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Understanding wastewater treatment mechanisms: A review on detection, removal and purification efficiencies of faecal bacteria indicators across constructed wetlands

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Abstract

The specific mechanisms of faecal bacterial removal by constructed wetland (CW) mechanisms are inadequately understood. In several circumstances, constructed wetlands have been compared to “black box” systems involving poorly understood waste removal mechanisms despite being an emerging environment friendly waste management approach. This has therefore attracted numerous scientific studies to further unlock CWs’ functional mechanisms and to increase its efficiencies. This review paper covers detailed information on the status of detection techniques and removal efficiencies of faecal coliforms, with an emphasis on *Escherichia coli*. A comprehensive literature search was undertaken which involved a comparative review of various study results and critical analysis of previous scientific and review papers. The ultimate objective is to shed further light on the role of wetlands on wastewater purification for improved aquatic ecosystem health and clean water for humans and other organisms.

Key Words: Contamination; Efficiencies; Faecal; Indicators; Constructed wetlands.

Introduction

Constructed Wetland (CW) is an emerging technology designed to simulate saturated substrates, emergent and submerged vegetation, animal life, water and purification functions of natural wetlands (Ezeah *et al* 2015) (Fig. 1). The use of CW is quickly spreading in wastewater

treatment as it often requires minimum energy input and provides many ecosystem services (Vymazal, 2005; Vymazal, 2010; Wu *et al.*, 2015). The main goal of a constructed wetland is to purify different types of wastewater, including those from dairy and swine operations (Cronk, 1996), potato processing plants (Burgoon *et al.*, 1999), waste water polluted with enteric bacteria (Vymazal, 2005; 2010), as well as waste water polluted with human pathogens (Diemont, 2006; Wu *et al.*, 2015; 2016). This will ensure proper restoration of aquatic and terrestrial systems whose degradation are on the increase at the present (Donde and Onditi, 2017). There is also need to modify the technology for its increased efficacy (Akarat *et al.*, 2015; Sara *et al.*, 2015). CWs are of various types, with their classification based primarily on their hydrology (Kela and Raymond, 2008). These include Free Water Surface CW (FWS CW) and Subsurface Flow CW (SSF CW), the latter can have horizontal or vertical flows, resulting in three general types of CWs: Free Surface Water (FSW), Horizontal Subsurface Flow (HSSF) and Vertical Flow (VF) CWs (Col and Asim 2015; Natalia *et al.*, 2016; Penghe *et al.*, 2016). The specifics of faecal bacteria removal mechanisms and efficiencies by constructed wetland as well as appropriate detection techniques across a wetland treatment system are still not adequately understood (Stottmeister *et al.* 2003; Akarat *et al.*, 2015). As a result, this technique is often compared to a “black box” system, leading to numerous scientific studies and debates for a better understanding (Haberl *et al.*, 2003; Flood *et al.*, 2011; Lee *et al.*, 2014; Morató *et al.*, 2014; Ridley *et al.*, 2014). The success of CWs application requires the need to understand its dynamics in pathogen removal from water (Lee *et al.*, 2014; Wu *et al.*, 2016; Donde *et al.*, 2017). However, some of the issues which have not been adequately addressed as far as its role in pathogen removal are; the types of microorganisms that are present, their abundance, how their activities shape the environment or influence other organisms, their possible effects on human health as well as how

the environment influences the structure and function of the microbial communities (Tilman, 2001; Morato *et al.*, 2014; Waller and Bruland, 2016). This review paper therefore seeks to shed more light on the “black box”, by providing detailed information on the removal mechanisms, purification efficiencies by different wetland systems and appropriate detection techniques of faecal bacterial pollution indicator- *Escherichia coli* as a known member of faecal coliform bacteria. A comprehensive literature search was undertaken which involved comparative reviews of various study results and critical analysis of previous scientific and review papers, with great emphasis put on studies and reviews published after the year 2010.

