INVESTIGATION OF PATHOGENS REMOVAL FROM WASTEWATER USING A HYDROPONIC SYSTEM

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A dissertation submitted for the fulfillment of a degree Master of Science (Microbiology), in the Department of Biochemistry and Microbiology, Faculty of Science and Agriculture, University of Zululand, South Africa.

By

Sinqobile Fanele Ndulini

DECLARATION

"I declare that the thesis herewith submitted for the degree Master of Science: Microbiology at the University Of Zululand is my original work and has not been previously submitted for a degree at any other institution of higher education, and that its only prior publication was in the form of conference papers. I further declare that all the sources cited or quoted are acknowledged and indicated by means of a comprehensive list of references"

………………..	....
Ndulini SF	Date

I hereby approve the submission of this thesis…

....	………………..
Dr MS Mthembu (UZ)	Prof. AK Basson (UZ)

On this day …….. Of ………………………… 2017, at the University Of Zululand (UZ)

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ABSTRACT

Microbial contamination in water sources is a threat to public health and environment. It may lead to distribution of pathogenic microorganisms in water, which may lead to waterborne diseases outbreaks. The current municipal wastewater treatment systems employ the chlorination process for disinfection and inactivation of pathogenic microorganisms. This process has been associated with life threatening drawbacks, which has necessitated the application of green technology in wastewater treatment sector. This work was aiming at investigating the reduction of pathogens in the hydroponic system. A vertical flow constructed wetland planted with emergent macrophytes (*Bidens pilosa L* and *Amaranthus hybridus L*) was used to treat wastewater treatment from the secondary stage of the conventional sewage treatment process. Wastewater samples were collected at different hydraulic retention times and analyzed for physiochemical parameters (temperature, pH and dissolved oxygen), organic material and pathogens concentrations. A field multimeter probe was used for measuring physiochemical parameters. The respective cell tests and Aqualytic Thermostatic Cabinet was used to measure organic material content. The real-time qPCR was used for the quantification of pathogens.

The removal efficiency of pathogens in the system after treatment was 59%, 73% and 18% for *E. coli*, *Salmonella spp*. and *Shigella spp*. respectively. The removal was not statistically significant (p=0.06), which indicated that the system was able to efficiently remove pathogens from wastewater. The physiochemical parameters were correlated with the reduction of pathogens. The temperature had a positive correlation with pathogens removal ($0.15 \leq r \leq .083$). While pH had a negative correlation with pathogens removal ($-0.90 \leq r \leq 0.09$). Dissolved oxygen also had a negative correlation with pathogens removal ($-0.99 \leq r \leq -0.65$), and COD had a positive correlation with pathogens removal ($0.19 \leq r \leq 0.99$). These correlations indicated that there was some influences of physiochemical parameters on the pathogens reduction in the system.

Pathogens removal mechanism in the system was also investigated through investigation of antimicrobial production by macrophytes. The plant extract was prepared and screened for phytochemical compounds, which revealed the presence of saponins, alkaloids, tannins and terpenoids. *Salmonella*, *Shigella* and *E. coli* were selected as the pathogens of public concerns and tested against the plant extracts of macrophytes using the method described by Eloff 1998. Where the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the methanol extracts were determined. The MIC of *B. pilosa L* extract against pathogens ranged between 25-50 mg/ml while *A. hybridus L* had the MIC at 25 mg/ml only. The MBC results showed that the extracts inhibition activity was not lethal but bacteriostatic to the pathogens of interest. This has shown that macrophytes are capable of inactivating and ultimately reducing pathogens in the system. Thus the study found that the hydroponics have a potential of being used to remove pathogens in wastewater treatment. This technology could be used in a demanding area of the water sector if properly constructed and optimized.

DEDICATION

I would like to dedicate this work to my lovely family, especially my son. Zanokuhle, you put a smile on my face every time and keep me ambitious and inspired to do more in life. My mom, your prayers and support is the fuel that kept the fire of success burning in me…. I love you. This is the product of your unconditional love, support and prayers.

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PREFACE

CONFERENCE ATTENDED

Papers presented at national conferences:

- **Ndulini S.**F., Makhoba, X.H., Sithole, G.M. and Mthembu, M.S. Antimicrobial activity of *Bidens Pilosa L* and *Amaranthus Hybridus L* on bacterial pathogens in domestic wastewater. *Faculty of Science and Agriculture Symposium*. 29 September 2017, University of Zululand, Main Campus, South Africa.

- Sithole, G.M**.**, Mthembu, M.S., **Ndulini, S.F**. and Makhoba X.H. Responses of microbial communities to seasonal changes in natural wetlands. *Faculty of Science and Agriculture Symposium.* 29 September 2017, University of Zululand, Main Campus, South Africa.

- Nyandeni, N., **Ndulini**, **S.F.,** Sithole, G.M., Zulu, N.C.T. and Mthembu, M.S. Nutrient Removal in Domestic Wastewater Using Microbial Biofilms. *30th Annual SASM-KZN Symposium*, 27-29 October 2017, UNIZULU, Science Center, South Africa.

- Ntuli, Y.T., **Ndulini, S.F**., Zulu, N.C.T. and Mthembu, M.S. The Effect of Microalgae in Nutrients Removal from Domestic Wastewater. *30th Annual SASM-KZN Symposium*, 27-29 October 2017, UNIZULU, Science Center, South Africa.

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- **Ndulini, SF**., Mthembu, MS., Sithole, G.M. and Makhoba, X.H. Investigation of nutrients and fecal coliforms removal in the hydroponic system for wastewater treatment. *17th WaterNet/WAFSA/GWP-SA Symposium*. 26 – 28 October 2016, Gaborone, Botswana**.**

- Sithole, G.M., Mthembu, M.S., **Ndulini, S.F**. and Makhoba, X.H. Effects of nutrients levels on water quality of Lake Cubhu and Nhlabane estuary during extensive drought period. *17th WaterNet/WAFSA/GWP-SA Symposium*. 26 – 28 October 2016, Gaborone, Botswana.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Human health must be protected from contaminated water by prevention of microbial contamination in drinking water sources. Microbial contamination may occur in different ways in natural water sources and in water supply system. This may lead to the introduction of pathogenic microorganisms in water which their occurrence may be problematic to public health. Occurrence of pathogens in water threatens the human health, environmental ecosystems as well as the agricultural sector. South Africa is a semi-arid country with low rainfall and high evaporation rate. It is also experiencing fresh water scarcity due to draught resulting from high variability and spatial distribution of rainfall (Morato *et al*., 2014).

The improper disposal of wastewaters of any kind is accelerated by the rapid increasing population which compels the fast operations with regards to industrialization, urbanization and food security. These accelerate deforestation to provide space and other living needs, shortage of water supply in South Africa that is already experiencing fresh water scarcity and high usage of fertilizers to cater for agriculture. Although water covers about 71% of earth's surface which is vital for all forms of life, only 2.5% is fresh water. Out of the 2.5% fresh water available on the planet account only 0.01% is available for human utilization. The available freshwater is threatened by rapid population growth, urbanization and unsustainable water consumption in industries and agriculture (Azizulla *et al*., 2011). Freshwater scarcity has been a growing problem that leads to a significant impact on economic development, human livelihood and environmental quality on developing countries (Rajasulochana and Preethy, 2016). Fresh water scarcity, and the other previously mentioned factors accelerate favorable conditions for the microbial pathogens to prevail in water sources and in water supply systems, thus causing waterborne diseases outbreaks.

Waterborne diseases result into deaths, and such outbreaks have been reported in developing countries with lower levels of sanitation accompanied by less public health awareness. In developing countries, water shortage is accelerated by lack of proper management, unavailability of professionals and financial constrains (Azizulla *et al.*, 2011). In 2013 more than 325 protozoan outbreaks were reported, with the majority of cases reported in United States and Europe (Botes *et al*., 2013). In the United States alone, over 58 000 cases of waterborne diseases associated with cryptosporidium had occurred between 1995 and 2007. While in 1993, over 400 000 cases of cryptosporidium were reported in Milwaukee (Staggs *et al*., 2013). In India, in 2004, 535 000 people were killed by diarrhea from contaminated water consumption, while 21% of communicable diseases were reported to be water related (Rajasulochana and Preethy, 2016).

This does not only result in diseases outbreaks but also water and land pollution. Water pollution results in the eutrophication process which is caused by excessive growth of aquatic plants which cause depletion of oxygen in water, thus resulting in the death of aquatic life. Land pollution occur as a result of alteration of microbial community structures, disturbance of ecosystems and occurrence of excessive nutrients which degrades plants and grass. Proper sanitation and effective wastewater treatment systems are of critical importance. Therefore, there are two fundamental reasons that necessitates the treatment of wastewater before disposal. The first is to secure public health and secondly, prevention of pollution in receiving waters (Ramírez-Castillo *et al.*, 2015).

Natural water bodies have their way of treating water through filtration of pathogens in sand particles. However, these cannot function independently. Therefore, before disposal of wastewater to these water bodies, water must first be treated to meet the standards of disposal set by

Department of Water and Sanitation. The currently used disinfection methods are physical and chemical. The physical disinfection includes ultraviolet (UV) and solar radiation. These involve the exposure of wastewater to radiations which damages their DNA material and hinder reproduction. However, there are disadvantages associated with the application of these methods. The first being the fact that they are not effective in turbid wastewater. Secondly, they leave no residuals which may cause regrowth of microbial pathogens in water supplies (Olaolu *et al.,* 2014).

Chemical disinfection processes include chlorination, chlorimation and ozonation. These employ the usage of chemicals that penetrate the microbial cell walls and obstructs their metabolism. These processes have some drawbacks, including the high corrosiveness of chlorine and production of harmful by-products (trihalomethanes) during disinfection process. On the other hand, the ozonation disinfection method is expensive and is not effective in killing the cysts and larger organisms (Olaolu *et al.,* 2014). Therefore, adequate wastewater treatment is essential to sustain natural environment and ensures the safety of human beings.

Alternative method may be used in wastewater treatment to overcome eutrophication and microbial pathogens contamination of natural water bodies as well as ground water contamination. The application of green technology, which involves the growth of crops in a closed system using a nutrient solution (wastewater) for irrigation may solve the problem of domestic water disposal (El-Serehy *et al.*, 2014). These systems are called hydroponics and have been applied mostly in the United States and China and their application has increased due to their success rate. Considerable attention towards wastewater treatment using wetlands consisting of bed filters usually planted with emergent plants and constructed wetlands have been gradually developed in the past 20 years. They have a low energy and maintenance requirements suited for rural areas and require no use of chemicals, thus being environmentally friendly and sustainable. The gravel bed hydroponics are engineered systems with a potential of removing pathogens from wastewater in a variety of proposed mechanisms (El-Serehy *et al.*, 2014). These removal mechanisms include the filtration of pathogens by soils, the inactivation of pathogens by environmental stress (El-Serehy *et al*., 2014) and the production of antimicrobial substances by macrophytes (Werker *et al*., 2002).

The research aiming at investing pathogens removal in domestic wastewater using hydroponic system and establishment of their removal mechanisms was conducted. It was of critical importance for this study to be conducted in order to introduce and emphasize the importance of applying green technology in wastewater treatment. In this study, the use of a hydroponic system constructed for wastewater treatment was tested and its ability to remove pathogens was evaluated. The ability of these systems to reduce or completely remove pathogens will solve problems associated with disinfection methods currently used. Apart from that, it may offer guaranteed safety of environmental ecosystems, aquatic life as well as human health.

1.2 Aim and Objectives

1.2.1 Aim

The aim of the study was to investigate the pathogens removal in wastewater using hydroponic system and establish the mechanisms of pathogens removal from the system

1.2.2 The objectives of the study were:

(i) To determine physiochemical parameters (Temperature, pH, DO, COD and BOD_5) in a hydroponic system and model these against pathogen removals

(ii) To isolate and identify bacterial pathogens (*Salmonella*, *Shigella*, and *E. coli 0157:H7*) and establish their reduction potential in a hydroponic system

(iii)To establish the mechanism of pathogens removal from hydroponic system

1.3 Literature Review

1.3.1 Introduction

The microbial pathogens contamination in water sources is a threat to public health. Pathogens are defined as disease causing organisms and are found ubiquitous in the environment. The major groups of pathogens found in wastewater are bacteria, viruses, fungi and protozoa. Viruses are reported to be the most dangerous wastewater pathogen due to their resistance to treatment (Olaolu, 2014). They are more infectious and not easily detected in water (Olaolu, 2014). Wastewater primarily receive pathogenic contamination through faecal contamination that originates from sewage disposal. This introduces pathogenic microbial contamination in water resources that occurs as a result of disposal of improperly treated or untreated water from domestic, industrial and agricultural uses. The alternative wastewater treatment systems can form part of an important strategy in water demand management (Morato *et al*., 2014).

Hydroponics are potential biological systems that have a potential and have been recommended for wastewater treatment (Adrover *et al.,* 2013). These systems mimic natural wetlands by employing biological processes through the interactions between macrophytes, microorganisms and wastewater (Garfi *et al*., 2012). There are biological, physical and chemical processes that occur within these systems that result in pathogens removal. These processes may be affected by a number of factors. While some of these factors may enhance the removal efficiency, some may limit their functioning. The treatment efficiency of these biological systems varies with types as well as with respect to flow directions, granular size and types of macrophytes used in the system.

1.3.2 Microbial Contamination in Water

Water primarily receive pathogenic contamination through faecal contamination that originates from sewage disposal. The introduced pathogenic microbial contamination in water resources occur as a result of disposal of improperly treated or untreated water from domestic, industrial and agricultural uses. This also includes the disposal of industrial and agricultural wastes into water resources. There are various factors that accelerate the spread of microbial pathogens in water resources as well as in water supply systems. These include the occurrence of leaks in the supply system leading to low pressure events and during their repair, these pathogen gains entrance into the system. Weather is the main factor that may influence the spread of pathogens. Heavy rains or floods and runoffs into water resources may introduce microbial contamination. The change of temperature may also alter dynamics of microbes in pipes and plankton microbes may be trapped in biofilms while pathogens in biofilms may be released into flowing water in the supply system pipes (El-Serehy *et al.*, 2014).

In rural areas where there are no proper water supply systems. People fetch water directly from water bodies for consumption. These communities are at risk of being affected by waterborne diseases from consumption of contaminated water derived from contaminated natural water sources and contaminated water supply systems. There are two fundamental reasons that necessitate the treatment of wastewater before disposal. The first is to secure the public health and secondly, prevention of pollution in receiving waters (Ramírez-Castillo *et al.*, 2015).

1.3.3 Pathogens Transmitted Through Contaminated Water

The major pathogenic microorganism found in wastewater includes bacteria, viruses, fungi, protozoa (Olaolu, 2014) and helmiths (Ajonina *et al.*, 2015). Microbial pathogens enter in water through faecal contamination. Wastewater discharges are regarded as the main sources of environmental pollution, while the reuse of wastewater is questioned due to microbiological quality of water, thus possibly enhancing the spread of waterborne diseases (Ajonina *et al.*, 2015). The main causes of waterborne diseases are discussed below:

1.3.3.1 Bacterial Pathogens

Bacteria are the most commonly found waterborne pathogens contracted through wastewater. Pathogens commonly found in wastewater include species of *Salmonella*, *Shigella*, *Vibrios*, *Yersenia*, and pathogenic strains of *Escherichia coli*. Waterborne diseases contracted from ingestion of contaminated water includes shigellosis, salmonellosis, cholera and dysentery. Waterborne diseases caused by bacteria are mostly diarrheal infections. Cholera is caused by *Vibrio cholerae* and primarily transmitted through contaminated water. The pathogenicity of this microorganism involves the secretion of exotoxins that attach to intestinal mucous membrane of cells resulting in intense diarrhea (Olaolu *et al*., 2014).

Salmonellosis is caused by *Salmonella* species. This species produces two types of infections which include typhoid and paratyphoid fever. Salmonellosis is also transmitted through ingestion of contaminated water. Shigellosis is also a waterborne disease caused by *Shigella* species. Its pathogenicity involves the production of shiga toxins in the epithelial cells of intestinal tract of humans. Infection manifestation is characterized by anorexia, fever and fatigue. Apart from the above mentioned pathogens of bacterial origin, there are also pathogens of protozoan origin found in contaminated waters.

1.3.3.2 Protozoan pathogens

Protozoans are single celled organisms with membrane bound organelles and are ten times larger than bacteria. These pathogens are capable of causing life threatening diseases which include giardiasis, cryptosporidiosis, dysentery and amoebic meningoencephalitis. Protozoan are able to survive without a host and are capable to thrive under adverse conditions in a form of cysts or oocysts of 3-14 µm in diameter (Hai *et al*., 2014). Protozoan are introduced into aquatic environments through discharge of untreated or inadequately treated sewage, runoffs and discharges of manure from agricultural lands (Girones *et al.*, 2010).

Major protozoa pathogens are species of *Cryptosporidium* and *Giardia*. They are detected in a form of cysts or oocysts. Protozoa infections emerge from the ingestion of cysts or oocysts, resulting in either dysentery or diarrhea. These pathogens are mostly associated with intestinal diseases, dysentery and ulceration of liver and intestines (Olaolu, 2014; Azizullah *et al.,* 2011). They can cause life threatening illnesses to unborn children and immunosuppressed individuals.

Giardiasis occurs when *Giardia* is consumed and adheres to intestinal epithelial cell walls. This disease is not fatal. However, diarrhea, abdominal cramps, weight loss, nausea and gastrointestinal distress are experienced. Cryptosporidiosis infection occurs when water contaminated with *Cryptosporidium* is ingested. The oocysts attach into the gastrointestinal tract of humans, and headache, nausea, vomiting, diarrhea, abdominal cramps are experienced (Olaolu, 2014). Removal of these pathogens in hydroponics occur as a result of the interactions that take place in the rhizosphere which includes macrophytes-rhizomicrobia interactions. The water scarcity and contamination by pathogens do not only affect health but it also has negative influence in the environment and agricultural sector.

1.3.4 Impacts of Water Scarcity and Contaminated Water Disposal in General Health, Environment and Agriculture

The emergence of waterborne infections negatively affects the public health. The lack of reliable data for the estimation of diseases and lack of monitoring programs result in underdiagnoses of such infections. Despite the prevalence of parasitic diseases, they may potentially result in greater economic burden due to high costs for income, medical treatment and diagnosis. Only few developing countries have protozoa included in their operational surveillance systems, as major focus with bacterial and viral infections (Fletcher *et al.*, 2012). Apart from lack of prevalence, protozoa underdiagnose may also occur due to failure of conventional staining and microscopy methods. This may occur as a result of protozoa evolution, which may require sensitive, accurate and simultaneous detection though molecular methods. Although these may be costly and labor intensive, they will benefit water industry and public health (Fletcher *et al.*, 2012).

Water is an important element to agricultural production and is a key to all socioeconomic development as well as healthy ecosystem maintenance (Khan, 2014). The shortage of water lowers food production which has a worldwide negative impact on food security. In a study conducted by the World Wide Fund for Nature South Africa in early 1990`s looking at the latest agricultural trends, findings have indicated that most farms have been converted to other land uses due to water shortages. Water scarcity has left South Africa with less than two thirds of the food producing farms compared to about three decades ago (Khan, 2014).

