coli* values. Indeed, this trend was confirmed by the respective high copy numbers for all the studied genes. Various effluent quality regulation agencies such as the World Health Organization (WHO), United States Environmental Protection Agency (US-EPA) and National Environmental Management Authority Kenya (NEMA-Kenya) have all laid down stringent measure for effluent quality discharge (Donde *et al.* 2013). Based on bacteriological quality of the effluent, all these agencies have recommended 0 cfu/100 mL for both the total coliform and *E. coli* prior to its discharge to drinking water source such as the Njoro River. This study therefore confirmed a failure of the wetland in meeting such standards and that the river that receives such effluent is only fit for agricultural use. This was confirmed by unacceptable higher bacterial values within baffle 4 than the outlet,

indicating deteriorating wastewater quality between the two compartments. Such unexpected rise in bacterial values may be due to inconsistency in dredging and macrophyte harvesting from the system, resulting to back flash of faecal related bacteria and other organic wastes into the effluent, especially between baffle 4 and outlet points. Furthermore, knowing the bacterial purification performance ability of specific macrophyte has not been well document within the sub-Saharan Africa as opposed to the tropics (Donde *et al.* 2018).

CONCLUSION AND RECOMMENDATIONS

Based on this study, it can be concluded that the integrating MFT-qPCR in faecal bacterial purification efficiencies within a CW system provide a more accurate and reliable outcome. Considering the regulation on the bacterial quality standards of effluent discharge to drinking water sources by WHO, US-EPA and NEMA-Kenya, the study showed that the system was of poor purification efficiency. Indeed, the final effluent at the outlet point was of poor bacterial quality, indicating a shortfall in the treatment system that could be attributed to low dredging frequency. The wetland purification efficiency for faecal bacterial colonies and genes was very low and dissolved oxygen had the strongest influence on faecal pathogenic bacterial purification trends across the wetland. This study therefore recommends a more frequent dredging and macrophyte harvesting frequency of more than three episodes per year. It has also proved the need to use an holistic and integrative approaches such as MFT-qPCR in monitoring the purification performance of CWs in faecal pathogen eradication and achievement of proper effluent standards prior to its discharge to drinking water source. Further study should be undertaken to understand the macrophyte with the highest bacterial purification efficiency for use in industrial and domestic wastewater treatment within the tropics.

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