Constructed wetlands and faecal pathogens detection techniques

Faecal Pathogens

Water borne and water related illnesses have remained a persistent worldwide challenge, (WHO, 2013; Erick and Robert 2016). Despite the fact that developing countries are particularly more susceptible to these outbreaks due to their poor sanitation and water quality control measures, the developed countries are also affected by emerging pathogens resistant to water treatment, technological failure and/or inappropriate detection techniques (De Man *et al.*, 2014; Rathinasabapathi *et al.*, 2016). Many bacterial communities are natural inhabitants of the digestive tracts of animals and humans and pass into water through faeces (Donde *et al.*, 2014; 2015). Bacterial members such as coliforms, *E. coli* and *Enterococcus* spp., are used as hygiene indicators. The detection of these hygiene indicators in water reveals poor quality of water as their presence often indicate presence of faecal and sewage pollution (Noble *et al.*, 2003). At present, the most preferred indicators are Total Coliforms (TC), Faecal Coliforms (FC), and Enterococci (EC) (Frahm *et al.*, 2003; Pote *et al.*, 2009; Tallon *et al.*, 2005; Lam *et al.*, 2014; Rop *et al.*, 2014; Donde *et al.*, 2015; Madec *et al.*, 2015; Naville *et al.*, 2015).

The genus *Escherichia* is the dominant coliform bacteria that are able to ferment lactose at 44 – 45 °C and are therefore commonly referred to as thermotolerant coliforms, in addition to a few members of *Citrobacter*, *Klebsiella* and *Enterobacter* (Sueiro, 2001). *E. coli* are considered the most suitable index of faecal contamination (Donde *et al.*, 2015; Madec *et al.*, 2015). They are found in high numbers in human and animal faeces (10^9 per gram of faeces), sewage and water that is subject to recent faecal pollution (George, 2001; Gouthami *et al.*, 2015; Wu *et al.*, 2016). The detection of *E. coli* provides evidence of recent faecal contamination hence warrants further investigation of their potential sources and the necessary control measures (Ashbolt *et al.*, 2001; George, 2001, Sueiro, 2001 and Donde *et al.*, 2015; Wu *et al.* 2016).

Faecal pollution detection techniques

Different cultured-dependent and culture-independent-techniques have been used to study CWs purification mechanisms and efficiencies (Flood *et al.*, 2011; Ridley *et al.*, 2014). Culture-dependent methods are still used in detection and enumeration of faecal coliforms as a procedure for studying efficiencies of CWs in purifying wastewater as it provides data at a reasonable cost (Flood *et al.*, 2011). However, this method provides limited information about the total microbial community (encompassing < 1% of the diversity) and changes therein (Nnadozie, 2015). For improved accuracy, modern research on CWs removal efficiencies has incorporated the application of culture-independent techniques to overcome these limitations and has since revealed a new and improved view of microbial community studies (Lee *et al.*, 2014; Waller and Bruland 2016). The implementation of these techniques as the method of choice to investigate microbial community dynamics in wetland systems is slow, since they require more specialized equipment, trained personnel and are more expensive than the culture-dependent methods (Nnadozie, 2015).

1. Culture-dependent Methods

I. Membrane Filtration techniques

Membrane Filtration Technique (MFT) involves membranes with a pore size that will retain bacteria but allow water or diluents to pass through, that are then placed on an appropriate medium, incubated at the correct temperature, and colonies counted after a given time duration (Jay, 1992; Ropre *et al.*, 2002; APHA, 2005; Buckalew *et al* 2006; Seham *et al.*, 2010; Om-kolthoum *et al.*, 2016). The preference of this method is based on its ability to offer quantitative enumeration and flexibility with different sample volumes for increased sensitivity (Flood *et al.*, 2011). Under this method, growth inhibitors such as water-soluble impurities can be removed at the prefiltration stage, resulting in a precise quantitative result with no further cultivation step needed thereafter (Om-kolthoum *et al.*, 2016). Furthermore, this approach makes isolation for further confirmatory tests easier (Flood *et al.*, 2011). However, it may not be highly reliable especially when the quality of membranes is poor (Nnadozie, 2015). Additionally, chemicals adsorbed from the sample to the membrane during filtration may also interfere with the growth of the target organism. It is also not applicable to turbid samples and scoring of typical colonies is always challenging (APHA, 2005; Waller and Bruland 2016). Total coliforms are generally measured in 100ml samples of water. A variety of relatively simple procedures are available based on the production of acid from lactose or the production of the enzyme β -galactosidase (APHA 2005). *E. coli* are also measured in 100ml samples of water based on their production of acid and gas from lactose or the production of the enzyme β -glucuronidase. (APHA, 2005; Rop *et al.*, 2014; Donde *et al.*, 2015).