The occurrence of pollutants in aquatic environments has been associated with a number of negative effects, which include short and long term toxicity, endocrine disruption effects and antibiotic resistance of microorganisms (Luo *et al.*, 2014). The release of contaminants and wastewater constituents into surface water expose pathogens to natural attenuation. Natural attenuation may include dilution in surface water, sorption into suspended sediments, direct and indirect photolysis and aerobic degradation (Luo *et al*., 2014). This may alter microbial community structures in the environment, disturb living ecosystems and limit the biodiversity.

The disposal of contaminated water does not only negatively influence human health through emergence of waterborne diseases but also in agricultural production. Macarisn *et al.* (2014) reported that fresh produce has been associated with human enteric pathogens worldwide. Foodborne illnesses associated with *E. coli* 0157:H7 linked with leafy vegetables have increased. Some organisms are capable of internalizing the plant tissue through roots and then spread upward the edible part in the plant (Macarisn *et al*., 2014). All these negative impacts of wastewater scarcity and contaminated water disposal on the environment and health necessitates the efficient wastewater treatment operations that are environmentally friendly and sustainable. These operations may have a potential to be achieved through employment of a low cost eco-friendly constructed wetland for wastewater treatment.

1.3.5 Constructed Wetlands in Wastewater Treatment

Constructed wetlands are complex biological systems that mimics natural wetlands with natural self-cleansing processes by reducing the level of pollutants to dischargeable limits (Bhatia and Goyal, 2014). They are made up of four components. These are water, soil, vegetation and microorganisms (Shelef *et al.*, 2013). These wetlands provide a wide range of benefits as a treatment system. Their usage may generate huge economics savings since they solely depend on natural treatment routes with minimal energy requirement cost and less labor intensiveness accompanied by low construction and maintenance costs (Lee *et al*., 2009). Wastewater treatment in treatment wetlands is accomplished through an integrated combination of physical, chemical and biological interactions among macrophytes, substrate and synergistically microbial community (Korboulewsky *et al*., 2012). They offer flexible site selection, easy to operate and maintain and also a wildlife habitat as well as high stability under changing environmental conditions. The treated water from constructed wetlands can be used for irrigation. The efficiency of the constructed wetland to remove pathogens depends largely on the type of macrophytes used and granular medium (Morato *et al*., 2014). The usage of constructed wetlands in wastewater treatment has been documented to be advantageous through being environmentally friendly (Lee *et al*., 2009) compared to currently used conventional wastewater treatment systems.

Wetlands possess several properties that make them attractive to manage pollutants. These include high plant productivity, large adsorptive capacity of sedimentation and high rates of oxidation by microflora associated with plant biomass. There are different types of constructed wetlands, and are classified according to the water levels and flow directions. Subsurface constructed wetlands are regarded as most efficient in contaminants removal because they provide both aerobic and anaerobic conditions which makes them yield high removal in terms of nutrients and pathogens from wastewater (Lee *et al*., 2009). These types of wetlands may be applied in a form of hydroponic systems, which are also potentially efficient in wastewater treatment for both nutrients and pathogen removal. These systems are highly recognized because of their ability to remove various contaminants such as biological oxygen demand (BOD), chemical oxygen demand (COD), suspended solids, nitrogen, phosphorus as well as pathogens (Lee *et al*., 2009). Hydroponics are a type of subsurface flow constructed wetlands that are essential for wastewater treatment.

1.3.6 Hydroponics in Wastewater Treatment

Hydroponics systems may be defined as cultivation technologies that employ nutrient solutions rather than soil substrates (Kumar and Cho, 2014; Lee and Lee, 2015). The Unites States (US) and China are two leading countries in application of hydroponics. They are mostly used in studies of plant nutrition due to homogenous root medium. These systems are also useful for nutrients and pathogens removal from wastewater (Adrover *et al.*, 2013). In hydroponics, the growing macrophytes receive nutrients directly from water and the gravel is used to support growing macrophytes and to provide anchoring surfaces for roots as well as surface area for biofilm development. In hydroponics the correct functioning is defined by the interaction between macrophytes, substrate and microorganisms (Leto *et al.*, 2013). The employment of these systems offers a lot of benefits, especially to the environment.

Hydroponics offer the ability of water and nutrients reuse as well as easy environmental control through prevention of soil borne diseases and pests (Haddad and Mizyed, 2011; Lee and Lee, 2015). They require a small space (Haddad and Mizyed, 2011) and can offer high yields and high quality products (Lee and Lee, 2015) with growth rate increase between 30-50% than that of soil gardens (Haddad and Mizyed, 2011). These systems offers good treatment performance at low operation costs and results in improved environmental and public health conditions (Garfí *et al.*, 2012). This occurs as a results of employing biological processes which are facilitated by the interaction of macrophytes and microbes with wastewater.

1.3.7 The Role of Macrophytes in Hydroponic Systems

Macrophytes have unique characteristics that make them suitable for wastewater treatment processes. These properties enable them to play multiple roles in the removal of contaminants and unwanted wastewater constituents. Although macrophytes have special features to perform wastewater treatment processes, there are factors that affects their functioning. Those factors include photosynthetic rate, radial oxygen loss and microbial biomass. The macrophytes adaptations, limitations and processes in mediating wastewater treatment in hydroponic systems are presented below:

1.3.7.1 Macrophytes adaptations to Wastewater Treatment

In wetlands, macrophytes are regarded as one of “big three” wetland components together with hydrology and soils (Vymazal, 2013). Gupta *et al.* (2012) reported that macrophytes were first recognized in 1960s and 1970s in water quality improvement and play a vital role in wastewater treatment. Macrophytes exhibits several properties that enable them to be essential for wastewater treatment processes. They possess a wide variety of morphological and physiological adaptations and responses to waterlogged environments. Radial oxygen loss (ROL) is the excess oxygen released by plant roots to their surrounding and is regarded as the most important feature of macrophytes (Li *et al.*, 2013). This is the major anatomical feature of macrophytes, the presence of development of air spaces in different parts of leaves, stems, rhizomes and roots. The tolerance to high organic matter and nutrients, reach below ground organs, high above ground biomass for winter insulation in cold and temperate regions and nutrient removal via harvesting (Vymazal, 2013).

The removal of pollutants by macrophytes depends on photosynthetic rate, ROL, as well as microbial biomass (Li *et al.*, 2013). The photosynthesis process and radial oxygen loss mediated the presence of important gases (oxygen and carbon dioxide) into treatment wetlands. The biomass content is directly proportional to the removal of pollutants. Adsorption and absorption of nutrients and colonization of microorganisms on the surface area enhances the removal of pollutants in hydroponics. However, physiochemical processes and microbial processes are just as important than the presence of macrophytes in treatment wetlands (Li *et al.*, 2013). This has created a debate regarding the significance and role of macrophytes and it clearly shows that the eco-physiological characteristics of macrophytes as well as their influence are unclear (Li *et al.*, 2013), though Chen *et al.* (2016) has demonstrated that the presence of vegetation in treatment wetland significantly increase the removal of contaminants.

1.3.7.2 Processes Mediated by Macrophytes in Hydroponic systems

The remediation processes are mediated by macrophytes and may occur in different routes depending on the type of wastewater being treated as well as its composition. Macrophytes in treatment wetlands reduce methane emissions in the atmosphere. They are also capable of removing nutrients from wastewater. Macrophytes produce antimicrobial compounds in wastewater treatment wetlands which are believed to have bactericidal and or bacteriostatic effect on pathogens. They also indirectly stimulate the microbial activities which enhances the treatment efficiency of the wastewater treatment wetland. The macrophytes mediated processes are:

- Remediation processes

Depending on the composition of wastewater being treated the remediation processes occurs under different conditions. Five main processes have been identified in remediation processes and includes phytoextraction, phytodegradation, rhizofiltration, phytovolatilization and phytostabilization (Bhatia and Goyal, 2014). While some of the processes may coexist, some occur in varying proportions during phytoremediation. However, the macrophytes remediation has been coupled with microbial remediation in the form of rhizospheric bacteria, rhizoremediation. This is where organics and nutrients removal are facilitated by microbiota. Rhizoremediation involves the interaction of plant roots associated with microbes to reduce concentration of pollutants present in water (Bhatia and Goyal, 2014)

- Reduction of Methane Emissions

Wetlands reduce methane emissions. Methane is produced in anaerobic soils and sediments whereas well drained soils sink atmospheric methane. This occurs as a result of either ammonia oxidizers or methanotrophs. In wastewater treatment wetlands methane is produced during the aerobic biodegradation of organic matter present in effluents or accumulated as a result of plant litter (Mander *et al*., 2014). There are factors affecting methane emissions reduction in treatment wetlands. They include the physical and ecological structure (Zhou *et al*., 2016). Macrophytes form part of the ecological structure in natural as well as constructed wetlands for wastewater treatment. Decomposition of macrophytes favors the occurrence of organic matter which later undergoes reduction to methane. This process requires anoxic conditions and low oxidation reduction potential. The macrophytes root can release oxygen that increases the methane emission by altering the soil oxidation reduction potential status. Optimization of ecological structure of wastewater treatment wetlands is of great significance in order to reduce methane emissions (Zhou *et al*., 2016).

- Nitrogen reduction

Macrophytes facilitates the reduction of nitrogen in treatment wetlands. Their role may be defined in two ways. First being the enhancement of nitrogen removal through plant uptake. Macrophytes assimilate nitrogen, even though the amount of nitrogen removed by plants is less compared to the total nitrogen metabolized within vegetated wetlands (Pierobon *et al.*, 2013). Secondly, by indirectly enhancing microbial activities through releasing oxygen and organic matter. The studies conducted on the relationships between the functioning of the plant diversity and ecosystem revealed that plant species' diversity increases the nitrogen removal efficiency in treatment wetlands (Zhou *et al*., 2016).

- Production of antimicrobial compounds

Macrophytes in treatment wetlands influence contaminants removal due to the presence of roots exudates which have a strong effect on the rhizomicrobia. Exudates allows macrophytes to produce more metabolic products under stressful conditions (Chen *et al.*, 2016). Macrophytes accumulate armory antimicrobial secondary metabolites which they produce to protect themselves if under attack by pathogens. There are inducible antimicrobials which are known as phytoalexins and phytoanticipins which are constitutive chemical barriers to microbial attack (González-Lamothe *et al.*, 2009). The rhizobacteria found close to plant roots, are able to control diseases caused by soil pathogens. The antimicrobial secondary metabolites produced by macrophytes may be bactericidal (Morato *et al*., 2014).

- Stimulation of microbial activities

Macrophytes play an indirect role in stimulating microbial activities in wastewater treatment wetlands. They achieve this through provision of large surface area for microbial attachment (Korboulewsky *et al.*, 2012). The presence of macrophytes in treatment wetlands is the main factor affecting microbial activity. It has been reported that macrophytes have the ability to translocate oxygen from leaves to roots which is then released into the rhizosphere (Li *et al.*, 2013). The released oxygen has the effect in the redox potential of the substrate. Oxygen releases in the rhizosphere result in oxidized conditions which can stimulate both aerobic and aerobic decomposition of organic matter and growth of nitrifying bacteria. Macrophytes roots also release a number of organic compounds which include enzymes, organic acids and amino acids. The organic carbon released by roots may be a carbon source for the denitrifying bacteria, thus increasing nitrite removal in treatment wetlands. The indirect role of macrophytes in treatment wetlands includes provision of the surface area, oxygen and organic compounds release may also result in high efficiencies of nutrients removal (Korboulewsky *et al.*, 2012).

1.4 Macrophytes Used in the Study

Wastewater treatment wetlands planted with macrophytes are regarded as more efficient in treating wastewater than unplanted wetlands. The presence of macrophytes in these systems enhance the biological processes that improves the treatment efficiency of wetlands. Macrophytes can be emergent, sub-mergent or free floating. In this study emergent macrophytes *Bidens pilosa L* and *Amaranthus hybridus L* were used. These were selected because they are indigenous plants in South Africa, Empangeni (KwaZulu-Natal) and were easily accessible. Their ability to survive in high organic matter soils as well as their waterlogged tolerance made them suitable for wastewater treatment processes.

1.4.1 *Amaranthus hybridus L*

Amaranthus hybridus L is used as a leafy vegetable and a pot herb. It grows in temperate and tropical regions in South Africa. It grows best in soil temperatures between 18-25^{0}C on well drained fertile soils and deeper soils at pH 6.4 (Amaranthus Production Guideline; 2010). *Amaranthus hybridus L* is a member of *Amaranthaceae* family, the *Caryophylales*. This plant is widely used in developing countries like Burkina Faso as a traditional medicine for treating various kind of diseases, including malaria, fever, pain, hepatic disorder, nervous system disorders, cancer and cardiovascular diseases (Nana *et al*., 2012). *Amaranthus spp*. were also important to pre-Colombian American people's diet. Their consumption was recommended to patients with celiac diseases, liver infection, knee pain and diabetic persons (Nana *et al*., 2012) as well as convalescent patients.

Amaranthus have also been used for diarrhea and dysentery due to their laxative, diuretic and cicatrisation properties. *Amaranthus hybridus L* together with *Amaranthus cruentus L* are regarded as two species under *Amaranthus* family that promotes health and prolonged shelf life (Nana *et al*., 2012). Apart from the medicinal uses of this plant, *Amaranthus hybridus* L together with other Amarantheceae family species have been reported to have an ability to accumulate heavy metals (Chinmayee *et al*., 2012, Nwaogu *et al*., 2012). Its chemical composition contains alkaloids, flavonoids, saponins, taninns, phenols, hydocyanin acid and phytic acid. Alkaloids are known to play a role in metabolism and they control development of living system and have a protective role in animals (Akubugwo *et al*., 2007).

1.4.2 *Bidens pilosa L* (Black Jack)

Bidens pilosa L is used as a leafy vegetable or pot herb in African countries. This plant is distributed in tropical areas and grows naturally in homestead gardens in KwaZulu Natal and Limpopo provinces. It requires temperatures between 25-38^{0}C to grow, and grows best in fertile soils which are well drained at pH 4-9 (Blackjack Production Guideline; 2011). There are 200 known species of *Bidens* with *Bidens Pilosa L* being a representative of a perennial herb that is globally distributed in tropical and temperate regions. It is one of the 1 200 macrophytes that has been reported to have antidiabetic activity. It has been used in Asia, America and Africa event though its safety has not yet been evaluated (Lai *et al.,* 2014). This plant species has also shown to have hypoglycemic activity in diabetic and alloxan-treated mice (Bartolome *et al.,* 2013). The current uses of this plant involves traditional usage for food and medicine without any known or noticed adverse effects (Bartolome *et al.,* 2013). This plant has been used to treat more than 40 disorders such as inflammation, immunological disorders, digestive disorders, infectious disorders, cancer metabolic syndrome, wounds and many others (Bartolome *et al.,* 2013). The present studies conducted about *Bidens Pilosa L* have only focused on botanical, traditional usage, phytochemistry, pharmacology, as well as toxicology. However, the ability of this macrophytes for display medicinal uses makes this plant potential for production of antimicrobial effect against pathogens. The occurrence of antimicrobial production may lead to reduction or complete removal of pathogens in wastewater by this macrophytes. The discovery of pathogen reduction potential by

Bidens pilosa L may add substantial information on the body of knowledge in wastewater treatment wetland macrophytes.

Bidens pilosa L has proven to have great tolerance to high concentrations of lead in soil (Graziani *et al*., 2016). This was reported by Graziani *et al* (2016) where this plant was exposed to high concentrations of lead. This macrophyte also presented a higher root development in the presence of elevated lead concentrations with the uptake as well as the translocation of the lead (Graziani *et al*., 2016). *Bidens pilosa L* has been considered to possess an ability to take up and degrade xenobiotic compounds through detoxification (Chen *et al.*, 2016). This was reported when this native macrophyte was investigated for biotransformation of propanil herbicide. *Bidens pilosa L* is an annual species which makes it suitable for biodegradation (Chen *et al.*, 2016).

1.3.9 The Role of Microorganisms in Hydroponic Systems

The functional group of bacterial communities in wetlands are determined by environmental conditions as well as the system feed. The dynamics of bacterial communities in constructed wetlands is important for the understanding of their diversity and functionality. Water habitats are dominated by microorganisms and they control all biochemical processes in water systems. Proper functioning of any water body including treatment wetlands relies on rich microbial diversity (Srivastava *et al.*, 2016). There are a number of factors that affect the formation of microflora. They include water temperature, organic matter concentration and hydraulic conditions (flow, aspect ratio and granular media type) (Morato *et al*., 2014). Microorganisms in hydroponics are observed as biofilms on solid and on macrophytes surfaces (Bhatia and Goyal, 2014). Wastewater composition and concentration varies in terms of nutrients, toxins and microbial loads. This can either promote or inhibit bacterial activity or growth (Calherios *et al.*, 2010). The direct degradation of organic chemicals is mediated by microorganisms despite capability of macrophytes to detoxify xenobiotic compounds. Bacteria achieve this by synergistically interacting under aerobic and anaerobic conditions on roots and substrate surfaces.

There are a number of biological processes taking place in hydroponics. These include photosynthesis, respiration, fermentation, nitrification, denitrification and microbial phosphorus removal. Photosynthesis occurs in macrophytes in wetlands and this process adds carbon dioxide and oxygen into the system. Both of these gases are essential for nitrification process. The oxidation of carbon is respiration. This process is facilitated by living microorganisms. Fermentation process involves anaerobic decomposition of organic carbon, leading to production of methane. This process is microbial driven. Wetlands microflora remove soluble organic matter coagulates cell tissue. Microflora in constructed wetlands has specific tolerances and requirements for dissolved oxygen, temperature ranges and nutrients (Calherios *et al.*, 2010).

Microorganisms require nutrients for their growth. They take up phosphorus which makes 1-2% of phosphorus in their biomass (Shilton *et al*., 2012). Phosphorus in cellular components exist as membrane phospholipids and DNA. During wastewater treatment, microorganisms feeds on the nutrients available in sludge to grow, and phosphorus is incorporated in their biomass. This brings about the reduction of phosphorus in wastewater (Shilton *et al*., 2012). Microorganisms plays a huge role in removal of pathogens and nutrients in wastewater treatment systems. They achieve this through predation and production of toxins by rhizobia against pathogens (Martin *et al*., 2012). However, their interactions with macrophytes elevates the pathogens removal efficiency in treatment wetlands (Jillson *et al*., 2001).

1.3.10 Macrophytes-Microbes Interaction in a Hydroponic Systems

Plant species have major effects on the bacterial community structures in constructed wetlands for wastewater treatment. Macrophytes and biota possess a strong functional linkage as producers and decomposers of terrestrial ecosystem. They produce organic carbon for soil microorganisms through root exudates and microorganisms decomposes organic matter and releases mineralized nitrogen and phosphorus essential for plant growth (Zhang *et al*., 2010). The ecological structure richness is directly proportional to the microbial diversity. This is due to high heterogeneity of resource environment.

The rhizosphere/rhizoplanes is regarded as the most important active zone (Bhatia and Goyal, 2014) because of the presence of microbial communities that carry out contaminants removal processes. Macrophytes do not affect the microbial community structure in the microcosms but rather enhances their activities to establish macrophytes-microbe interaction even in sediments (Srivastava *et al.*, 2016). Rhizobia are microbes found in the rhizosphere. They promote the health of macrophytes by stimulating root growth, enhancing water and mineral uptake and inhibiting the growth of pathogenic and non- pathogenic soil microbes (Bhatia and Goyal, 2014). Macrophytes roots provide surface area for "benthic" microbial community to rest and provide continuous nutrient supply, organic carbon and oxygen. On the other hand, macrophytes get mineral nutrients and defensive immunity from direct microbes, forming firm interrelationships between macrophytes and microbes (Srivastava *et al.*, 2016). Water quality improvement studies has focused in removal of environmental pollutants either by macrophytes or microbes alone (Srivastava *et al.*, 2016). Studies reporting on the possible influence of macrophytes-microbes interaction on water quality are available (Li *et al.*, 2013; Srivastava *et al.*, 2016).