II. Most Probable number

Most Probable Number (MPN) determines the average number of bacteria in a sample through probability tests (Sutton, 2010). It consists of inoculating a series of tubes with appropriate decimal dilutions of the sample (APHA 2005). Production of gas, acid formation or abundant growth in the test tube after incubation at 35 °C for a certain period of time constitutes a positive presumptive reaction. The sample is subsequently subjected to a confirmation test where the formation of gas in a Brilliant Green Lactose Bile (BGLB) broth fermentation tube at any time within 48 hours at 35 °C constitutes a positive confirmation test (Rompré *et al.*, 2002). A test using an *E. coli* medium can be applied to determine Total Coliforms (TC) that are Faecal Coliforms (FC), with the production of gas after 24 hours of incubation at 44.5 °C in an *E. coli* broth medium is considered a positive result (Osman *et al.*, 2016; Tiwari *et al.*, 2016). Though the method has been in use for several decades, its main setback is that it only provides estimates rather than real values (Rompré *et al.*, 2002).

2. Culture-independent Methods

I. Cytometry

Flow Cytometry (FC) is an alternative fast and reliable method to monitor bacterial abundance and viability of planktonic cells or cells in suspension (Xue *et al.*, 2016). This technique involves the use of fluorescent dyes to stain the water samples before being detected quantitatively, based on the fluorescence intensity and the scattering generated (Hammes *et al.*, 2008; Bazatu *et al.*, 2014; Anna *et al.*, 2015). The size, shape, density of bacteria as well as the type of dye that are used either independently or bound to specific antibodies or oligonucleotides, plays a role in the detection efficiency (Hammes *et al.*, 2008; Bazatu *et al.*, 2014; Xue *et al.*, 2016). The photomultiplier or photodiode in combination with light splitters collects and filters (dicroic

145 mirrors) the light (Vesey *et al.*, 1994; Xue *et al.*, 2016). In CWs studies, FC has been used with
146 different fluorescent dyes to estimate total bacterial counts (Hammes *et al.*, 2008), virus-like
147 particles (Rinta-Kanto *et al.*, 2004) and *Cryptosporidium* and *Giardia* (Ferrari *et al.*, 2000) in
148 water samples. This method has continued to provide a much more realistic quantification of the
149 total number of cells in water samples than traditional plate counts (El-Chakhtoura *et al.*, 2015;
150 Lee *et al.*, 2014; Waller and Bruland 2016).

151 The main limitation of this technique is its susceptibility to errors due to the formation of cell
152 clusters and the attachment of cells to inorganic compounds (Van der Kooij *et al.*, 2014). In
153 addition, it is expensive to acquire, requires skilled operators and may not adequately detect
154 pathogenic microbes that occur at very low or very high concentrations in wastewaters (Vesey *et*
155 *al.*, 1994; Xue *et al.*, 2016). To address some of these shortcomings of FC, a solid phase laser
156 scanning analyzer that has higher detection at low concentrations is being used as an alternative
157 (Deere *et al.*, 2002). Despite it having higher detection capabilities, it is expensive and complex
158 to operate (Helmi *et al.*, 2015). Cell Sorting Flow Cytometer (CSFC) has also been incorporated
159 in CW studies (Doherty *et al.*, 2010; Guez-Barber *et al.*, 2012). CSFC has made it possible to
160 retrieve populations of interest from a heterogeneous population for more detailed studies
161 (Doherty *et al.*, 2010). This technique is called Fluorescence Activated Cell Sorting (FACS)
162 (Ibrahim and van den Engh, 2007; Verónica *et al.*, 2014). With this technique, a population of
163 cells is separated into sub-populations based on fluorescent labeling (Ibrahim and van den Engh,
164 2007). Cells stained using fluorophore-conjugated antibodies can be separated from one another
165 depending on which fluorophore they have been stained with, for example, a cell expressing one
166 cell marker may be detected using a "Fluorescein Isothiocyanate (FITC)-conjugated antibody
167 that recognizes the marker, while another cell type expressing a different marker could be

detected using a Phycoerythrin (PE)-conjugated antibody specific for that marker (Guez-Barber *et al.*, 2012; Veronica *et al.*, 2014). Even though the use of both the CSFC and FACS have improved FC, they remain expensive and complicated to operate (Van der Kooij *et al.*, 2014).

II. Enzymatic Assays

Enzymatic Assay is a rapid assay that has been used in the study of the beta-D-glucuronidase (GLUase) activity of *E. coli* (Naga *et al.*, 2016). This has been achieved in different aquatic systems including seawaters (Caruso *et al.* 2002), freshwaters (Farnleitner *et al.*, 2002; George *et al.*, 2004) and wastewaters (George *et al.*, 2002). The technique involves the use of the substrate 4-methylumbelliferyl-b-D-glucuronide (MU-b-D-GLU) measured as the rate of production of fluorescent methylumbelliferone (MU) and hydrolysis of the substrate which is estimated by fluorometry. This technique is preferred due to its fast speed and high reliability (George *et al.*, 2004; Caruso *et al.* 2002; Farnleitner *et al.*, 2002). Significant correlations between GLUas activity and *E. coli* abundance (estimated by plate counts) demonstrates its reliability (Farnleitner *et al.*, 2001).

III. Fluorescence In-situ Hybridization (FISH)

Fluorescent In-Situ Hybridization (FISH) technique effectively extends epifluorescence microscopy to allow for the fast detection and enumeration of specific microorganisms (Tamara and Piere, 2004; Wagner *et al.*, 2006; Jonach *et al.*, 2014). FISH has been used in CWs' microbial studies to detect the presence of faecal bacteria cells such as *E. coli* in water samples (Rudolf *et al.*, 2008; Baudart *et al.*, 2009; Almeda *et al.*, 2013). The technique uses fluorescent labelled oligonucleotides probes which bind specifically to microbial DNA in the sample, allowing the visualization of the cells using an epifluorescence or confocal laser scanning microscope (CLSM) (Gilbride *et al.*, 2006; Kongsted *et al.*, 2013). The use of FISH to

191 characterize microorganisms within biofilms and to detect pathogens in drinking water samples
192 has been successful (Wilhartitz *et al.*, 2007; Almeda *et al.*, 2013). To further improve on FISH,
193 Catalyzed Reporter Deposition-FISH (CARD-FISH) has emerged (Claudia *et al.*, 2015; Chen *et*
194 *al.*, 2017). This advancement uses oligonucleotides probes labelled with a horseradish peroxidase
195 (HRP) to amplify the intensity of the signal obtained from the microorganisms being studied
196 (Schauer *et al.*, 2012). CARD-FISH technology has been more appropriately applied to water
197 quality studies since it can improve the fluorescent signal from cells in samples with low
198 microbial concentrations (Pernthaler *et al.*, 2002a; Dorigo *et al.*, 2005). This technology can
199 provide fast and reliable results when studying CWs' microbial purification efficacy since it has
200 widely been used in aquatic microbial studies (Aurell *et al.*, 2004; Deines *et al.*, 2010; Baudart
201 and Lebaron, 2010).

202 FISH is commonly used in conjunction with other methods as it is not preferably used as a stand-
203 alone technique (Lehtola *et al.*, 2007; Savichtcheva *et al.*, 2005). For instance, it has been used
204 together with higher affinity peptide nucleic acid (PNA)-FISH) to study pathogens in biofilms
205 due to the enhanced capability of the probe to penetrate through the Extracellular Polymeric
206 Substance (EPS) matrix (Lehtola *et al.*, 2007). Additionally, it has been combined with
207 LIVE/DEADeFISH which combines the cell viability kit with FISH (Savichtcheva *et al.*, 2005)
208 to assess the efficiency of disinfection in drinking water systems (Hoefel *et al.*, 2003). Despite
209 the numerous advantages of FISH as compared to other culture dependent techniques, it requires
210 knowledge of the nucleotide sequence of the target organisms and the design of new probes, and
211 the optimization of the hybridization conditions is time consuming and complex (Sanz and
212 K€ochling, 2007). Additionally, the hybridization efficiency might also be influenced by the

physiological state of the cells, and the signal emitted by auto-fluorescence cells can interfere with the signal emitted by the target microorganisms (Dorigo *et al.*, 2005; Jonach *et al.*, 2014)