The presence of macrophytes enhances microbial activity which plays the main role in removal of pollutants. The macrophytes species, root morphology and roots development seem to be the key factors affecting microbial-plant interactions (Calherios *et al.,* 2010). Macrophytes forms two types of symbiotic relationships with microorganisms. The first is endophytic. This involves the colonization of internal tissue of macrophytes with nitrogen (N_2) fixing diazotrophs and other nutrients assimilators. Secondly, the ectophytic relationship where microorganisms remains outside the macrophytes, like ammonia oxidizing bacteria and methanotrophic bacteria (Srivastava *et al.*, 2016). This type involves interaction of roots and leaves and is regarded as important because there are several biochemical interactions occurring at the interactive surface (Srivastava *et al.*, 2016).

1.3.11 Proposed Mechanisms of Pathogens Removal from Wastewater in a Hydroponic System Planted with Macrophytes

The removal of pathogens in constructed wetland system is achieved by physical, biological and chemical processes that occurs through interaction of macrophytes, microorganisms and a substrate. Different plant species have different potential in pathogen removal. The bactericidal effect of macrophytes differs with plant species. However, previously conducted studies reveal that bactericidal effect is an active process in the presence of macrophytes (González-Lamothe *et al*., 2009; Morato *et al.,* 2014). Macrophytes are proposed to play a significant role in pathogens removal. It is proposed that the rhizosphere enhance the level of anaerobic degradation and this is where antimicrobial compounds are secreted (Werker *et al*., 2002). This is where the physical filtration of pathogens occurs. There are arguments surrounding the pathogens removal in planted and unplanted wetlands. Where planted wetlands are being pointed out as harboring high microbial population due to roots development with many removal mechanisms leading to pathogens removal compared to unplanted wetlands. However, microorganism's predation by ciliated protozoa appears to be greater in planted wetlands (Werker *et al.*, 2002). Macrophytes take up pathogens through endophysim. This is where pathogenic organisms from wastewater internalize through roots and spread upward the plant to addible portions of the plant (Macarisin *et al.*, 2014).

Biological mechanisms include natural die off, predation by protozoa, lytic bacteria, aggregation and retention of biofilms, potential production of bactericidal compounds or antimicrobial activity of roots exudates. They also include predation by nematodes and protists attack by lytic bacteria and viruses as well as completion of nutrients or trace elements (Stottmeister *et al.*, 2003). In closed systems, microorganisms compete for nutrients and oxygen and some die during this process. The inability of macrophytes to translocate enough oxygen to the rhizosphere may also lead to the death of some pathogens. Ciliates are also able to flocculate suspended matter in sludge effluents (El-Serehy *et al.*, 2014). The chemical processes include oxidation and adsorption to organic matter as well the production of toxins from the living bacteria in the rhizosphere or macrophytes (Martin *et al.*, 2012).

The physical removal processes include filtration, sedimentation, adsorption and aggregation. Another physical mechanism proposed by El-Serehy *et al.* (2013) is the inactivation of pathogens due to environmental stress. Sand filters has been used in water purification and pathogens elimination for over 200 years (Oki *et al.*, 2017). These systems have assisted in prevention of cholera outbreaks in Altona, Germany in 1892 (Oki *et al.*, 2017). Their pathogen removal relies on microbial diversity establishment on sand surface consisting of protozoa, fungi, algae, rotifers, nematodes and flatworms. The microbial community in sand is referred to as "filter skin". This is where pathogens are trapped and broken down by microbes in biological systems (Wand *et al.*, 2007; Oki *et al.,* 2017). The sand filters systems require slow flow rates to allow the prolonged hydraulic retention periods for effective functioning of the filter skin. The maturity of the filter skin increases the removal efficiency of the pathogens (Oki *et al.,* 2017).

1.3.12 Factors Influencing Pathogen Removal in Hydroponic Systems for Wastewater Treatment

Different plant species have different potential in pathogen removal. The bactericidal effect of macrophytes differs with plant species. However, previously conducted research reveals that bactericidal effect is an active process in the presence of macrophytes (González-Lamothe *et al*., 2009 and Morato *et al.,* 2014). The rich ecological structure is directly proportional to the microbiota development which enhances the removal efficiency in wastewater treatment wetlands. Temperature influences the removal of pathogens in wastewater treatment wetlands. Cold temperatures result in poor performances of wetlands due to reduced dissolved oxygen concentration in roots zone. This occurs due to low metabolic activities in roots zones resulting in reduced levels of antibiosis and bacterial predation. The effect of temperature varies with the plant type and bacterial species (Werker *et al*., 2002). Roots oxygen release also may be low due to cold temperatures.

Wetlands may be affected by the solar radiation and ambient temperatures that cycles on annual and daily basis. The abiotic factors mediate wetland environment, causing cycling patterns in evapotranspiration, photosynthesis and microbial activity (Picard *et al.,* 2005). Biological processes slow down and cease at 5^0C and the nitrification process is said to be inhibited at temperatures about 10^0C (Picard *et al.,* 2005). In temperate environments, the treatment wetlands functions at low levels in cold seasons. This occur as a result of the decrease in oxygen availability. High temperatures has also been reported to inhibit the growth of nitrogen reducing bacteria.

The hydrology (size of soil grains used and the flow direction) of the wetland also affect the wetlands ability or potential to remove pathogens. Sand and gravel with vertical flow has been proposed to yield higher removal of pathogens and other contaminants. Horizontal flow is not efficient as vertical flow (Stottmeister *et al*., 2003; García *et al*., 2013). This is because vertical flow systems are less prone to clogging due to uniform wastewater distribution with equal loading rate, resulting in alternating aerobic and anaerobic conditions for maximum pathogens removal (Brunch *et al*., 2014). Small soil particles that are not well drained result in hydraulic problems like clogging and this reduces the potential efficiency. Pebbly soils are not good for filtration of pathogens because they are large in size, thus leaving large spaces in between particles. Previous studies have shown that wetlands with smaller particle sizes have higher surfaces to protect microbial growth and produce adsorptive environment for closer interactions of microorganisms and their decomposition products (Wu *et al*., 2016 and Zhao *et al*., 2016). Gravel particles are known to emit more methane than smaller particle sizes like sand soils. However, the issue of sand substrate particle size on methane emissions is still in question (Zhou *et al*., 2016).

1.3.13 Conclusion

The removal of pathogenic microorganisms in water is of critical importance because it prevents waterborne diseases outbreaks as well as loss of lives. Microbial contamination in water sources and supply systems cannot be controlled since it is caused, and affected by a variety of environmental factors as well as anthropogenic activities. Although the usage of constructed wetlands with macrophytes may offer solution, some of the removal mechanisms are not fully understood. Even though these systems are made to mimic natural wetlands, with many factors influencing their functioning, the understanding of removal mechanisms may allow their optimization, thus lead to efficient pathogens removal from wastewater. This may yield complete removal of contaminants or wastewater constituents at short hydraulic retention periods, decrease the waterborne diseases outbreaks and lower environmental pollution. In this way improvement of public health conditions may be achieved.

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CHAPTER 2

DETERMINATION OF THE REDUCTION POTENTIAL OF THE PATHOGENS IN THE HYDROPONIC SYSTEM USING qPCR

2.1 Introduction

Inadequately treated domestic water discharges cause a serious public health concern due to potential transmission of infectious diseases in form of waterborne pathogens. Majority of pathogens are of enteric origin and are present in domestic wastewater. Bacteria are the most commonly found waterborne pathogens contracted through wastewater. Pathogens commonly found in wastewater includes species of *Salmonella*, *Shigella*, *Vibrios*, *Yersinia*, and pathogenic strains of *Escherichia coli*. Waterborne diseases contracted from ingestion of contaminated water includes shigellosis, salmonellosis, cholera and dysentery. Waterborne diseases caused by bacteria are mostly diarrheal infections. This pollutes the environment and threatens public health. Therefore, it is necessary to remove pathogenic microorganisms from wastewater before disposal.

Municipal wastewater treatment systems employ disinfection technologies to get rid of such pathogenic organisms. These technologies include chlorination, ozonation and ultraviolet (UV), where chlorination is the most utilized disinfection method. Chlorination process produces harmful by products which are carcinogenic to humans. However, the drawbacks associated with the application of these technologies have necessitated the invention of environmental friendly technologies for pathogens removal.

The subsurface flow constructed wetlands are regarded as the best technique to remove pathogenic organisms in wastewater since they provide a combination of physical (filtration through substrate, adsorption, sedimentation and aggregation), biological (antibiosis, predation, attack by lytic bacteria and viruses, natural die off and completion for nutrients and trace elements) and chemical mechanisms (oxidation and biocides exposure) (Headley *et al.*, 2013; Gruyer *et al.*, 2013; Morató *et al.*, 2014). These systems can function under non-optimized conditions where they mimic natural wetlands. Their proper functioning is defined by interaction between substrate, water, microorganisms and macrophytes. Their functioning is also largely affected by environmental factors such as temperature, pH, vegetation type and density, carbon availability and redox potential (Gruyer *et al*., 2013). Constructed wetlands offer a number of advantages in wastewater treatment sector because they are simple, affordable, easy to maintain and operate (Headly *et al*., 2013). They are cost effective as well as environmentally friendly. A vertical flow constructed wetland was evaluated for the removal of pathogens, where the study aim was to determine the pathogens reduction potential by the system was conducted.

2.2 Aim, Hypothesis and Objectives

2.2.1 Aim

This chapter aimed at determine if the hydroponic system was able to significantly reduce pathogens to allowed discharge limits set by the Department of Water and Sanitation for disposal of wastewater into natural water bodies.

2.2.2 Hypothesis

There will be a reduction of pathogens in wastewater after treatment by the hydroponic system

2.2.3 Objectives

- To quantify the sizes of DNA present in water samples at different hydraulic retention times in order to determine the reduction potential of pathogens of interest

- To amplify the 16S RNA of the pathogens of interest in order to confirm the presence of pathogens in wastewater samples

2.3 Methodology

2.3.1 Introduction

In determining the reduction potential of pathogens in wastewater by the hydroponic system, a previous system was employed. Wastewater from the secondary stage of the conventional sewage treatment system were introduced into the system. Samples were collected prior, during and post treatment in order to isolate DNA which was further visualized with gel electrophoresis to investigate the differences of microorganism's concentrations at different hydraulic retention times. To evaluate the reduction of specific pathogens, the virulence genes were amplified using the real-time qPCR. For identification and quantification of *E. coli*, the translocation intimin receptor (*tir*) gene was amplified. This gene enables the characteristic adherence of this organism in the human epithelia cells (Kyle *et al.*, 2012; Mellor *et al.*, 2013). In quantifying *Salmonella spp.*, the invasion protein (inA) gene was amplified. Salmonella invasion proteins (A, B, C, D and E) are virulence genes responsible for the pathogenesis of this organism (Kaur and Jain, 2012). *Shigella spp*. were quantified through amplification of the invasion plasmid antigen H gene.

2.3.2 The Construction of the Hydroponic System

The planter box and the water tank made of fibre glass were used for setting up the hydroponic system. The planter box was designed to rest on top of the steel frame and this was ideal for installation in order to the save space. The planter box had a 32 mm predrilled hole at the bottom and it was pre-fitted with a 25 mm uniseal where the standpipe was fitted. The water tank was placed below the planter box (on a steel frame) so that the downpipe of the bell siphon feeds back into the water tank. The stand pipe was secured with a siphon pipe and the gravel guard large pipe was fitted over siphon pipe to prevent the growth media from blocking the water intake channels on the siphon pipe as indicated on Figure 2.1. The constructed hydroponic system is shown in Figure 2.2.

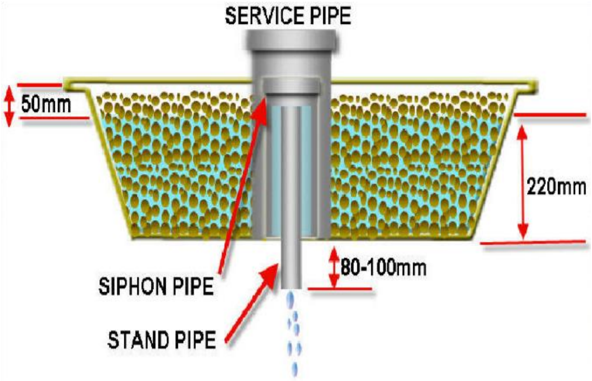


Figure 2.1: The bell siphon pipe installation during the setting up of the hydroponic system.

The combination of sand and gravel was used as growth medium in the system. The base of the planter box was covered with sand and the bell siphon pipe was surrounded with sand. The top of the planter box where macrophytes were planted was covered with gravel aggregates. This was done to ensure that the macrophytes receive nutrients direct from wastewater and that the gravel supports the growing of macrophytes by holding their roots. These types of soils are recommended as they provided the best hydraulic conditions and high removal of contaminants. Sand is also known as natural filter and gravel provided the surface area for roots to adhere, biofilms formation, and microbial activity (Stottmeister *et al.*, 2003). After the growth medium was added into a planter box. Clean water was added into a water tank and the submersible pump with a flow rate of 1400 litres per hour was connected to the nearest power source and immersed into water tank. The system was left circulating for five days in order to ensure the removal of floods residues from the growth medium.

Figure 2.2: The constructed hydroponic system used in the study

The subsurface vertical flow system was planted with emergent macrophytes, *Bidens pilosa L* and *Amaranthus hybridus L* (Figure 2.2). These macrophytes were randomly propagated by seeds in the surface of the hydroponic system covered with gravel particles. These plants were selected because they are indigenous plants in South Africa, Empangeni (KwaZulu-Natal) and they were easily accessible. They were both used because they both have never been used for wastewater treatment purposes and this was done to enrich the physiological structure for better performance of the system.

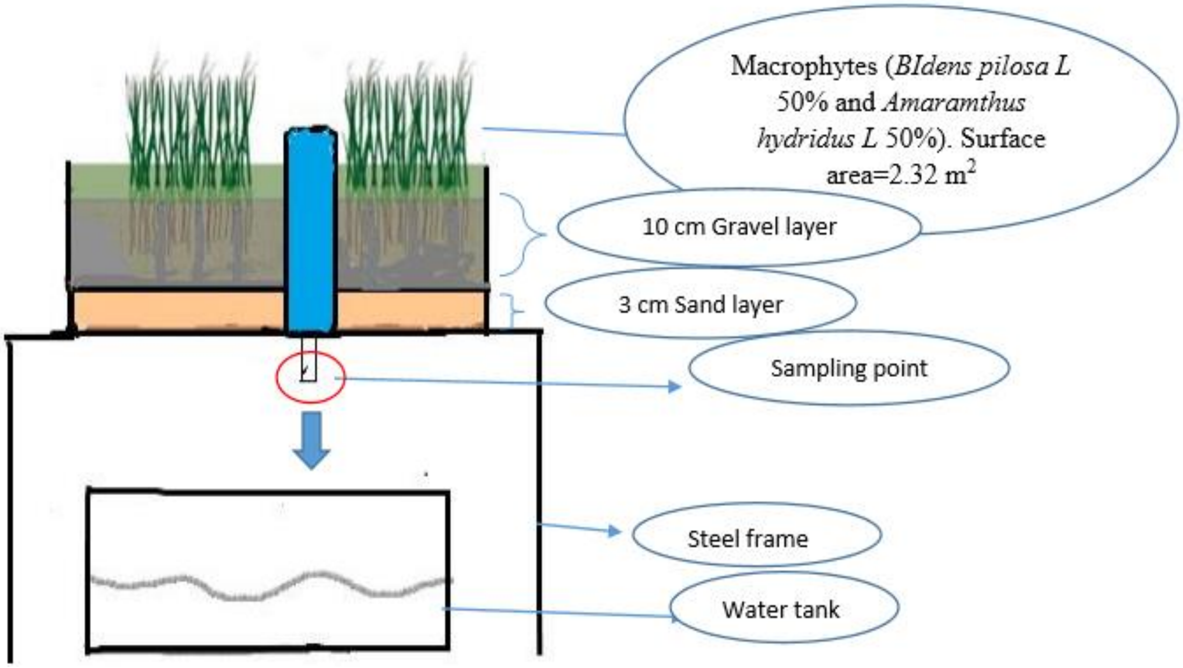


Figure 2.3: The Schematic Diagram of the Hydroponic System Used in the Study

2.3.3 Samples Collection

Water samples were collected at different hydraulic retention times. This was done to establish the efficient hydraulic retention time for pathogens removal in order to develop a model. Water samples were collected every 24 hours using sterile plastic bags. The samples were placed on ice in a cooler box and transported to the lab where the DNA extraction was performed. The sampling was done from the sampling point shown on (Figure 2.3).

2.3.4 DNA Extraction from Wastewater

The ZR Fungal/Bacterial DNA MiniPrep kit (D6005) from Zymo research was used to extract DNA from water samples following its protocol. This kit allows the extraction of DNA from environmental biological samples containing bacteria, fungi and protozoa. Following the protocol, water sample of 200 µl was introduced into ZR bashing beads lysis tube where 750 µl of lysis solution was added and vortexes. After vortexing, the bashing bead lysis tube was centrifuged in a microcentrifuge at 10000g for 1 minute. The supernatant of 400 µl was transferred into a zymo-spin filter in a collection tube and centrifuged at 7000g for a minute. The dinging buffer of 1 200 microliters was added into a filtrate in the collection tube. Eight hundred microliters of the mixture from the collection tube was transferred into a zymo-spin IIC column in a collection tube and centrifuged for 10000g for a minute. The flow through in the collection tube was discarded and the previous step was repeated. The DNA pre-wash buffer of 200 µl was then added into a zymo-spin IIC column in a new collection tube and centrifuged at 10000g for a minute. The flow through was discarded from the collection tube and the wash buffer of 500 microliters was added into a zymo-spin IIC and centrifuged at 10000g for a minute. The zymo-spin IIC column was transferred into a sterile 1.5 ml microcentrifuge tube, where 100 µl of DNA elution buffer was added directly into the column matrix. The zymo-spin was then centrifuged at 10000g for 30 seconds to elute the DNA. The DNA was stored at -85 ^{0}C and later used for PCR. Before the PCR was conducted the success of an extraction of DNA was confirmed by its visualizing using the gel electrophoresis.

2.3.5 Gel Electrophoresis

For the gel electrophoresis, 1% agarose gel was prepared by dissolving 1.5 g of agarose in 150 ml of 1x TAE buffer (4.84 g of Tris base, 2 ml of 0.5 M EDTA and 1.142 ml of glacial acetic acid) and boiled in a microwave for 5 minutes. After boiling, 15 µl of ethidium bromide (EtBr) was added, the gel was poured in a gel tray with 20 well-comb for making wells and cooled at a room temperature. After cooling, DNA was loaded in wells where, 5 µl was mixed with 1 µl DNA loading dye (Thermofisher). The DNA ladder (Thermofisher) of 3 µl was also loaded as a maker. The gel was run at the voltage of 100 for 30 minutes. The gel was then viewed using the IN genus syngen bioimaging (Vacutec).

2.3.6 Real-time Quantitative Polymerase Chain Reaction (qPCR**)**

The quantitative polymerase chain reaction (q-PCR) involves the simultaneous amplification detection and quantification of specific targets nucleic acid in a biological sample (Botes *et al*., 2013). The q-PCR has two methods namely, the end point qPCR and real-time qPCR. The end-point qPRC take the measurement after the completion of the entire PRC reaction while the real time qPCR takes measurement at the exponential phase of the quantitative PCR reaction and is regarded as a more sensitive and reproducible method (Botes *et al.*, 2013). In this work real-time qPCR was employed because quantification occurred at early exponential phase since the aim was to detect and quantify the initial DNA. The real-time qPCR was more accurate and less time consuming. It also required no post-treatment of PCR products and reduced contamination (Botes *et al*., 2013). This method proved highly sensitive and small amounts of DNA in biological samples were quantified (Shannon *et al*., 2007).

2.3.6.1 Pathogens detection in wastewater samples

In detecting the most prevalent bacterial pathogens in wastewater, namely *Escherichia coli*, *Shigella spp* and *Salmonella spp*, the real-time qPCR was employed. Specific genes coding for targeted genes were amplified using specific TaqMan probes and primers (Table 2.1). The 2X DreamTaq green mastermix was used. It contained Taq DNA polymerase, dATP, dCPT, dGDP, 0.04 Mm of each and 4 Mm of magnesium chloride. The real-time qPCR reaction set up was conducted following the order of components as listed in Table 2.2. The reaction was conducted under the conditions stated in Table 2.3 in the Eppendorf thermal cycler. The qPCR products were visualized using gel electrophoresis.

Table 2.1: The primer set and probe designed by (Kong, 2002; Shannon *et al.*, 2007) that were used for quantification of selected bacterial pathogens in wastewater

	Target gene	**Primer**	**Sequance (5′-3′)**
Shigella spp.	Invasion plasmid Antigen H	*IpaH*-F	CCTTGACCGCCTTTCCGATA
		IpaH-R	CAGCCACCCTCTGAGGTACT
		IpaH-PR	CGC CTT TCC GAT ACC GTC TCT GCA
E. coli O157:H7	Translocated intimin receptor (*tir*)	EcoOH-F	TCGAGCGGACCATGATCA
		EcoOH-R	GGCGGCGTCTGAGATAACA
		EcoOH-PR	AGAACTTCAAATCCATCATT
Salmonella sp.	Invasion protein (*invA*)	Sal-F	CGTTTCCTGCGGTACTGTTAATT
		Sal-R	AGACGGCTGGTACTGATCGATAA
		Sal-probe	CCACGCTCTTTCGTCT

Table 2.2: The concentrations and volumes of qPCR components used for amplifying genes of interest in order to detect the quantify the pathogens in wastewater prior and post treatment

qPCR components	**Volume (µl)**
DreamTaq green PCR mastermix (2X)	25
Forward primer	1.5
Reverse primer	1.5
Probe	2
DNA template	2
Nuclease free water	18
Total volume	**50**

Table 2.3: The thermal cycling conditions that were employed during the real-time qPCR for detection and quantification of bacterial pathogens in wastewater samples prior and post treatment

Process	**Temperature (^{0}C)**	**Time**	**Number of cycles**
Initial denaturation	50	2 minutes	1
Denaturation	95	10 minutes	1
Annealing	95	15 seconds	45
Extension	60	60 seconds	1

(i) Visualization and Interpretation of qPCR Products

The qPCR products quality and quantity was evaluated by loading samples on agarose gel stained with ethidium bromine which controlled the fluorescence emitted. This method allow detection of double stranded DNA, thus displayed the initial DNA quantity of the present pathogens in the sample prior and post treatment in the hydroponic system. This method was advantageous because the gel was easily poured and does not denature the samples. The DNA visualized through gel electrophoresis can also be recovered and used for further analysis if required. The fluorescence emitted was visualized using Bio-Vision software. This method allowed detection of double stranded DNA, thus displayed the initial DNA quantity. The gel electrophoresis was conducted as explained in 2.5.4.

2.3.7 Data Analysis

The Bio-Vision software version 17.06 was used to analyze the data. The gel electrophoresis image was visualized using the three dimensional view. The 3D scan images provided direct information regarding the image dynamic, background level and the quantity of DNA. The images were further analyzed for quantification where the background subtraction approach was used. This approach allowed the elimination of the gel opaque, random signal noise and opacity of the corner medium that may have interfered with quantification.

2.4 Results and Discussion

The DNA extracted from wastewater was presented in gel scan images, where two samples were used and the extraction was conducted and presented in duplicates. In the quantification of pathogens, *E. coli, Salmonella spp. and Shigella spp*. at different hydraulic retention times (0, 24 and 72 hours), and the findings were presented in gel scan images and 3D scans. The qPCR also was conducted in duplicates and the results were viewed and presented in duplicates at each hydraulic retention time for all pathogens investigated.

2.4.1 DNA Extracted from Wastewater Samples

The DNA extracted from wastewater samples prior treatment in the hydroponic system are shown in gel electrophoresis scan image (Figure 2.4 A). The samples were analyzed in duplicates for statistical reasons. Lanes M represents the DNA ladder and lanes 1, 2, 3 and 4 are DNA extracted from water from the clarifier prior introduction to the hydroponic system for treatment. Small amounts of DNA were observed prior treatment. This did not necessarily imply less amount of pathogenic contamination since environmental biological samples are known to have small DNA. The extracted DNA was also observed with a three dimensional scan image (Figure 2.4 B), where different peaks were considered as DNA quantities. The highest DNA peak was observed at 241 Kb, which was obtained in lanes 2 (Figure 2.4 A). The highest peak indicated the high quantity of DNA in a water sample, which clearly indicated that wastewater from the secondary stage of the conventional sewage treatment system contained microorganisms. Sewage water contained fecal material of humans and animals, therefore they were rich in enteric microorganisms. Figure 2.4 B is a 3D scan image representing the peaks of the bands obtained in lanes M, 1, 2, 3 and 4 respectively from Figure 2.4 A. Lane 2 in Figure 2.4 A had a thick band, which had a higher quantity of DNA peak in Figure 2.4 B.

Figure 2.5 shows the DNA extracted from wastewater samples after treatment in the hydroponic system. Few distinct bands were observed, however this did not mean there was no pathogens in wastewater. Therefore, the qPCR was carried out to amplify desired genes in order to quantify specific pathogens. The 3D scan image shows the peak of the DNA ladder in lane M (Figure 2.5 B) and a very small peak in lane 1. This indicated that the concentration of microbial load decreased as the hydraulic retention time was elongated in the system. Other samples showed no peaks at all, which was an indication of very low concentrations of microorganisms.

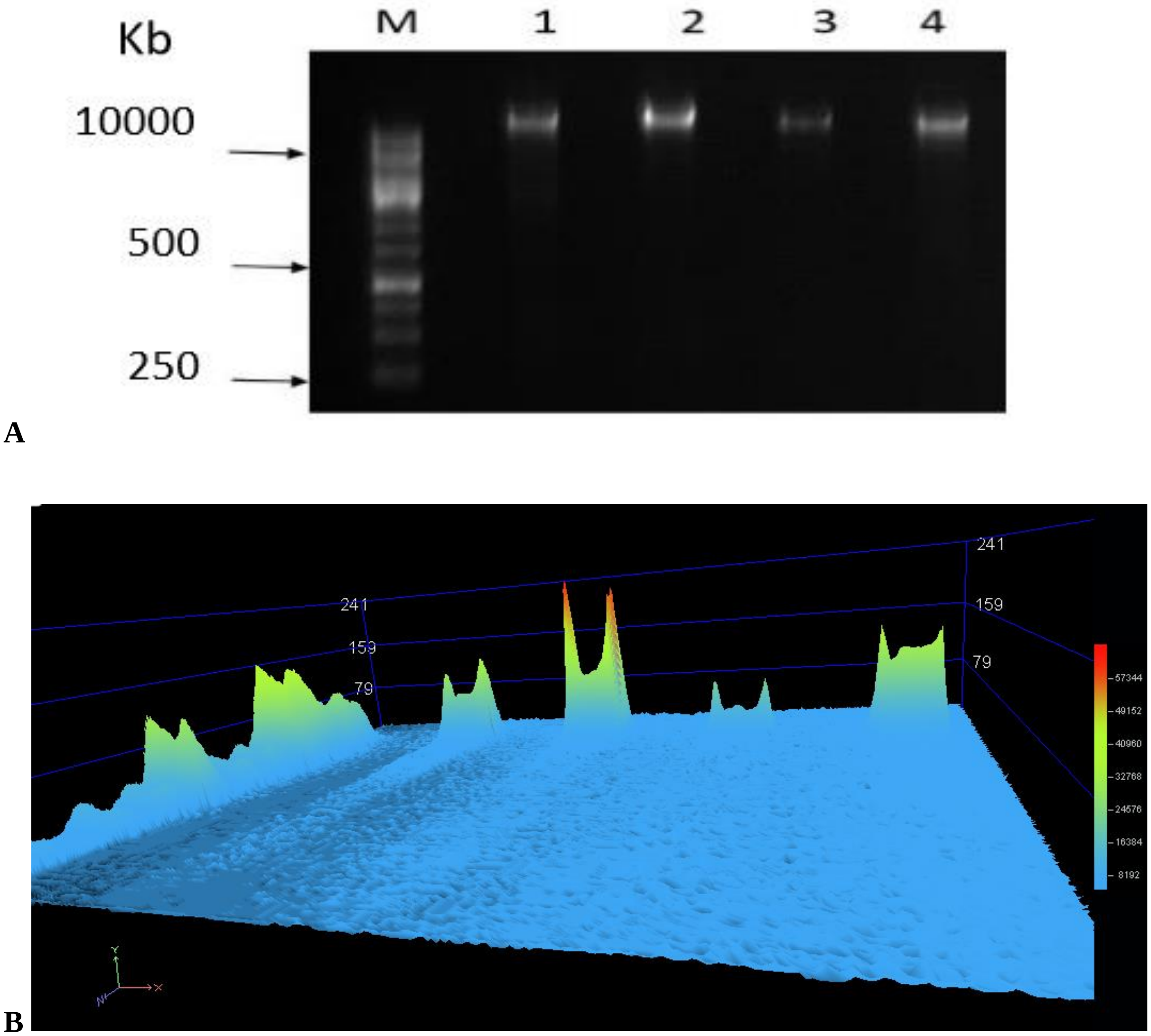


Figure 2.4: A, is the genomic DNA of wastewater samples collected from the hydroponic system prior treatment. Line M is the DNA ladder with 10000 kb, where line 1-4 represent the genomic DNA obtained from wastewater samples prior treatment in the hydroponic system. B, is the 3D image scan of the DNA extracted from wastewater samples prior treatment in the hydroponic system. B, is showing the DNA quantity peaks in A, in lanes M, 1,2,3 and 4 respectively

The results of DNA axtracted prior and post treatment in the hydroponic system showed huge differences in their quantities, which indicated that the microbial load declined significantly during the treatment in the system. However, the aim was to quantify specific pathogens thus, the real-time qPCR conducted also showed the significant decline of pathogens after treatment. The decrease of microbial load showed by the extracted DNA was considered as the result of the removal of pathogens in the system.

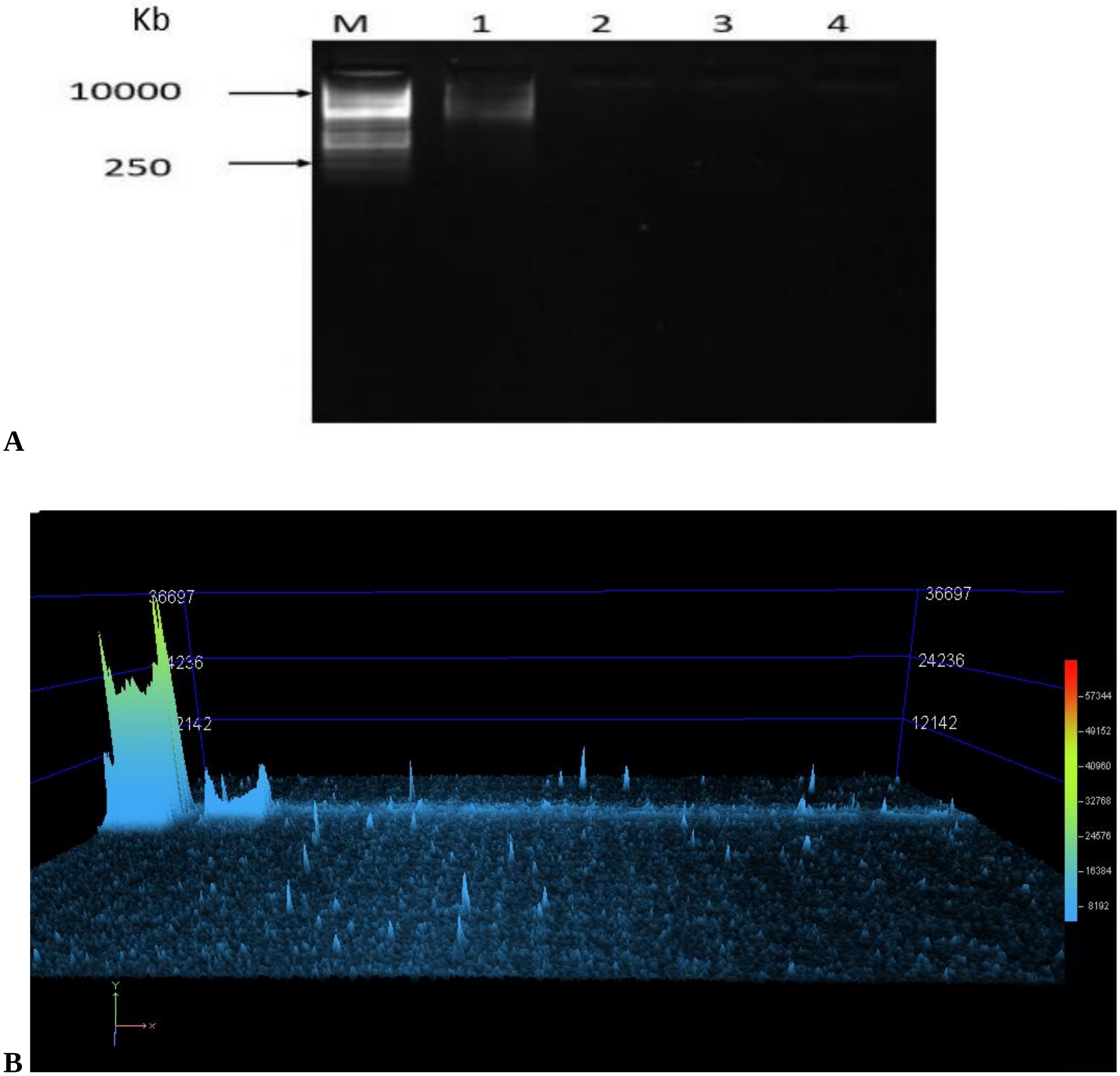


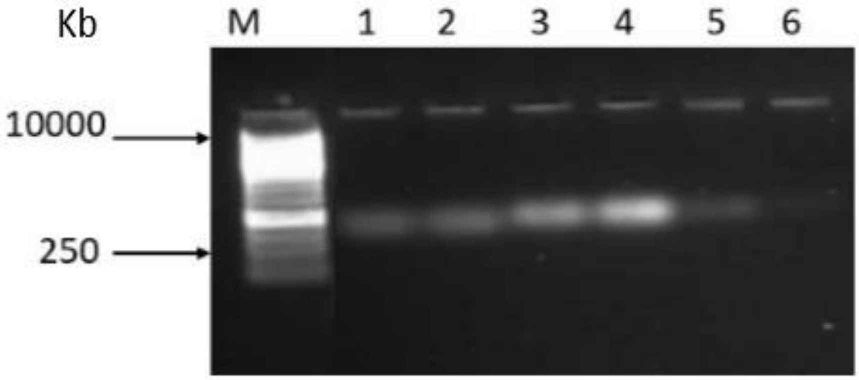
Figure 2.5: A, is the DNA extracted from waste samples post treatment in the hydroponic system, B, is the 3D scan image showing the DNA extracted from wastewater samples after treatment in the hydroponic system. B is showing the peaks of bands obtained in A, where the first peak represents the DNA ladder in lane M (A), Lane 1 is also represented by the small peak in B.

2.4.2 Real-time qPCR Products

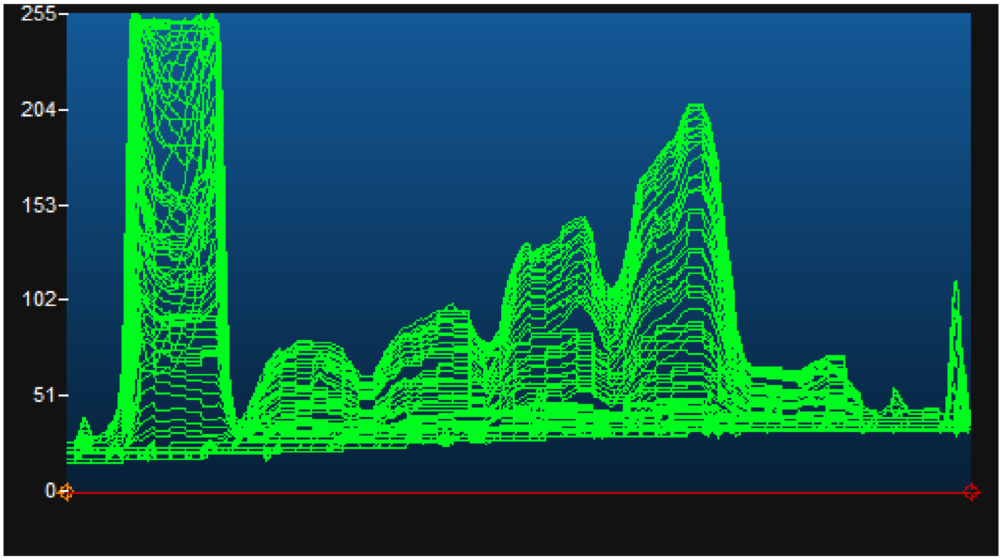
After the amplification of pathogens virulence genes, the qPCR products were observed in the gel scan images for each pathogen at different hydraulic retention times. The gel electrophoresis scan images were presented in form of 3D and quantification bars to show the differences in DNA quantities.

(i) *Escherichia coli* removal in the hydroponic system

The translocation intimin receptor gene (*tir*) amplification is shown in the gel electrophoresis scan images (Figure 2.6) in A and B. These images displayed the quantity of DNA present in wastewater samples that was obtained through real-time qPCR. The quantity of DNA at 24 hours showed DNA increase in lanes 3 and 4 (Figure 2.6 B), representing the presence of this organism in higher quantities in the system. The quantification analysis through background subtraction approach (Figure 2.6 B), showed the highest quantity of *E. coli* at 24 hours (lanes 3 and 4) represented by the highest peaks in the scan image. However, these peaks drastically declined at 72 hours (lanes 5 and 6), which indicated the reduction of this organism in the system. The declining of peaks indicated that the system was able to reduce *E. coli* concentration during treatment.



A



B

Figure 2.6: A, shows the *Escherichia coli* reduction in wastewater where lanes M indicate the DNA ladder, lanes 1 and 2 shows the quantity of *E. coli* prior treatment, lanes 3 and 4 shows the quantity of *E. coli* at 24 hours and lanes 5 and 6 shows the *E. coli* quantity after 72 hours in the hydroponic system. B indicates the 3D scan image of *E. coli* quantification, it shows the DNA quantification peaks form the DNA bands obtained in A, in lanes M, 1, 2, 3, 4, 5 and 6 respectively.