IV. Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) technology involves the amplification (i.e. obtain multiple copies) of the fragments of DNA (Jesica *et al.*, 2011; Diana *et al.*, 2015; Chandra *et al.*, 2016). PCR has helped to increase precision in microbial studies, and is achieved through the extraction of nucleic acids (DNA/RNA), the amplification of a target gene or genes via PCR and the post-PCR analysis (Omar *et al.*, 2014; Bożena *et al.*, 2015). MultiplexqPCR and quantitative real time (qPCR) are the most useful PCR-based techniques in detecting faecal bacterial pathogens in wastewater (Jason *et al.*, 2014; Diana *et al.*, 2015; Dolapo *et al.*, 2015; Chandra *et al.*, 2016). Multiplex-PCR that uses several oligonucleotide probes to simultaneously detect different microorganisms has increased precision in detecting faecal indicators and/or pathogens in CWs (Bej *et al.*, 1991; Ibekwe *et al.*, 2002; Felipe *et al.*, 2015). To count the microorganisms in CWs, q-PCR has been put into use to quantify the number of target gene copies present in a sample (Hui *et al.*, 2014; Delapo *et al.*, 2015). This technique monitors the amount of PCR product formed at the exponential phase of the PCR reaction by quantifying a fluorescent reporter (Ibekwe *et al.*, 2002; Omar *et al.*, 2014). Correlating the detected reporter with the initial amount of target template allows for the quantification of the target organism (Kubista *et al.*, 2006; Hui *et al.*, 2014; Delapo *et al.*, 2015). Even though the technology is costly, it is considered to be one of the most accurate and highly reliable techniques for enumerating bacterial pathogens in CWs. (McDaniels *et al.*, 2005; Bożena *et al.*, 2015).

V. Amplicon sequencing and Shotgun Metagenomics

The application of amplicon sequencing and shotgun metagenomics in constructed wetlands (CW) microbial studies is gaining ground (Stanley *et al.*, 2014). Basic amplicon sequencing involves library preparation (designing of primers), Emulsion Polymerase Chain Reaction (emPCR) amplification, sequencing, data processing and data analysis using either Gene Sequencing (GS), Amplicon Variant Analyzer (AVA), or GS Reference Mapper (Torsen *et al.*, 2012). However, metagenomics incorporates the sequencing of samples using appropriate primer sets, hence making it possible for constructed wetlands' microbiologists to evaluate bacterial diversity and detect the abundance of numerous forms of faecal bacterial communities in wastewater, where unculturable microorganisms can be studied through shotgun metagenomics (Gourmelon *et al.*, 2010). Indeed, Next Generation Sequencing (NGS)-based metagenomic sequencing can even detect very low abundance members of the microbial community in constructed wetlands that may go undetected or are too expensive to identify using other methods (Daryl *et al.*, 2016).

Gene composition of microbial communities in constructed wetlands can also be explored through metagenomics which is the analysis of genetic materials recovered from the environment (Torsten *et al.*, 2012). Metagenomics entails more analysis of the gene than the phylogenetic-based surveys, which only relies on the diversity of one gene as in the case of the 16S rRNA gene (Torsten *et al.*, 2012). Deep amplicon sequencing of the 16S rRNA has been conducted using gene and shotgun metagenomics to study bacterial communities in aquatic systems (Fisher *et al.*, 2015). Even though this technique is expensive and complex, it is highly efficient in identifying microbial signatures of water quality by relying upon the analysis of a specific gene or microbial fraction (mainly bacteria) hence making them highly appropriate in the detection

and enumeration of faecal bacterial pathogen indicators such as *E. coli* in constructed wetlands (Gourmelon *et al.*, 2010; Dary *et al.*, 2016).