(ii) *Salmonella spp.* reduction in wastewater

The removal of *Salmonella spp*. in wastewater was observed (Figure 2.7 A and B), where the concentration of the amplified DNA decreased with the prolongation of the hydraulic retention time. The DNA observed in the gel electrophoresis was an indication of the *Salmonella spp*. Invasion plasmid antigen H gene that was amplified as the virulence gene to quantify *Salmonella spp*. The removal of this pathogen showed the same pattern with the removal of *E. coli*. Where there was a slight increase of the organism concentration at 24 hours (lanes 3 and 4) that significantly declined at 72 hours (lanes 5 and 6) as shown in Figure 2.7 B, where the DNA quantity peaks represented the bands obtained in Figure 2.7 A.

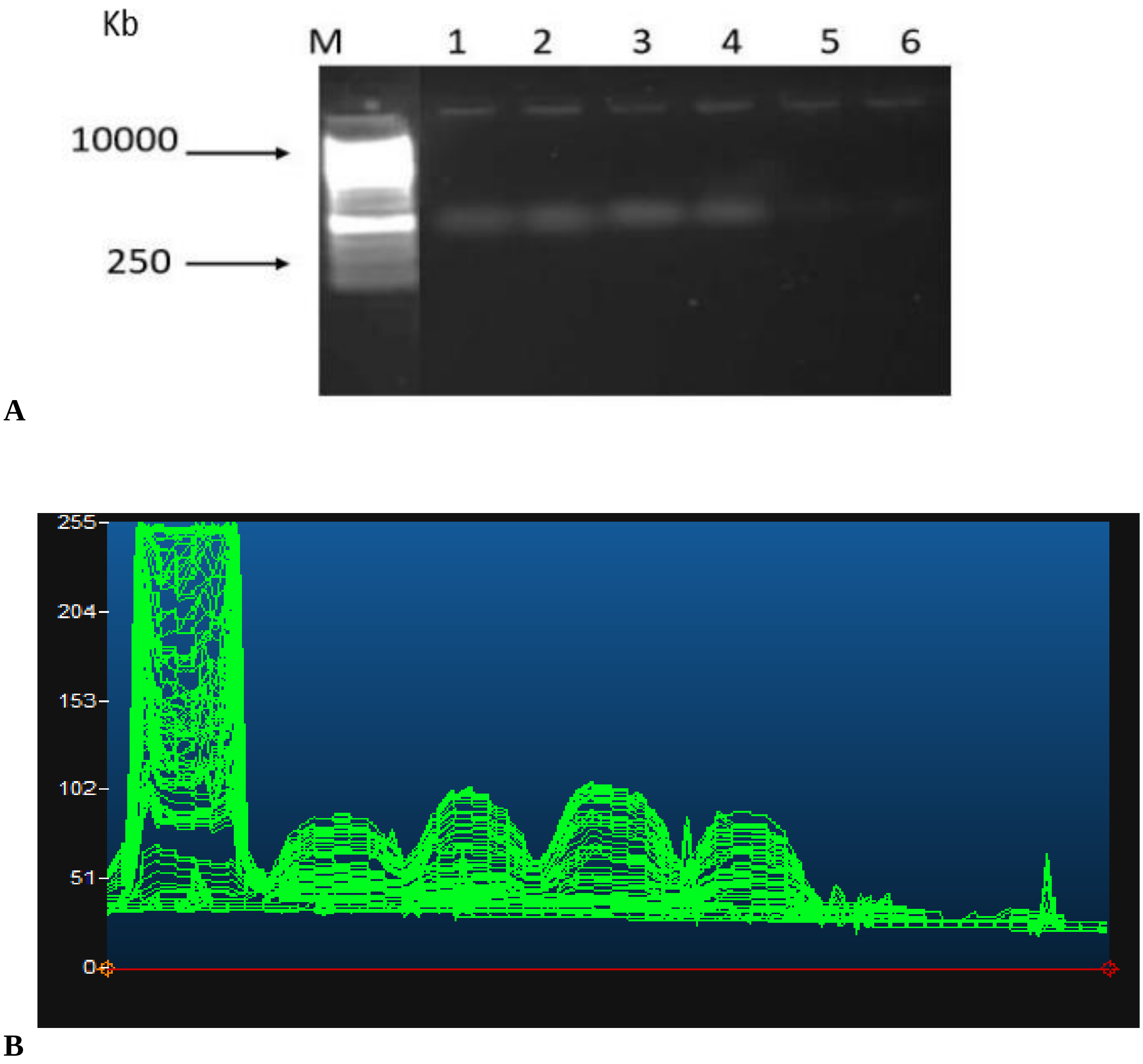
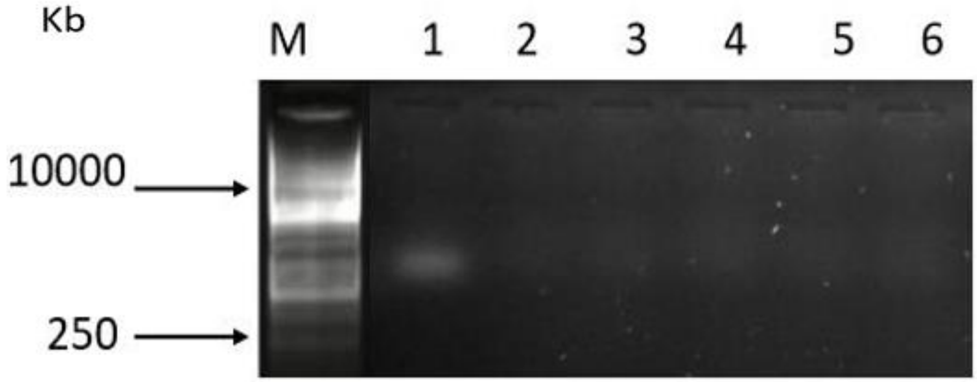


Figure 2.7: A, shows the *Salmonella spp.* reduction in wastewater where lanes M shows the DNA ladder, lanes 1 and 2 show the quantity of *Salmonella* prior treatment, lanes 3 and 4 shows the quantity of this organism at 24 hours and lanes 5 and 6 displays the *Salmonella* quantity after 72 hours in the hydroponic system. B, is the 3D scan image of *Salmonella spp.* quantification, the DNA quantification peaks represents the DNA bands shown in lanes M, 1, 2, 3, 4, 5 and 6 in A, that were obtained at different hydraulic retention times.

Mthembu (2015), reported that constructed wetlands with macrophytes are known to have high density of microbial population which occur as a result of aerobic and anaerobic conditions that are established through oxygen transportation by macrophytes. Therefore, presence of macrophytes which provided a substrate for microbial growth and aeration that caused the elevation of microbial load, which later was reduced. Hence, at 72 hours *E. coli* and *Salmonella spp.* declined drastically. This might have caused by the biological removal mechanisms such as predation and competition for nutrients and trace elements as a result of high population of the organism. Gruyer *et al*. (2013) reported that biological methods preserve natural microflora that influence the achievement of higher removals of pathogens. Srivastava *et al*. (2016) also argued that abundance of microorganisms in water habitats, numerically and biochemically result in proper functioning of the water body. This might have created a consortium in the rhizosphere that might have elevated the competition and predation in the system, thus the reduction of pathogens. In wastewater treatment wetlands, pathogens compete with the consortium and intestinal organisms are destroyed during predation (Morató *et al.*, 2014).

(iii) *Shigella sp*. removal in wastewater

The amplification of the invasion plasmid antigen H virulence gene for the detection of *Shigella spp*. was observed in gel scan images (Figure 2.8 A). The *Shigella spp*. were removed as the hydraulic retention time prolonged. This was indicated by the quantification image (Figure 2.7 B) where the visible peak was only observed prior treatment (Lane 1). In lanes 2 to 6 no visible peaks were noted. This indicated that there was a huge reduction of this organism in the hydroponic system. Thus, the ability of the system to reduce this organism from wastewater.



A

B

Figure 2.8: A, shows the *Shigella spp.* reduction in wastewater where lanes M shows the DNA ladder, lanes 1 and 2 shows the quantity of *Shigella spp*. prior treatment, lanes 3 and 4 shows the quantity of this organism at 24 hours and lanes 5 and 6 displays the *Shigella spp.* quantity after 72 hours in the hydroponic system. B displays the 3D scan image of *Shigella spp.* quantification, the DNA peaks represents the DNA bands shown in A, in lanes M, 1, 2, 3, 4, 5 and 6 respectively.

The paired *t*-test analysis was carried out to evaluate the significance of pathogens removal. When the mean values of the influent and effluent were compared. The removal of pathogens was found to be significant ($p \leq 0.05$), even though the difference was too small ($p = 0.059$). However, the *Salmonella spp*. removal achieved the highest removal efficiency in the system compare to other pathogens. *E. coli* had a removal of 59% and the lowest removal was obtained by *Shigella spp*. The lowest removal efficiencies of pathogens occurred as a result of their small quantities in wastewater treated in the system.

The reduction of these pathogens in the system occurred as a result of physical, biological and chemical process that might have played a role, synergistically or individually. The removal of pathogen in the hydroponic system occurred as a result of filtration in the substrate since the treatment beds composed of sand particles and gravel layer. El-Sehery *et al*. (2013) reported that environmental stress also inactivated pathogens through damaging their DNA. While Wand *et al*, (2007) and Motaro *et al*. (2017), referred to sand as a “filter skin” because it is where the pathogens are trapped and biologically broken down during wastewater treatment. The biological process that included natural die-off, predation and production of antibiotics also played a role in pathogens reduction. Macrophytes produced compounds that eliminated pathogens. Gonzalez-Lamonte *et al*. (2009) and Morato *et al*. (2017), reported that bactericidal and bacteriostatic effect are active process in treatment wetlands with macrophytes. Apart from physical and biological processes, chemical reactions within the system by either macrophytes or microorganisms might also have attributed to pathogens removal. The above mentioned mechanisms, synergistically or individually led to pathogens removal in the system. Oki *et al.* (2017) reported that, these systems (constructed wetlands) assisted in preventing cholera outbreaks in Altona, Germany in 1892.

2.5 Conclusion

The hydroponic system was able to reduce pathogens in our study. *Salmonella spp*. was the most removed pathogen in the system with the highest removal efficiency. While pathogens were reduced in the system, they were not completely removed, thus further disinfection method may be necessary before wastewater can be disposed in the environment. While the system may not have completely removed all pathogens, the quantification obtained after treatment indicated improvement when compared to traditional wastewater treatment system. Thus, it could be concluded that our system was effective in the reduction and removal of pathogens from wastewater.

2.6 Recommendations

It is recommended that the prolongation of the hydraulic retention time may further remove remaining pathogens in wastewater in the hydroponic system. Thus, in future, it is recommended that the hydraulic retention time be prolonged up to the level of duration where there will be no pathogens left in treated water.

2.7 References

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CHAPTER 3

EFFECT OF PHYSIOCHEMICAL PARAMETERS ON PATHOGENS REMOVAL IN A HYDROPONIC SYSTEM

3.1 Introduction

The biological processes in wetlands for wastewater treatment are carried out by microorganisms which their functioning is greatly affected by a number of factors. These biological processes lead to reduction and removal of unwanted wastewater constituents and pathogens. Temperature is the principal factor that drives microbial activities in treatment wetlands and in hydroponics. Temperature has a direct and indirect influence, which affects the microbial activity through controlling the diffusion of oxygen into treatment wetlands and enzymatic activity of microorganisms. The alternating aerobic and anoxic conditions in treatment hydroponics results in high removals of wastewater constituents as well as pathogens. The aerobic conditions in subsurface flow treatment wetlands result in high removal of wastewater constituents which may be COD, BOD_5 and nitrogen (Headley *et al.*, 2013; Wu *et al.*, 2016).

In treatment wetland systems, the oxygen demand exerted by influents may exceed the oxygen demand inside the system. This may cause oxygen transfer to be the limiting process. Oxygen may be transferred to water itself or to the surfaces of the biofilms. However, the oxygen transfer pathways in subsurface systems involves atmospheric diffusion, plant mediated oxygen transfer and convective flow of air within the media pore space (Nivala *et al.*, 2013). Atmospheric diffusion largely depends on water and air temperature as well as the treatment bed saturation. The plant mediated oxygen transfer involves radial oxygen loss where there is an internal oxygen transfer through convective flow of air through the plants erenchyma. Oxygen transfer by plants varies with plants species type, seasons and oxygen demanded by the surrounding environment (Nivala *et al.,* 2013). The availability and transportation of oxygen is the major driving factor for biological processes that removes pathogens in hydroponics. Warm temperatures favor high oxygen diffusion rate, while low temperatures result in very low oxygen diffusion rates (Nivala *et al.,* 2013). The predation of microorganisms either by protozoa or lytic bacteria largely depend on the presence of oxygen. The process of biofilm formation, aggregation as well as the production of antimicrobial substances from roots nematodes also depend on the presence of oxygen which controls microbial activities.

The significant of temperature, constant carbon supply and sufficient oxygen supply with diverse distribution in wastewater treatment wetlands maximizes the treatment efficiency. In most cases the pH is usually not a limiting factor. This is because in treatment wetlands nothing is added, thus the treatment process is spontaneous and natural. This means the organisms that carry out microbial activities during the treatment period may be fully adapted to surviving in that particular pH. Although the biochemical processes occurring with time may slightly change while the functioning of microbial activities may not be affected. Microorganisms require carbon source to perform microbial activities in wastewater treatment wetlands. Organic matter in treatment wetlands supply carbon source for microorganisms. The physiochemical parameters directly or indirectly influence the performance of the system. Therefore, it is necessary for these to be studied during treatment.

3.2 Aim, Hypothesis and Objectives

3.2.1 Aim

This chapter aimed at determining the effect of physiochemical parameters on the removal of pathogens in the hydroponic system. The physiochemical parameters were modelled against pathogens removal in the system.

3.2.2 Hypothesis

Physiochemical parameters have a positive influence on the removal of pathogens in the hydroponic system

3.2.3 Objectives

- To determine the temperature, pH and dissolved oxygen (DO) prior and post treatment and model them against pathogens removal in the hydroponic system
- To determine the organic matter content prior and post treatment in order to model it against the pathogens removal in the hydroponic system

3.3 Methodology

3.3.1 Introduction

In determination of physiochemical parameters, the sterile bottles were used for sample collection and samples were transported to the lab where organic material concentrations were analyzed. This was conducted in order to correlate these parameters with pathogens removal obtained from the previous chapter (Chapter 2), to develop a model for the effect of physiochemical parameters in pathogens removal in a hydroponic system.

3.3.2 Water Sampling

Water samples were collected aseptically using sterile sampling bottles of 250 ml volume. This was done following general guidelines of sample collection and refrigeration. The World Health Organization guidelines prescribe 200 ml per sample, while Standards Methods for the Examination of Water and Wastewater prescribe 100 ml per sample. In this study 250 ml of water samples were collected because some of the physiochemical parameters (BOD_5) that were analyzed required more than 100 ml of a sample. The air space of 2.5 cm was maintained in order to allow adequate space for mixing of sample prior to analysis. Water samples were collected prior to introduction into the hydroponic system, during the treatment period at different hydraulic retention times (24, 48, and 72 hours) as well as post treatment as indicated in Figure 2.3. This was done in order to determine the most effective hydraulic retention time for efficient treatment. Samples were analyzed immediately on site for temperature, pH and dissolved oxygen, then placed in a cooler box with ice, and transported to the laboratory for refrigeration below 10^0C before further analysis.

3.3.3 Temperature, pH and dissolved oxygen (DO) Measurements

The pH, temperature and dissolved oxygen was measured on site using a field multiparameter instrument (InoLab_IDS multi 9310) (Merk). Measurements were conducted in triplicates for statistical purposes. All water samples were transported to the laboratory and stored in the refrigerator for further analysis.

3.3.4 Determination of Organic Matter in Wastewater

Organic matter may be defined as the remains of anything that contains carbon compounds that were formed by living substances. In wastewater, this may occur as a result of the presence of human and animal wastes. However, in wastewater treatment wetlands with macrophytes, the decomposing plant material may also form part of organic material. Even though this is a wastewater constituent that may cause serious adverse effects in aquatic systems. If it is not reduced, it has a positive influence towards optimizing the removal efficiency of wastewater treatment wetlands. Organic matter may be estimated in water through the measurement of the amount of oxygen required to breakdown biodegradable materials. This was achieved through measuring COD and BOD_5.

3.3.4.1 Measurement of COD

Chemical oxygen demand was measured using spectroscopic method (14541) (Merck) following the manufactures protocols. The spectrophotometer used was the spectroquant® Pharo 100 (Merck). The COD is the amount of oxygen originating from the potassium dichromate that reacts with the oxidizable substances contained in an analyzed water under the working conditions of the procedure. Samples were oxidized with hot sulphuric solution of potassium dichromate that reacted with silver sulfate as a catalyst. The concentration of green chromium ion (Cr^{3+}) was then determined by a spectrophotometer at 650 nm.

3.3.4.2 Measurement of BOD_5

Biological oxygen demand (BOD) is the amount of oxygen taken up by bacteria during oxidation of organic matter or degradable material. It provides information about the biodegradable fraction of the organic load in water. The United Kingdom Royal Commission selected BOD as the organic pollution indicator in water in 1908 (Jouanneau *et al.,* 2014). The BOD was measured using a method developed by Caldwell and Langelier (1948) based on measuring the pressure drop due to oxygen consumption by organic matter oxidizing microorganisms (Jouanneau *et al.,* 2014). This occurs under controlled conditions in the Aqualytic Thermostatic Cabinet (AL606 Merck), where temperature was 20^0C with constant agitation. The microbes inside the sample used available oxygen in the sample to break down biodegradable material. The carbon dioxide produced by metabolic reactions of organisms was chemically bounded by the potassium hydroxide solution contained in the seal cup of the bottle. The pressure drop was generated within the system and it was detected by the Aqualytic BOD sensor and displayed as the BOD value in mg/l of oxygen.

In the Aqualytic thermostatic cabinet the biodegradation of biodegradable materials in water by microorganisms had two distinct phases. The first phase involved the practical breakdown of carbons. The second phase involved the nitrification process where oxygen was consumed. This process involved two groups of microorganisms of nitrifying bacteria. The first group oxidized ammonia to nitrite and the second group further oxidized the nitrite into nitrate.

3.3.5 Statistical Data Analysis

The data was analyzed with inferential statistics, where the *t-test* (paired) was carried out through comparing the readings of organic matter in wastewater prior and post treatment. The removal was considered significant at $p \leq 0.05$. The Persons coefficient (r) was used to determine the correlations of physiochemical parameters with the removal of organic matter and pathogens in wastewater. The correlations were further explored using the multi-regression analysis where each impact of each physiochemical parameters was determined. This also determined the most contributing physiochemical parameter in the treatment of wastewater in the hydroponic system, especially in pathogens removal.

3.4 Results and Discussion

The results of the physiochemical parameters are presented in Figures 3.1-3.5. The temperature and pH were expressed line graphs with error bars, which was an indication of the variation in the triplicates readings at each hydraulic retention time. The dissolved oxygen (DO) was expressed in a line graphs and the analysis of column analysis and correlation analysis was carried out to define the impact of the hydraulic retention time (HRT) as well as the significance of DO increment efficiency (IF). The COD results were also expressed in a line graph, together with the column statistics and correlation analysis were carried out in a similar way as it was done for DO, but here they were done to determine the removal efficiency (RF) of COD. The BOD_5 results were recorded and plotted in a line graph as per Aqualytic Thermostatic Cabinet manual. The BOD_5 results were also interpreted as per manual. The reviewed literature revealed the relationships to exist between the physiochemical and pathogens removal, that some of them are important as they promote the high removal efficiencies in treatment wetlands. The correlations of physiochemical parameters were carried out in order to understand the functioning of the model.

3.4.1 Performance of the Hydroponic System

The hydroponic system used during the period of study operated 24 hours a day. Good hydraulic conductivity conditions were maintained throughout the study period. There were no overflow experiences, thus indicating that the inflow did not exceed the outflow, and provided enough hydraulic retention time. The treatment bed saturation, thickness and arrangement of soil types allowed enough HRT without causing overflow of water in the system. This was achieved by good choice of soil types used that positively influenced the functioning of the entire system. Good hydraulic permeability was achieved through selection of good substrate that prevented clogging in the system. Hydrology is one of the controlling primary factors in wetlands functioning (Wu *et al.*, 2016). Although there was development of biofilms in between soil particles, they did not alter nor extremely slower the flow in the system. The hydroponic system was constructed to provide a vertical flow, besides the positioning of the discharging pipe downwards the gravity also played a role in the flow direction. This may also have assisted the system to overcome flow obstacles that may have slowed or altered the hydraulic conductivity conditions in the system. The macrophytes (*Amaranthus hybridus* and *L Bidens pilosa L*) used in this study grew well due to the high level of nutrients that were being absorbed from wastewater that was circulated in the closed system by the electric pump for the purpose of treatment.

3.4.2 Temperature, pH and Dissolved Oxygen

The temperature ranged between 16-20^{0}C during the study period with a fluctuating pattern at different hydraulic retention times. This parameter was not controlled during the study period since the hydroponic system used was situated outside the Vulindela Wastewater Treatment plant in the open environment. The pH ranged between 6.2 and 7.6 at different hydraulic retention times. There was a little variation observed as shown by the error bars (Figure 3.1). The hydroponic system used was situated in an open area outside the building. The environment was not controlled to optimize microbial activity for biochemical activities. Instead the system was made to mimic the natural wetlands. The changes in temperature occurred mostly as a results of a day change in environmental temperature of the surrounding environment. The average high temperatures ranged between 24^0C and 29^0C, and average low temperature ranges between 23.80 and 27.80^0C. The trend of temperature fluctuations followed the same trend with changes in pH. This was because temperature is the major factor controlling biochemical reactions which were observed by the change in pH being alkaline, acidic nor neutral (Figure 3.2).

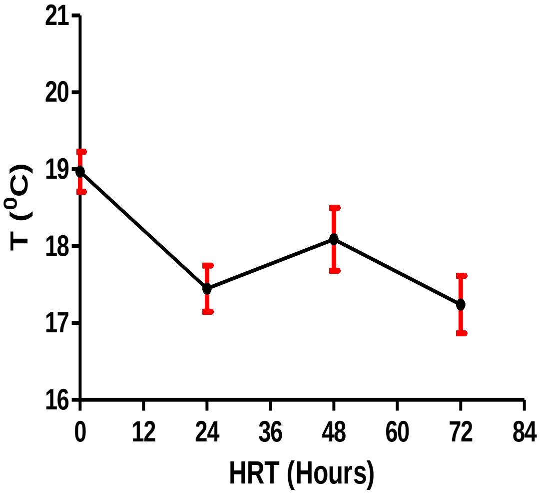

Figure 3.1: Temperature variations in the hydroponic system

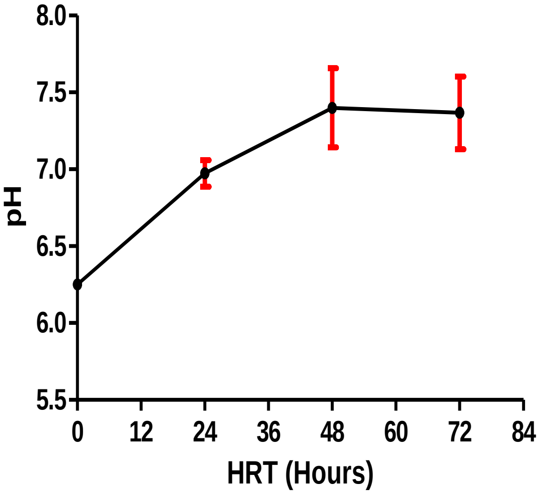

Figure 3.2: pH variations in the hydroponic system

The dissolved oxygen concentration ranged between 9.99-12.14 mg/l and increased as water circulated in the system. The elongation of hydraulic retention time was found to be directly proportional to the increment efficiency of the dissolved oxygen (Figure 3.3). This was also evidenced by the coefficient of determination (R^2=0.90), when the correlation analysis was carried out to evaluate the significance of elongation of HRT in the increment of DO efficiency (r=0.95).

This indicated that the HRT contributed to almost 90% in the increase of DO during the wastewater treatment in the hydroponic system.

When the small size vertical flow constructed wetland was used to treat domestic wastewater in Greece, the pH mean value was found to be around neutral between 7.4 and 7.7 (Gikas and Tsihrintzis, 2012). Gikas and Tsihrintzis, (2012), also found the wastewater temperature to change seasonally and daily. The mean increase of DO occurred as a result of oxygenation by the plants as they transported oxygen to the porous media through their extensive root system (Gikas and Tsihrintzis, 2012) and this increase of DO favored the yield of high COD removals. Plants function as carbon suppliers for microorganism's metabolism (Dong *et al*., 2012). The increase of temperature, increased the removal efficiency in planted wetlands.

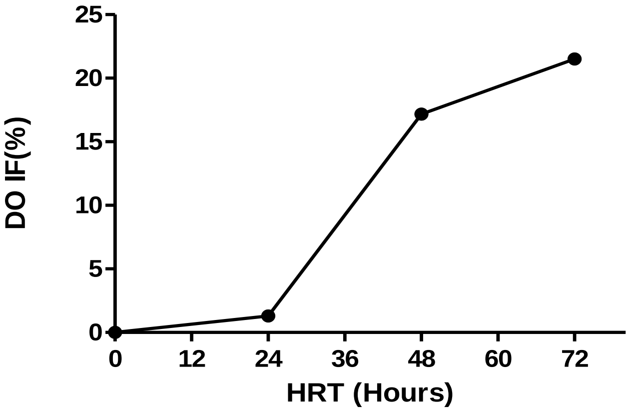

Figure 3.3: Dissolved oxygen increment in the hydroponic system

3.4.3 Organic Matter

The wastewater treated by the system was turbid and overtime, there were decomposition of macrophytes in the system. This indicated the presence of organic material in wastewater. However, the system was able to reduce the organic matter content with treatment.

3.4.3.1 Chemical Oxygen Demand (COD)

The chemical oxygen demand was removed during the treatment, and its removal was directly proportional to the elongation of hydraulic retention time. This was also confirmed by the correlation analysis that indicated the HRT to have a strong positive correlation with COD removal efficiency (RF) (r=0.96) (Figure 3.4). The highest removal efficiency at 72 hours was 57%. The coefficient of variation showed a strong positive influence of the treatment duration the removal efficiency of the COD (R^2=0.93). The concentration of COD decreased as a result of significant organic matter removal. The microbial activity of aerobic and anoxic bacteria was found to be the main removal mechanism in the vertical flow constructed wetland used for domestic wastewater treatment. This showed the significant role of plants in the removal efficiencies as they are major transporters of oxygen to the porous medium through their deep root system and as media for microbial development. However, the removal of COD was found not to be statistical significance when the column statistics t-test was carried out (p=0.12). This was because the system was used as a tertiary treatment stage, where water treated was from the secondary stage of the treatment and initially contained less amounts of COD. The system was capable of treating wastewater of different concentrations of COD.

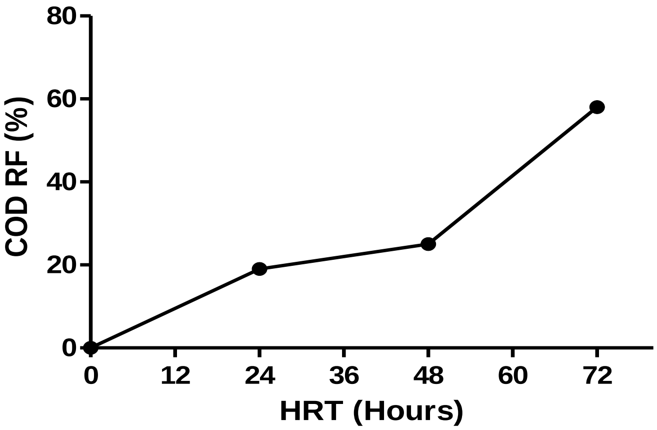

Figure 3.4: Chemical oxygen demand removal in the hydroponic system

The standards set by the Department of Water Affairs and Sanitation for disposal of treated wastewater into natural water bodies permits 75 mg/l and special standards recommends 30 mg/l of COD content. The water sample after treatment met the standards even though initially it contained lower COD amount (52 mg/l) because it was taken from the secondary stage of wastewater treatment process. Which was later (72 hours) reduced to 29 mg/l. The COD reduction occurred due to utilization of organic matter as a carbon source by microorganisms to break down wastewater constituents or contaminants in the system. Removal of COD might have occurred as a result of the filtration due to the presence of sand layer as part of the treatment bed. The forces of attraction might have also played a role of adsorption, where the organic matter might have adhered to the biofilm surfaces, the surfaces of the system walls or surfaces of supporting gravel particles. However, the system displayed a potential in the removal of COD (57%).

3.4.3.2 Biological Oxygen Demand (BOD_5)

The samples taken at different hydraulic retention times produced different increase patterns on the BOD_5 curve. The sample collected at zero HRT produced an increasing but fluctuating pattern of BOD_5. This was also seen on the samples collected at 24, and 72 HRT. The 48 HRT sample presented a linear increased between day 2 and day 5. The 24 HRT sample is the one that showed the highest BOD_5 at day 5, which was 5 mg/l. While the 48 HRT sample had the lowest BOD_5 value at day 5, which was 3 mg/l.

The biological oxygen demand values were very low in all samples. Most of the samples at day one read 0 mg/l which might have happened as a sign of severe inhibition in the sample. The system used contained emergent macrophytes which used nutrients for their growth. As a result, it might have happened that the effluent water samples may have contained little nutrients which could not sustain the growth of bacteria in the Aqualytic Thermostatic Cabinet. The inhibition of bacterial growth might have hindered the breakdown of biodegradable material in wastewater samples during this time.

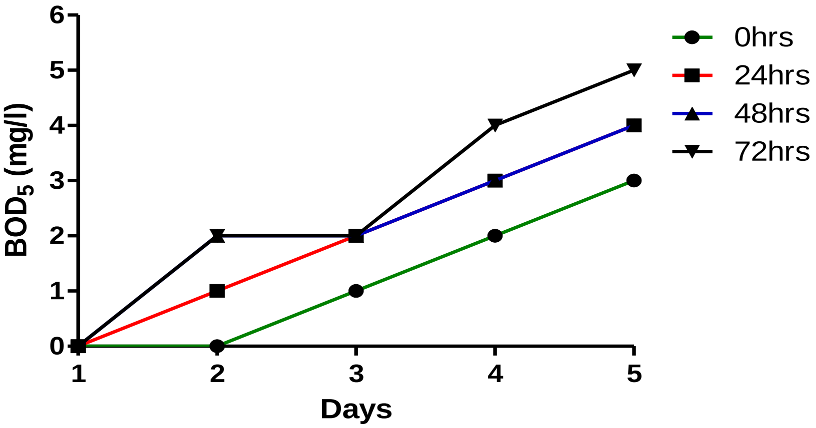

Figure 3.5: Biological oxygen demand (BOD_5) in the hydroponic system

The BOD value were within the standards set by the Department of Water Affairs and Sanitation for disposal of treated wastewater into natural water sources which allows 25 mg/l of oxygen. The

BOD results indicated that water from the system was ready to be disposed to natural water sources after treatment.

3.4.4 Correlation of Physiochemical Parameters with Pathogens Removal In The Hydroponic System

3.4.4.1 Effect of Temperature and pH on Against Pathogens Removal in the hydroponic system

The correlation of temperature with the removal of pathogens varied with the type of an organism. *E. coli* removal had a negative moderate (*r*=-0.51) correlation with the temperature, *Salmonella spp.* (*r*=0.15*)* and *Shigella spp.* (*r*=0.83) had a weak positive correlation and a strong positive correlation respectively (Figure 3.6). The pH had an inverse correlation with the removal of pathogens (Figure 3.7). The removal of pathogens and pH had a very weak negative correlation (*r*=-0.14) to a strong negative correlation (*r*=-0.74) and a very strong negative correlation (*r*=-0.90) for *E. coli, Salmonella spp.* and *Shigella spp.* respectively. This indicated that the increase in pH favored the decrease of pathogens in the system.

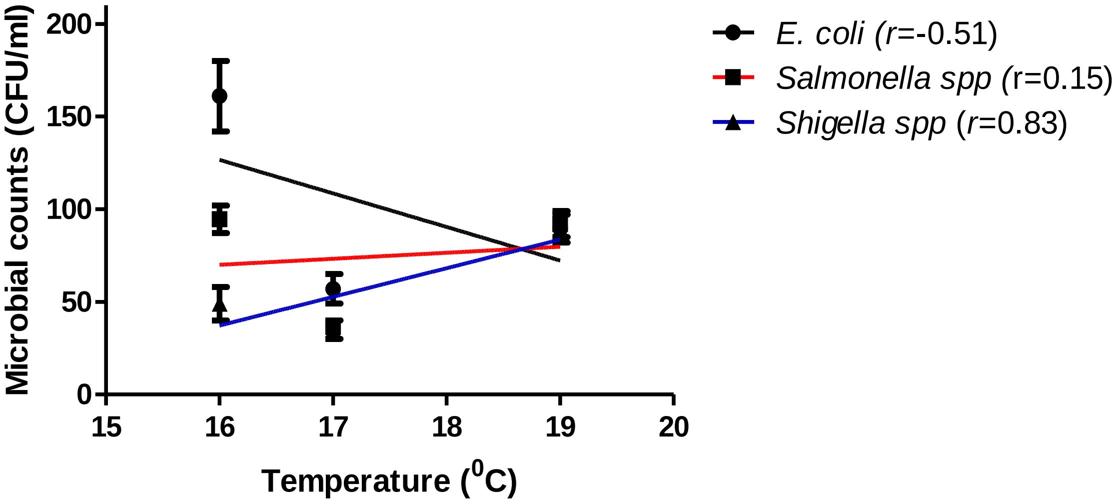

Figure 3.6: The correlation of temperature with pathogens removal in the hydroponic system

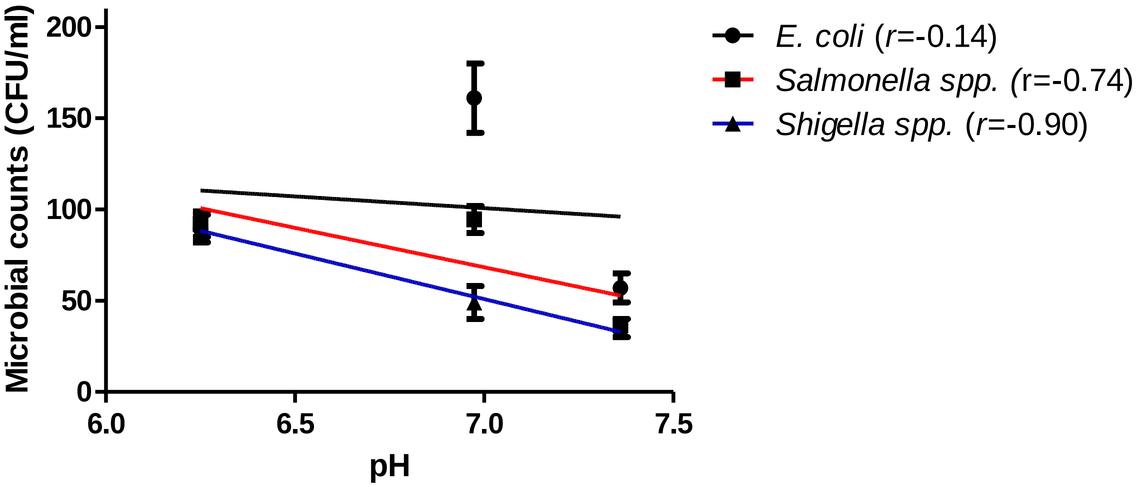

Figure 3.7: The inverse correlation of pH and pathogens removal in the hydroponic system

Gruyer *et al.* (2013), reported that the physiochemical parameters (temperature, pH, vegetation type and density and redox potential) influence the treatment wetland properties and determine the efficiency of pathogens elimination. Weker *et al.* (2002) also reported that low temperatures lowers roots oxygen release.

3.4.4.2 Effect of Dissolved Oxygen on Pathogens Removal

A negative correlation between dissolved oxygen and removal of pathogens was observed (Figure 3.8) in the system during treatment. The DO had an inverse moderate correlation (*r*=-0.68) with *E. coli* removal. A very strong negative correlation (*r*=-0.99) was observed between DO and *Salmonella spp*. removal, while *Shigella spp*. had a negative strong correlation with DO. This indicated that the removal of pathogens in the system caused an increase in the concentration of dissolved oxygen. Although the biological activities for removal of contaminants might have utilized the dissolved oxygen available, but the macrophytes kept the oxygen supply. Macrophytes are responsible for approximately 90% of oxygen transport and availability in the rhizosphere (Mthembu *et al.*, 2013). This caused the elevation of DO concentration in the decrease of microbial population in the system. Wu *et al*. (2016) reported that oxygen availability and vegetation presence increase the bacterial die-off.

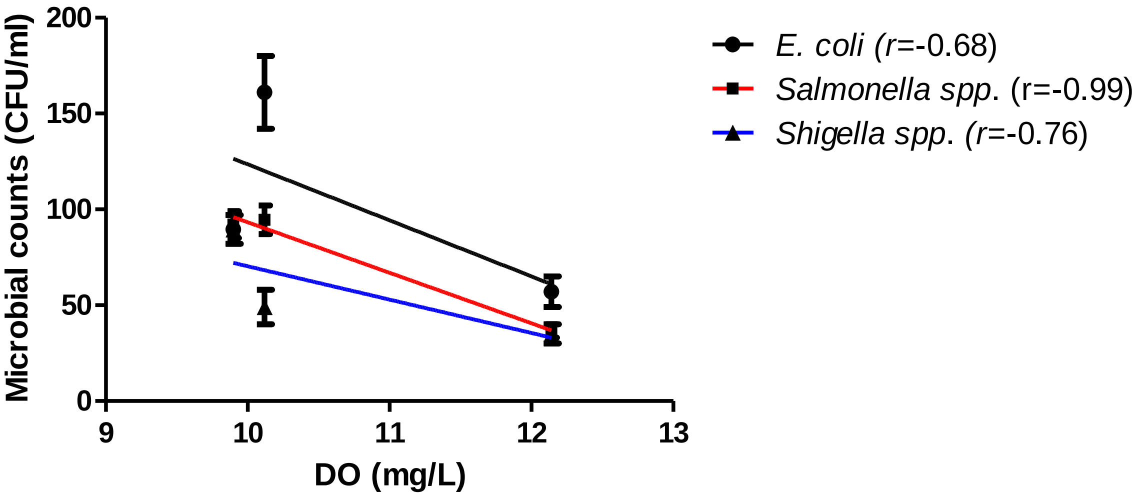

Figure 3.8: The inverse correlation of DO decrease and pathogens decrease

3.4.4.3 Effect of COD on Pathogens Removal

The COD had a positive correlation with the removal of pathogens (Figure 3.9). A very weak positive correlation (*r*=0.19), a strong positive (*r*=0.78) and a very strong positive correlation (*r*=0.99) with the removal of *E. coli*, *Salmonella spp*. and *Shigella spp*. in the system. This indicated that the COD has a positive influence in the removal of pathogens by the system. In hydroponic system, the microflora utilized the organic matter as a substrate for carrying out biological activities for removal of contaminants and other unwanted wastewater constituents.