Constructed wetlands faecal bacterial removal mechanisms

Major concerns with wastewater wetlands are their ability to remove pathogens of helminthes, protozoans, fungi, bacteria, and viruses (Solano *et al.*, 2004a). The constructed wetlands' faecal bacteria removal mechanisms include natural die-off due to starvation or predation, sedimentation, filtration, and adsorption (Quiñónez-Díaz *et al.*, 2001; Solano *et al.*, 2004a; Jasper *et al.*, 2013). The main process involved in pathogen removal is sedimentation (Quiñónez-Díaz *et al.*, 2001). Sediments of wetlands tend to accumulate a vast number of coliforms and bacteria. It has been found that river mud contains 100-1000 times higher faecal coliforms than the surface water, indeed, pathogens such as *salmonella* *sps* which highly accumulates in sediment are detected in higher number in muddy river waters (Jasper *et al.*, 2013). These sediments also provide some bacteria with the ability to survive longer (Solano *et al.*, 2004a). Viruses tend to attach to colloidal material which takes longer to settle out and eventually settle out in a loose layer above sediment. This layer can be disrupted by human activity or natural storm events which could cause the pathogens to enter the water column (Quiñónez-Díaz *et al.*, 2001; Solano *et al.*, 2004a; Jasper *et al.*, 2013). The grain size of the substrate is one determinant of removal efficacy, since a smaller grain size has a larger specific surface area for interactions (Cui *et al.*, 2010). Although bacteria removal mechanisms by CWs is still debatable, studies have compared processes such as filtration, adsorption, natural death and predation by other microorganisms (Bruch *et al.*, 2011; Wu *et al.*, 2016). There has been evidence to reveal that the presence of an active microbial community tremendously accelerates the inactivation and decay of pathogens in aquatic system (Nasser *et al.* 2002; Jed *et al.*, 2009; Fleming *et al.*, 2016).

Another pathogens filtration approach is thought the root system of wastewater wetland plants. A study by Karim *et al.*, (2004) that tested whether sedimentation plays a significant role in reducing pathogens in wetlands, observed that there were no large differences in the number of faecal coliforms and coliphages in the water column compared to the sediments. The attachment to the root system played a larger part with these pathogens. The report found that Giardia and Cryptosporidium had concentrations of two to three times larger in sediments than in the water column. A multispecies wetland showed 73 and 58 percent removal of giardia and cryptosporidium and a duckweed wetland showed 98 and 89 percent respectively (Karim *et al.*, 2004).

Faecal contamination indicators' removal efficiencies by constructed wetlands

Many studies have focused on the faecal bacteria removal efficiencies by CWs (Diemont, 2006; Vymazal, 2010; Ana *et al.*, 2014; Namratha *et al.*, 2016; Natalia *et al.*, 2016; Prabuddha *et al.*, 2016; Zheng *et al.*, 2016). However, due to the extensive and dynamic range of faecal bacterial pathogens, together with the current emergence of new bacterial strains, more research is required to better understand the removal efficiencies in constructed wetlands (Bruch *et al.*, 2011; Zheng *et al.*, 2016). A number of studies have shown higher removal rates, 96.6% to 98.9% removal of *Escherichia coli* (Decamp and Warren 2000), 99% removal of fecal coliforms (Burgos *et al.*, 2016; Zheng *et al.*, 2016) and fecal streptococci by constructed wetlands (Kalibala *et al.*, 2008).

Zheng *et al.*, (2016) also discussed the contribution of environmental factors on the performance of constructed wetlands, and found that there was higher removal efficiency at higher temperatures than at low temperatures. Studies done on bacteria removal in either Free Water Surface Flow CWs (FWSCWs) or Horizontal Subsurface Flow CWs (HSBFCWs) have indicated

304 high removal capacity of faecal bacteria (Roberto *et al.*, 2008; Galvão *et al.*, 2009). Although
305 such findings though are exponentially related to the loading rates, there is still need for
306 incorporating different wetland types to function as a unit in an Integrated Constructed Wetland
307 system (ICW) (García *et al.*, 2013). Wu *et al.*, (2016) also highlighted that that horizontal
308 subsurface flow CWs have better capacity than free water surface flow constructed wetlands for
309 the removal of bacteria. In this study, an average of removal efficiencies by different wetlands
310 types from mainly Europe, America and Asia were calculated. The 24 wetlands had an average
311 water retention time of 5 days and an average influent concentration in log units of 3, 5, 3, 2, 3,
312 and 6 for *E. coli*, faecal coliforms, faecal streptococci, *Clostridium perfringens*, staphylococcus
313 and total coliforms respectively. The average removal efficiencies for *E. Coli* was +1.1 log10
314 CFU/ 100 ml, faecal Coliforms was +0.2 log10 CFU/100 ml, faecal streptococci was +0.9 log10
315 CFU/100 ml, *C. perfringens* was +0.6 log10 CFU/100 ml, staphylococcus was +0.8 log10
316 CFU/100 ml, and with the exception of total coliforms which was -0.9 log10 CFU/100 ml).
317 Indeed, the combined CWs can improve the removal of *E. coli*, TC and FC by 1.5, 1.2, 0.3 log10
318 CFU/100 mL, (García *et al.*, 2013). WHO (2006) guidelines for the safe use of wastewater in
319 agriculture took into account fecal coliforms to be less than 1000 CFU/100 mL for food crops
320 eaten raw. However, the United States Environmental Protection Agency (USEPA) and the
321 United States Agency for International development (USAID) (1992) placed stricter guidelines
322 for wastewater reuse in the USA by recommending no detectable FC/100 mL in wastewater for
323 irrigation of crops that are likely to be eaten uncooked. Therefore, where space is inadequate for
324 increasing the wetland dimensions, either further improvements of the wetland technology or a
325 combination with subsequent treatment of the wetland effluent are needed to eliminate the health
326 risks associated with wastewater used for crop irrigation and landscaping (Richter and Weaver,

2003b). Aeration of the wetland bed has also enhanced constructed wetlands purification efficacy. For example, in an aerated horizontal flow constructed wetlands, a 3.3 log₁₀ CFU reduction per 100 mL of *E. coli* was achieved whereas in a non-aerated horizontal flow CWs had a log₁₀ CFU reduction/ 100 mL of around 1.4. (Headley *et al.*, 2013).

Conclusion

The advantages and limitations of approaches currently used for the detection and removal of faecal bacteria in constructed wetland have been discussed in relation to their applicability to wastewater management (Lee *et al.*, 2014; Waller and Bruland 2016). The ultimate choice of technique depends on the objective of the research, the required level of resolution/restoration, the availability of specialized equipment and the available funding (Lee *et al.*, 2014). Despite culture-dependent techniques still being used in constructed wetlands' microbiology studies, the modern molecular methods are slowly gaining ground (Božena *et al.*, 2015). These new techniques have greatly expanded the existing knowledge on the diversity and structure of microbial communities as well as providing a more detailed understanding of removal mechanisms. The future automatization of molecular methods might be indispensable for the development of online devices to detect pathogens in drinking water and wastewater networks. The major knowledge gap in understanding the microbiology of constructed wetlands is the lack of information that is required to link microbial diversity and function (Akarat *et al.*, 2015). Approaches that can fill this gap include microarrays (collection of microscopic DNA spots attached to a solid surface), metabolomics (set of life-sustaining chemical transformations within cells) and meta-proteomics (all protein samples recovered from the environment) which have been extensively used in environmental microbiology (Carla *et al.*, 2009). Unfortunately, their use has not yet been adequately explored in wastewater systems and constructed wetlands in

particular. Future research should explore the use of integrated approaches that will improve the current understanding of constructed wetlands microbiology. This can be achieved by combining a range of techniques that explore and link microbial diversity and activity that will ultimately lead to a better understanding of the relationship between microorganisms and system function that is based on overlapping influencing factors. Some of these influencing factors can be attributed to the fact that the faecal indicator bacteria in CWs may be derived from different sources; influent wastewaters, regrowth within the constructed wetlands, and animal activities. These factors pose a big challenge in understanding and improving the working conditions of constructed wetlands (Xue *et al.*, 2016). Therefore, even though constructed wetland is a promising technique in eliminating bacterial pathogens from various effluents, the possibility of pathogens re-emergence still exists, signaling that more work is needed to address the survivability of pathogens in wetlands.

Recommendations

The reuse and safety of properly treated wastewater has become more attractive due to water shortages caused by climate change, the rise in human populations and the demands of rapidly evolving industrial processes in recent years. Constructed wetlands as environmentally friendly ecosystems have been widely applied not only for various water pollution controls but also for faecal bacteria reduction and presumably for the elimination of human pathogens. Although the current literature contains a wealth of information on the overall removal of indicator bacteria in different types of constructed wetlands with many suggestions on mechanisms for their removal (e.g. adsorption, sedimentation and predation), the complex micro/macro nature of constructed wetlands environments result in the magnitude of the various processes and influencing factors being poorly understood. Therefore, further research, modifications and developments are

necessary to increase the efficiency of bacteria removal in wastewater by constructed wetlands. This will aid in managing the quality of water in rivers, lakes and ocean and help strive to solve the rampant incidences of water borne and water related illnesses.

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Figure 1: A typical constructed wetland system (Source: Author)