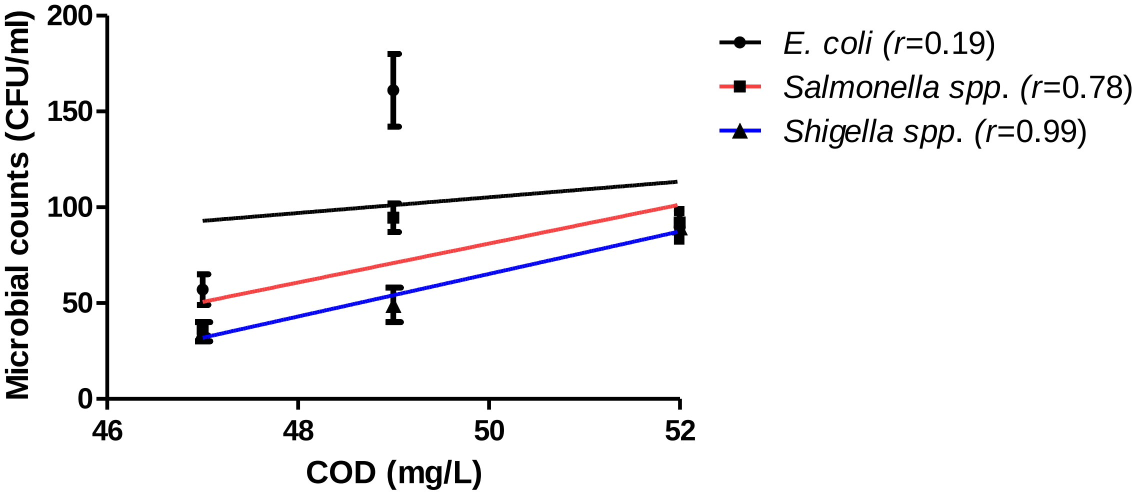

Figure 3.9: The positive correlation of COD and pathogens removal in the hydroponic system

3.5 Conclusion

The hydroponic system mimicked a natural wetland because it was able to reduce wastewater contaminants and constituents into the recommended levels at environmental conditions. The system was able to reduce wastewater constituents' physiochemical parameter to the desired levels at a short hydraulic retention times. The removal mechanisms were not hindered by the non-optimized conditions. The physiochemical parameters influenced the removal of pathogens in the system, negatively and positive depending at their different influences in the system. This affected the removal mechanisms in the system but however, the pathogens were removed in the system.

3.6 Recommendations

Optimization of physiochemical conditions in wastewater treatment systems result in high removal efficiencies. For future studies, it is recommended that the hydroponic system may have aeration pipes that could ensure the presence and efficient circulation of oxygen. This will yield maximum microbial activity and high removal efficiencies. There is also a need for installation of physiochemical sensors that would monitor the steady desired and efficient levels of essential parameters for wastewater treatment processes. This may not only result in high pathogens removal efficiencies but may also improve public health conditions

3.7 References

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CHAPTER 4

ANTIMICROBIAL ACTIVITY PRODUCTION BY MACROPHYTES IN THE HYDROPONIC SYSTEM

4.1 Introduction

Microbial contamination in water leads to the emergence of waterborne diseases outbreaks. According to the World Health Organization (WHO) (2010), death cases associated with waterborne diseases exceeds 5 million people per year. This threatens the public health. Therefore, the removal of pathogenic microorganisms is essential. Constructed wetlands planted with macrophytes have been reported to reduce pathogens in wastewater (Gruyer *et al.*, 2013). Plants produce a complex series of antimicrobial compounds when under pathogenic attack. Macrophytes are part of the important wetland components that is completed by soil and microorganisms, and play a significant role in pathogen removal in the system. Such mechanisms are capable of resulting in efficient removal of unwanted wastewater constituents including pathogens. The presence of macrophytes in treatment wetlands enhances the functioning, as well activates a variety of mechanisms. Macrophytes achieve this through their unique adaptations that make them ideal for wastewater treatment. These adaptations include photosynthetic rate, radial oxygen loss and microbial biomass. Through these characteristics, macrophytes play multiple roles in wastewater treatment processes. These processes include remediation, nutrients removal, microbial activity stimulation as well as antimicrobial compounds production. The pathogens removal may occur as result of synergistic or individual occurrence of these processes. However, focusing at the antimicrobial compounds production by macrophytes, they are known to be defensive against pathogens (Burketova *et al.*, 2015).

Macrophytes have natural resistance mechanism that they use against pathogenic attack (Oliveira *et al.,* 2016), which includes physical and chemical barriers such as cuticles, cell walls and antimicrobial anticipins (Burketova *et al*., 2015). The infection of a pathogen induces dramatic changes in the cell wall activity and around the invasion site. This causes the release of elicitor molecules during the plant-pathogen interaction (Oliveira *et al.*, 2016). The recognition of the microbe/pathogen association molecular pattern (MAMPs/PAMPs) by the plant immune receptor triggers immunity. PAMPs are conserved molecular structures for overall fitness of microbes. The enzymatic activities and toxins are externally recognized in damage-associated molecular patterns (DMAPs). The plasma membrane located pattern recognition receptors (PRRs) recognize the MAMPs and DAMPs and induce the defense mechanisms. The PRRs triggers a sequence of signaling events, including ion fluxes leading to plasma membrane depolarization, production of reactive oxygen species (ROS), nitric oxide and activation of mitogen-activated and calcium-dependent protein kinases (MAPKs and CDPKs) (Burketova *et al*., 2015). These signaling events exert the transcription factor (TF) activities, leading to massive transcriptional reprogramming of a related defense. The resulting defenses are referred to as pattern-triggered immunity (PTI).

Different enzymes acting as specific stress metabolites are accumulated after defense gene activation. These include pathogens related (PR) proteins, lignin and cellulose deposition to the cell wall for strengthening and compounds with antimicrobial activity such as phytoalexins (Burketova *et al*., 2015). The production of PR proteins in plants occur because of the pathogen invasion (Oliveira *et al.,* 2016). Phytoalexins are produced by healthy cells adjacent to localized damaged and necrotic cells. In uninfected plant parts, the PR proteins can prevent the further attack of the pathogen. These systems prevent pathogens entrance and inhibit their establishment. The plants system may acquire resistance against pathogens. The system acquired resistance (SAR) and induced systemic resistance (ISR) trigger defense mechanisms not only in infected parts but also in the whole plant (Oliveira *et al.,* 2016). The SAR develop systemically in response to necrotic pathogens. The resistance acquired is effective to a broad spectrum of pathogens. This is because the PR proteins trigger this type of resistance. On the other hand, the ISR usually is induced by rhizobacteria. In this case, the inducer is not necrotic but induce the plants systemic protection and there is no accumulation of proteins. The plants response to pathogen attack varies with plants species, age, part and its physiological state (Oliveira *et al.*, 2016).

This chapter aimed at investigating the production of antimicrobial compounds by *Amaranthus hybridus L* and *Bidens pilosa L* that were used in the hydroponic system. The knowledge of antimicrobial productivity status of the two macrophytes will add substantial knowledge in understanding the mechanisms of pathogens removal by macrophytes during hydroponic treatment process, and thus in the wastewater treatment sector. Discovering the ability of these plants to inhibit microbial growth will enhance their utilization in constructed wetlands for wastewater treatment, which will further result in the improvement of public health and environmental conditions. This is because it may decrease waterborne diseases outbreaks and eliminate environmental pollution through elimination of pathogens in wastewater. This information may be a solution to major drawbacks caused by currently used wastewater treatment technologies as well as the availability of pathogens in treated waters.

4.2 Aim, Hypothesis and Objectives

4.2.1 Aim

The aim is to establish the pathogen removal mechanism from the hydroponic system by investigating the production of antimicrobial activity by macrophytes in the system.

4.2.2 Hypothesis

- The macrophytes will have diverse phytochemical compounds that will enhance their inhibitory reaction on pathogens
- The macrophytes will inhibit the pathogens in the hydroponic system

4.2.3 The objectives were:

- To determine the phytochemical composition of the macrophytes and correlate them with the macrophytes extracts against pathogens
- To determine the reaction of the macrophytes extracts against the selected pathogens in order to determine their lowest inhibitory concentration
- To evaluate the strength of the inhibitory activity of the macrophytes extract

4.3 Methodology

4.3.1 Introduction

In order to evaluate the production of antimicrobial activity of macrophytes towards the pathogens. The phytochemicals were screened from macrophytes tested against pathogens. The wastewater treated in the hydroponic system was collected from the secondary stage of the conventional sewage treatment system. The microbial quantification of pathogens in Chapter 2 provided the indication that there were very low concentrations of pathogens in the system at this stage. This necessitated the spiking of pathogens in the system in order to increase their concentration, and thus evaluate the systems potential to efficiently remove pathogens in wastewater. Therefore, the pathogens of interest were purchased from Thermofisher scientific (Table 4.1) and spiked in wastewater prior treatment in the hydroponic system. This was done to increase the biomass of pathogens in wastewater. These microorganisms have been associated with diarrheal infections emerging after fecal contaminated water consumption in developing countries with poor drinking water quality and improper sanitation (Cabral, 2010). Even though there is a variety of microorganisms found in contaminated water sources, these pathogens are regarded as the most prevalent in causing waterborne diseases transmitted through water (Cabral, 2010).

Apart from preparation of purchased organisms for susceptibility testing, the macrophytes extract to be tested was also prepared and screened for phytochemical compounds. This was done to correlate the phytochemical compounds with the activity of the macrophytes extracts. The minimal inhibitory and bactericidal concentrations of the extracts was determined. Determination of these concentrations was conducted to test if the macrophytes extracts were able to remove pathogenic organisms from wastewater. Tests were conducted to determine if the extract were completely killing or inactivating the microorganisms.

Table 4.1: The pathogens that were used in the study during the testing of antimicrobial activity of macrophytes

Pathogens	
Salmonella enterica	ATCC 13311
Shigella flaxneri	ATCC 9199
Shigella sonnei	ATCC 25931
Escherichia coli	

4.3.2 Isolation and Spiking of Pathogens in Wastewater

Fecal coliforms including *E. coli* are common indicator of organisms that are used to access water quality as well as pathogenic organisms (Boutilier *et al.*, 2009). Therefore, *E. coli* was isolated from wastewater using a selective medium (Eosin Methylene Blue). Where green metallic sheen colonies were observed as an indication of *E coli*. For the isolation of *Shigella spp.* and *Salmonella spp.* the Desoxycholate citrate agar was used. This was used because it is a selective medium for isolation of *Shigella* and *Salmonella* from fecal material and environmental samples. These colonies were further tested with biochemical tests, which included gram-staining, methyl red, an indole and ammonium acetate test. The isolation was conducted to confirm the presence of these pathogens in wastewater prior spiking to increase their biomass in water.

During the spiking of pathogens in wastewater, a stock culture was prepared by inoculating the nutrient broth with purchased and isolated microorganisms. The inoculated broth was then incubated at 37^0C for 24 hours. After that, the undiluted spiking suspension was prepared by combining 99 ml of sterile broth in a sterile screw test tube with 1 ml of previously prepared stock culture. The inoculum was dispensed by vigorously shaking the broth culture that was then incubated at 37^0C for 24 hours in a shaker. This culture was referred to as undiluted spiking suspension and was estimated to contain $1.0X10^7$ to $1.0X10^8$ colony forming unit per milliliter (CFU/ml) of inoculated pathogen.

4.3.3 Plant extract preparation

The macrophytes were collected from the hydroponic system after wastewater was treated. The plants parts were used separately to determine the most effective parts in removing pathogens. In constructed wetlands, emergent macrophytes during the treatment only stays in contact with stems and roots in the rhizosphere. Therefore, stems and roots were prepared separately for both macrophytes. They were dried, grinded and then dissolved in methanol using the 1:5 ratios. The extract was shaken for 72 hours and evaporated at 40^oC using the rotary evaporator. Macrophytes were also tested for phytochemicals screening.

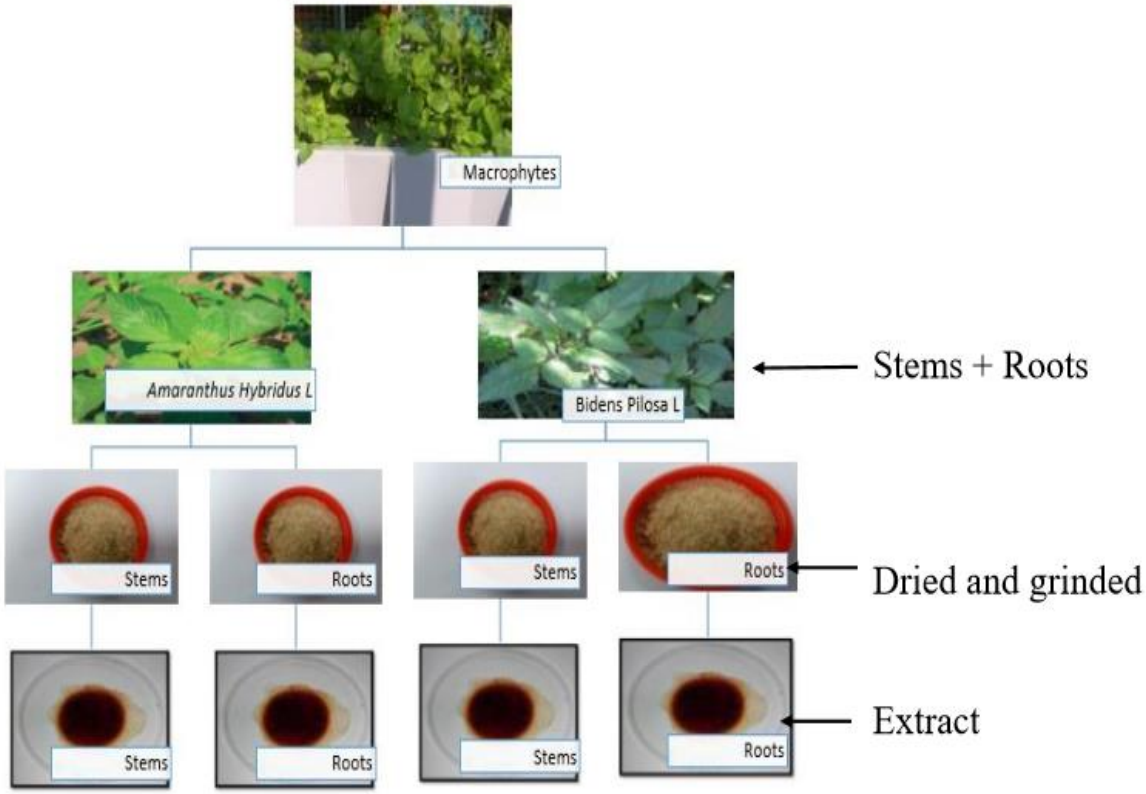

Figure 4.1: The processing of macrophytes for crude extract. This was conducted for both macrophytes used in this study. These crude extracts (100 mg/l) were further used for antimicrobial activity testing in the MIC and MBC determination

4.3.4 Phytochemicals screening

Phytochemicals may be defined as nonnutritive plant chemicals that contain protective and disease preventing compounds. These compounds are secondary metabolites such as tannins, steroids, phenols, flavonoids, saponins and alkaloids (Varsha *et al*., 2012). Plants produce these compounds to protect themselves from pathogenic attack (Maobe *et al.,* 2013). The phytochemical composition of both plants were also investigated. This was done to correlate the antimicrobial activity status of the plants against pathogens. Specific phytochemicals present in antimicrobial active plants were screened. They included flavonoids, saponins, tannins and alkaloids. The presence of these phytochemicals have been reported in plants with antimicrobial activity (Okoli *et al.*, 2002). The following procedures were used for phytochemical compounds screening.

- Flavonoids

The sodium hydroxide test was conducted where the plant material (1 g) was mixed with 1 ml of dilute sodium hydroxide (NaOH). A golden yellow precipitate was considered as evidence of the presence of flavonoids.

- Saponins

The plant material (2.5 g) was extracted with boiling water and was allowed to cool. The extract was vigorously shaken to froth and then allowed to stand for 15 – 20 minutes. The extract was then classified for saponins content as follows: no froth = negative (no saponins) and froth less than 1 cm = weakly positive (saponins present); froth 1-2 cm high = positive; and froth greater than 2 cm high = strongly positive

- Tannins

The plant material (0.5 g) was boiled with 10 ml of water for 15 minutes, filtered and made up to 10 ml with distilled water. Two milliliter of the filtrate was transferred into another test tube and a few drops of 0.1% ferric chloride ($FeCl_3$) solution was added to 2 ml filtrate. Black-blue, green or blue-green precipitate was considered as evidence of the presence of tannins

- Alkaloids

The plant material (0.5 g) was dissolved in 5 ml 1% HCl (aq.). The solution was stirred on steam bath and filtered. One milliliter of the filtrate was treated with Mayer's reagent. A precipitate was considered a preliminary evidence of the presence of alkaloids. Another 1 ml of the filtrate was treated with Dragendorff's reagent and the presence of turbidity or precipitate was also considered an evidence of the presence of alkaloids.

4.3.5 Determination of minimum inhibitory concentration (MIC)

Antimicrobial susceptibility testing can be conducted with a variety of methods. These methods include diffusion and dilution methods. The dilution method of minimum inhibitory concentration using both macrophytes was employed in this study. This method is widely used for testing susceptibility of microorganisms because it is able to test small numbers of isolates. This method was also advantageous because apart from measuring the inhibitory effect, it also determined the strength of inhibitory activity through minimum bactericidal concentration (MBC). In this method, the MBC was measures using the same tubes that were used in MIC determination. The dilution method also generated quantitative results, economize the usage of reagents and space through miniaturization of the tests.

A serial dilution method was performed as described by Eloff (1998) and Kamazeri (2012) to measure the lowest concentration of extract that inhibited the pathogens. The 96 well plate was used to quantify and determine the MIC for macrophytes extracts. The sterile nutrient broth of 50 µl was added to all the wells of the 96 well plates. Fifty microliters of extracts (100 mg/ml) was poured in the wells of the first raw and mixed on separate plates. Dilutions down the columns was prepared where 50 µl of extract mixture with broth was transferred. The selected microbial pathogens (50 µl) was pipetted into all wells in triplicates. Ciprofloxacin (20 µg/µm) was used as a positive control and 40% dimethyl sufloxide (DMSO) was used as negative control. The plates were covered and incubated at 37^{o}C overnight. To indicate microbial growth, 50 µl of p-iodonitrotetrazodium violet (INT) was added into microplates and incubated at 37^{o}C for 30 minutes. A production of a red colour was an indication of the microorganism's metabolic activity reducing INT to formazan. The clear colour was an indication of the absence of microbial activity (Eloff, 1998). Therefore, the production of red colour was used as an indication of the presence of microorganisms while the clear colour indicated their absence.

4.3.6 Determination of minimum bactericidal concentration (MBC)

From the 96 well plates where the MIC was determined, the wells that showed no microbial activity were grown on nutrient agar and incubated at 37^{o}C overnight. The microbial growth indicated that the antimicrobial substance was produced by macrophytes but it did not completely kill the pathogen but made it static. The absence of microbial growth was an indication of the production of antimicrobial substance that killed the microbial pathogen.

4.4 Results and Discussion

4.4.1 Plant extract phytochemical composition

The knowledge of the phytochemical composition of the macrophytes is of critical importance in correlating the antimicrobial activity reaction of the macrophytes with pathogens removal in the hydroponic system. The screening of the phytochemical compounds indicated the presence of saponins, tannins, flavonoids, and terpenoids in both macrophytes (Table 4.2). These secondary metabolites are known to exist in antimicrobial active macrophytes, Maobe *et al.,* (2013) demonstrated that macrophytes produce these phytochemicals to defend themselves from pathogenic microorganisms. Thus, the presence of these phytochemicals in macrophytes in our system synergistically played a role in protecting the macrophytes against pathogens attack, with their special properties that each compound possessed. This led to reduction of pathogens in the hydroponic system, and thus contributing to the treatment efficiency of the system.

Table 4.2: The phytochemical compounds that were found in the *Amaranthus hybridus L* and *Bidens pilosa L*

Phytochemicals	***Amaranthus hybridus L***	***Bidens pilosa L***
Saponins	++	+
Tannins	+	+
Flavanoids	++	++
Terpenoids	+	+
Alkaloids	+	-

++: -- Strong detection + : -- Low detection - :-- No detection

The phytochemicals are non-nutritive, naturally occurring biological active compounds that are found in the plant kingdom (Chang *et al.*, 2016). These secondary metabolites are produced by plants through sophisticated processes that leads to different responses. The secondary metabolites help the plants to survive through harsh conditions, which may be pathogenic and herbivores attack as well as harmful attack of ultraviolet light (Sahebi *et al.*, 2017).

The screening of phytochemical compounds revealed the presence of alkaloids in one of the macrophytes, which are a group of naturally occurring chemical compounds found in plants.

Alkaloids have been associated with cytotoxicity. Thus their absence in *Bidens pilosa L* may lower the risk of plant toxicity (Aiyegoro and Oko, 2010). However, their presence in *A. hybridus L* enhanced this macrophytes to have high activity in inhibiting the pathogens compared to *B. pilosa L* where alkaloids were not found. The chemical compounds contain nitrogen atoms, and are characterized by powerful physiologic activities in plants and animals. Saponins were also found to be present in both macrophytes. These are naturally occurring plant glycosides and a promising class of natural surfactants that could be used in dispersed system (Böttcher and Drusch, 2017). Flavonoids are class of plant and fungus secondary metabolites, which are also referred to as bioflavonoids. They are known to be secreted in the rhizobia during the infection stage. Tannins have been found in edible plants and were also present in the macrophytes used in this study. They had physiological effects and served as natural defense mechanism against microbial infections (Chung *et al.*, 1998). Terpenoids are regarded as one of the essential oils that has an antimicrobial activity (Bassolé and Juliani, 2012).

Based on the findings of the study, it can be said that the identified phytochemical compounds (saponins, tannins, flavonoids, terpenoids and flavonoids) attributed to the inhibitory activities of macrophytes against pathogens. Most of these phytochemicals are associated with antimicrobial defenses in plants. Therefore, the inhibitory activity of macrophytes occurred because of their individual or synergistic activities against the pathogens. Thus, the removal of pathogens after treatment in the hydroponic system occurred because of inhibition by antimicrobial compounds that were produced by macrophytes.

4.4.2 Minimal inhibitory concentration (MIC)

The macrophytes used in the study showed antimicrobial activity against pathogens, which contributed to the removal of pathogens in wastewater that was treated by the hydroponic system. This was observed in the MIC (Table 4.3) where the extracts displayed an inhibitory activity at different concentrations. The minimum concentration of *Bidens pilosa L* stems and its roots where *E. coli* was inhibited is 25 mg/ml. The *Amaranthus hybridus L* stems extract did not show any inhibitory effect on *E. coli*, while its roots extract had the minimum inhibitory activity at 25 mg/ml. An inhibitory activity of *Salmonella enterica* by *B. pilosa L* was observed at 12.5 and 50 mg/ml on stems and roots extracts respectively. *Amaranthus hybridus L* did not show any inhibitory activity on *S. enterica*. *Shigella flexneri* was inhibited at 25 and 50 mg/ml by *B. pilosa L* stems and roots extracts respectively. The *A. hybridus L* stems did not inhibit the growth of *S. flexneri*, but its roots extract had the inhibitory activity at 25 mg/ml. The *Shigella sonnei* was inhibited by *B. pilosa L* stems and roots at 25 and 50 mg/ml respectively. The ciprofloxacin was inhibited at 20 µg/µm. *Escherichia coli* is the only pathogen that was inhibited by the antibiotic at the lowest concentration of 1.25 µg/µm, while the other test organisms were only inhibited at the 10 µg/µm.

Table 4.3: The minimum inhibitory concentrations of macrophytes used in the study

Waterborne Pathogens	**Minimum Inhibitory Concentation**				
	Macrophytes extracts				Ciprofloxacin (µg/µm)
	B. pilosa L)		*A. hybridus L*		
	stems (mg/ml)	roots (mg/ml)	stems (mg/ml)	roots (mg/ml)	
E. coli	25	25	0	25	1.25
S. entetrica	12.5	50	0		10
S. flexneri	25	50	0	25	10
S. sonnei	25	50	0	25	10

Bidens pilosa L was found to have more inhibitory activity on pathogens than *A. hybridus L*. This was observed by inhibitory activity shown by this macrophytes in both extracts of stems and roots. While the *A. hybridus L* showed the inhibitory activity in its roots, the stems did not show any inhibitory effect. The inhibitory activity of the macrophytes might have occurred due to the presence of phytochemicals that these macrophytes possessed (Table 4.2). Even though these phytochemicals were not quantified in macrophytes, the inhibitory activity exhibited by macrophytes may have been due to the unequal distribution of phytochemicals in macrophytes. The inferential statistics analysis conducted on evaluating the inhibitory activity of *B. pilosa L* revealed that this macrophyte part inhibited microorganisms at different concentrations. This was observed when the *t-test* was conducted for mean comparisons on *B. pilosa L* stems and roots. The two tailed *t-test* showed that the means were statistically significant different ($p=0.02$), where the mean of stems inhibitory activity was found to be 43.75 while that of the roots was 21.88. The statistically difference of inhibitory means of different parts within the macrophyte was observed in different behaviors of *B pilosa L* stems and roots. The stems of *B pilosa L* were found to have more inhibitory activity compared to its roots. This was observed where this extract inhibited *S. enterica* at 12.5 mg/ml (Table 4.2). On the other hand, *A. hybridus L* had a poor inhibition activity compared to *B. pilosa L*. Only the roots of this macrophyte showed to possess an inhibitory effect. Although that inhibitory activity showed no variation. This might have occurred due to the nature of test microbial pathogens.

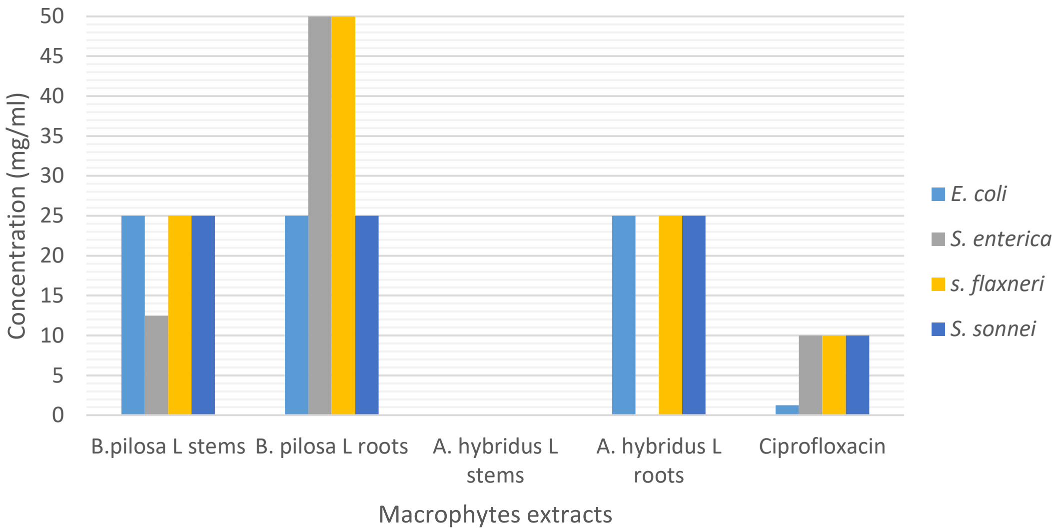

Figure 4.2: The MIC of macrophytes in pathogens

The pathogens used in this study were all gram negative. Gram-negative bacteria cause a spectrum of diseases. Although gram-negative bacteria possess thinner cell wall than gram-positive bacteria, they have an outer membrane that protects them from antibiotics, dyes and detergents that would damage their inner membrane (Miller, 2016). This outer membrane is composed of glycolipid polysaccharides (LPS) and glycerol phospholipids. The outer membrane protects the bacteria against damage from antimicrobial agents produced within multispecies microbial communities, where they function in microbial competition. It also promotes antimicrobial resistance and interpret bacterial signal from the membrane damaging agents, including antibiotic (Miller, 2016). However, the inhibitory activity of pathogens independent of extracts concentration might have played a significant role in reduction of pathogens in the hydroponic system.

Deba *et al*. (2008) showed that methanol roots extract of *B. pilosa L* had the antimicrobial activities against *B. cereus, E. coil, K. pneumonia* and *S. aureus*. Its leaf extract was also reported to have bactericidal and bacteriostatic effect on a gram negative and gram positive, where attempt to find natural products as food preservatives was conducted for microbial multiplication and food oxidation. *Bidens pilosa L* has also been found to possess antifungal activity against *Aspergillus niger, A. flavs and Penicillin nomatum. Bidens pilosa* L produces a variety of secondary metabolites that enables it to exhibit the antimicrobial activities (Deba *et al.*, 2008). Traditionally, *B. pilosa L* is used as an ingredient in herbal medicines and tea. It is consumed due to its good nutritional value and used to treat several disorders that includes, inflammation, immunological and digestive disorder. It also used to heal infectious diseases and wounds (Bartolome *et al*., 2013). *Amaranthus hybridus L* and other members of *Caryophillales* have been frequently reported to be used in traditional medicine to treat various kinds of diseases including malaria, fever and cardiovascular diseases (Nana *et al.*, 2012).

The activity of an extract might have limited by its temperature. Okoli *et al.* (2002) reported that the hot extract of *Harungana madagascariensis* inhibited the growth of *S. aureus,* while the cold extract did not inhibit this organism. The type of solvent that was used in this study might have limited the results obtained. The usage of variety of solvents might have greatly influenced the activity of phytochemical compounds, which may enhance the inhibitory activity of the macrophytes. The test pathogens in this study were all Gram negative and that might have limited the activity of the extracts. The composition of the cell walls greatly influenced the antimicrobial compounds from penetrating the cell. The usage of different macrophytes species in future, because the species type also influences the performance of the overall wetland system, may be recommended in order to increase the phytochemical variety in the system.

4.4.3 Minimal bactericidal concentration (MBC)

The inhibitory concentrations of macrophytes extracts in pathogens were all found to be bacteriostatic. The macrophytes extracts at certain concentrations inhibited the organism’s growth but it did not kill them. This happened due to the nature of pathogens possessing the resistance from their outer membrane, as they were all Gram-negative bacteria. The bactericidal effect was only observed on ciprofloxacin that was used as positive control. Its bactericidal effect was observed at 10 µg/µm in *E. coli, S. entetrica,* and *S. flexneri.* Even though the inhibitory effect of the macrophytes was bacteriostatic, these macrophytes have reduced the concentration of pathogens in the hydroponic system for wastewater treatment through antimicrobial compounds production.

4.5 Conclusion

The phytochemical compounds found in the macrophytes have influenced the inhibitory activity towards pathogens. This led to reduction of pathogens in the hydroponic system. The MIC findings revealed that *Bidens pilosa L* was more effective than *A. hybridus L*. The macrophytes used in the study showed the bacteriostatic effect in test pathogens of interest. This indicated that the macrophytes (phytochemicals) were able to inactivate the pathogens in the hydroponic system, which led to their reduction in the system. Although the macrophytes did not show the bactericidal effect on pathogens, the inhibitory activity reduced the microbial population in the effluent from the hydroponic system; thus, hypothesis was accepted. The antimicrobial compounds production by macrophytes for elimination of pathogens was proposed as one of the pathogens removal mechanism.

4.6 Recommendation

From the results and observations obtained from this Chapter, it can be recommended that phytochemicals be quantified. This information may be vital in correlating with the inhibitory activities of each macrophytes extract.

4.7 References

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CHAPTER 5

EXECUTIVE DISCUSSION AND CONCLUSION

5.1 Introduction

Microbial contamination is a major public health concern especially in developing countries. Disposal of contaminated water into natural water bodies creates waterborne pathogens reservoirs, where their consumption may lead to emergence of waterborne diseases outbreaks. The environmentally friendly and efficient technologies for removing pathogenic microorganisms in wastewater before disposal are needed (Mthembu *et al*., 2013; Wu *et al*., 2016).

Hydroponics are simple, cheap and easy to maintain constructed wetlands system technologies that have shown a great potential in the wastewater treatment (Haddad and Mizyed, 2011; Adrover *et al*., 2013; Lee and Lee, 2015). These are biological systems, that operate with the interactions of microorganisms, macrophytes, substrate and wastewater to treat wastewater though biological, chemical and physical processes in the rhizosphere (Bhatia and Goyal, 2014).

5.2 Pathogens Removal

Pathogens were removed in the system. All pathogens investigated in the system showed different levels of removal efficiencies. This clearly indicated that these organisms perfumed or acted differently in the system. The system composed of the substrate sand and gravel particles, where the sand influenced the physical mechanism of pathogens filtration in the system (Stottmeister *et al.*, 2003; Garcia *et al.,* 2013). The gravels provided a sufficient surface area for the microorganism’s attachment, growth and development which positively influenced the treatment processes by establishing the microflora of the system. The microflora created a consortium that competed with the pathogens in the system. This consortium defeated these enteric organisms and this led to their reduction in the system. The substrate in the system also promoted the growth of macrophytes through provision of roots attachment and development surfaces. Macrophytes optimized the pathogens removal processes in the system though provision of oxygen for microorganisms mediated processes and mediating different remediation processes. Apart from stimulating microbial activities, macrophytes also inhibited pathogens in the system through production of antimicrobial activity against pathogens. The synergistic effect of the above discussed processes caused the removal of pathogens in the system that ranged between 18 to 74% in the system.

5.3 Effect of Physiochemical Parameters on Pathogens Removal

The effect of temperature, pH, dissolved oxygen and chemical oxygen demand on pathogens removal was investigated in the hydroponic system. The system was situated in an open environment where its temperature depended on the day and night temperature variation. The samples were collected in July 2016. This system was made to mimic natural wetland. Temperatures ranged between 16 and 19^0C, these low temperatures negatively influenced the pathogens removal. The pH and dissolved oxygen also negatively affected the removal of pathogens in the system. The low temperatures decreased the oxygen diffusion rates which limited the aerobic reactions in the system that led to the removal of pathogens. However, the oxygen supply in the system was also maintained by macrophytes through the radial oxygen loss in roots exudates. Low temperatures did not limit the pathogens removal but rather it delayed the process because the high removal efficiencies in the system were achieved at prolonged hydraulic retention times. The levels of dissolved oxygen concentration in the system elevated as the pathogens were removed. This occurred as a result of the decrease of microbial population in the system with the macrophytes maintained the oxygen supply, even though the biological mechanisms might have used the dissolved oxygen. The chemical oxygen demand positively influenced the removal of pathogens in the system. The biological processes that led to pathogens removal were mediated by microbes used the organic matter in the system as a substrate. This led to decrease in the chemical oxygen demand concentrations as pathogens also decreased.

5.4 Antimicrobial Activity of Macrophytes

A macrophytes mediated pathogen removal mechanism was also investigated in the system. Macrophytes used in the hydroponic system had an ability to produce antimicrobial activity against pathogens and this also led to the removal of pathogens in the system. *Amaranthus hybridus* L inhibited the pathogens more than *Bidens pilosa L*, and this occurred due to their differences in phytochemical compositions.

5.5 Conclusion

The performance of the hydroponic system showed a great potential of removing pathogens in wastewater. The structural design, hydraulic conditions and ecological richness of the system made the hydropond an important wastewater treatment tool. Physiochemical parameters influenced the removal of pathogens in the system differently. High removal efficiencies were achieved at prolonged hydraulic retention times. The antimicrobial activity production by macrophytes was the pathogen removal mechanism that was confirmed in the system. This mechanism with other pathogens removal processes reduced pathogens in the system.

5.6 Recommendations

It is recommended that since this technology is relatively applied in other countries (China) due to their high success rate, therefore encouraging its application is important. There is also a need for more research and practical application of this technology in other types of wastewater (Industrial). There is also a need of optimization of physiochemical conditions in order to yield high removal efficiencies of pathogens. Studying of properties of different macrophytes is also a critical part on the optimization of such technologies. The understanding of hydroponic system complexes and dynamics based on exploring the microbial community structure and abundance.

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APPENDICES

Appendix 1: Physiochemical parameters in the hydroponic system during wastewater treatment

HRT	Temperature (^{0}C)		
0.	19.40	18.50	19.00
24.	17.34	18.01	16.99
48.	17.50	18.87	17.90
72.	17.50	17.72	16.50

Hydraulic Retention Time	pH		
0.	6.20	6.25	6.30
24.	7.07	7.05	6.80
48.	7.71	7.60	6.89
72.	7.66	7.54	6.90

Biological Oxygen Demand (BOD_5) (mg/L)

Days	0 hour	24 hours	48 hours	72 hours
1.	0.	0.	0.	0.
2.	0.	1.	2.	2.
3.	1.	2.	2.	2.
4.	2.	3.	3.	4.
5.	3.	4.	4.	5.

Appendix 2: Concentration of pathogens in the system during wastewater treatment

HRT	*E. coli* (CFU/ml)		*Salmonella spp.* (CFU/ml)		*Shigella spp.* (CFU/ml)	
0.	82.	97.	85.	99.	90.	89.
24.	142.	180.	102.	87.	58.	40.
48	120	150	80	50	50	35
72.	65.	49.	40.	33.	40.	30.

Appendix 3: Minimum inhibitory concentrations of the macrophytes on pathogens

Pathogen	*B.pilosa L stems* (mg/ml)	*B. pilosa L roots* (mg/ml)	*A. hybridus L stems* (mg/ml)	*A. hybridus* L roots (mg/ml)	Ciprofloxacin (µg/µm)
E. coli	25	25	0	25	1.25
S. enterica	12.5	50	0	0	10
S. flaxneri	25	50	0	25	10
S. sonnei	25	25	0	25	10