Analyses of the Impacts of Bacteriological Seepage Emanating from Pig Farming on the Natural Environment

by

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Submitted to the College of Agriculture and Environmental Sciences in fulfilment of the requirements for the degree of

MASTER OF SCIENCE

in the subject

ENVIRONMENTAL SCIENCES

at the

UNIVERSITY OF SOUTH AFRICA

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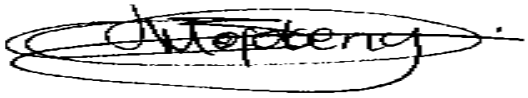
March 2014

Declaration

I declare that "Analyses of the Impacts of Bacteriological Seepage Emanating from Pig Farming on the Natural Environment" is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.



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Acknowledgements

The success of this work can be attributed to the assistance, guidance, and prayers of so many people who are hereby gratefully acknowledged. Firstly, I would like to give the Almighty God all the glory, honour and adoration for giving me the strength and wisdom through the Holy Spirit to complete this work.

To my supervisor: Dr. Olayinka Aiyegoro Ayobami, thank you for granting me the opportunity to learn high class science. I am sincerely grateful for your belief in me, your patience, support and encouragement and for allowing me to learn to the level I am today. I know it was not an easy road but it was worth it. You made it endurable throughout. I am grateful to you for everything, for assisting with all the work and the advices you gave really groomed me. Thank you for critically reading my research and finally for being patient with me, without which this thesis would not have been possible.

To Dr. Rasheed Adeleke: thank you so much for making me always feel at home at ARC-ISCW, your support and assistance is highly appreciated.

To the Soil health team in ARC-ISCW: to Cornelius, Maryam, Nomfundo, Maano and Rosinah. Thank you guys so much for all you taught me and for all the assistance in the lab you provided.

Thank you to ARC-API for funding my MSc studies and for granting me the opportunity to conduct my research in their institute. I am highly grateful.

To my Husband: Matladi Matjuda, honey thank you so much for all your support and encouragement you showed me. Even when I wanted to give up you were always there to give me strength and hope when I didn't have any left in me. Thank you so staying with me in the lab till late in the evening and even at weekends. For all that you are, I am blessed to have you in my life.

To my daughter: Mamello Matjuda, my little microbiologist, thank you so much for being my pillar of strength, thank you for not troubling mommy during my pregnancy. You grew up so fast I didn't even notice how time flew. Mommy loves you dearly.

To my family: The Mofokeng family: thank you for being a wonderful family to me. I really appreciate everything you did and have done for me, for giving me support, advice, and strength when I needed it the most. I am very grateful to all of you for supporting me emotionally,

spiritually, financially, and for all the long distances calls. Finally, for giving me a shoulder to cry on whenever I needed it, you are one in a million. The road we travelled together was not easy but it was worth every step. Through good times and the worst times, you have been there with me. I wouldn't have made it this far without you

To Mrs Elizabeth Mofokeng: Words can never describe how grateful and blessed I am to have a mother like you. You raised me up to be the woman I am today. You taught me that the first wisdom a woman should have is to fear God. I am what I am today because of you, you are my rock. Thank you for all the sacrifices you made in life to ensure that I become educated, for that I will forever be grateful to you. You are the world's best mom ever. Thank you for all your support and advices you gave me till now. Thank you for positioning me on the path of excellence and to challenge life when necessary. I have become who I am today because I am a product of your influence.

Dedications

I dedicate this thesis to my Husband, Victor Matladi Matjuda, my Daughter, Mamello Kganya Matjuda and my Mom, Elizabeth Mofokeng. Thank you so much for your love, encouragement and support. Your belief in me has allowed me to reach this point. Your constant encouragement and sustenance has enabled me to accomplish my dream. I love you always.

Table of Contents **Page**

List of Tables

List of Figures

Figures **Page**

List of Abbreviations

APHA	American Public Health Association
ARC	Agricultural Research Council
ARG	Antibiotic Resistance gene
Blast	Basic local alignment sequencing tool
BOD	Biological Oxygen Demand
cfu	Colony Forming Unit
CLSI	Clinical and Laboratory Standard Institute
cm	Centimetre
COD	Chemical Oxygen Demand
CW	Constructed Wetland
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide triphosphate
DO	Dissolved Oxygen
DWAF	Department of Water Affairs and Forestry
°C	Degree Celsius
EC	Electrical Conductivity
EDTA	Ethylenediaminetetra-acetate

EMB	Eosin methylene blue
g	Gram
HCl	Hydrochloric Acid
HNO_3	Nitric Acid
KOH	Potassium Hydroxide
L	Litre
m	Metre
mg	Milligram
mS	Micro Siemens
$MgCl_2$	Magnesium Chloride
µg	Microgram
µl	Microliter
µM	Micro Molar
NA	Nutrient agar
NO_2	Nitrite
NO_3	Nitrate
NTU	Nephelometric Turbidity Units
PCR	Polymerase Chain Reaction
PO4	Orthophosphate
RNA	Ribonucleic acid

rpm	Revolution per minute
TAE	Tris-acetic acid EDTA
TBE	Tris-boric acid EDTA
TDS	Total Dissolved Solids
TE	Tris EDTA
USDPIF	United State Department of Primary Industry and Fisheries
USGCRP	US Global Change Research Program
UV	Ultra Violet
vol	Volume
WRC	Water Research Council
XLD	Xylose lysine deoxycholate

Abstract

Modern pig farming production may over burden the environment with organic substances, exposure of bacterial pathogens and introduction of resistance gene. This may be caused by the pig's droppings, lack of seepage management or accidental spillage of seepage which may impact on the environment and its physicochemical parameters. The objective of this study is to determine and assess the level of bacteriological pollution emanating from the pig farm and their impact on the physicochemical parameters of soil and water as well as to identify the presence of antibiotic resistance gene of these prevailing bacteria. Soil and water samples were collected monthly for a period of six months (March- August 2013). Samples were collected at pig enclosures, soil 20 m and 100 m away from pig enclosures, constructed wetland used for treating pig farm wastewater, soil 20m and 100 m away from constructed wetland. Procedure followed for analysing soil and water samples includes physicochemical analyses, viable cell counts of 10^{-1} to 10^{-8} dilutions, identification of bacteria using API 20E test kit, antibiotic susceptibility analyses, and identification of resistance gene using molecular procedures. The media that were used for viable cell counts were, Nutrient agar, MacConkey Agar, Xylose Lysine Deoxycholate agar (XLD agar), and Eosin Methylene Blue (EMB). Physicochemical parameters of water showed unacceptable high levels of analysed parameters for BOD (163 mg/L to 3350 mg/L), TDS (0.77 g/L to 6.48 mg/L), COD (210 mg/L to 9400 mg/L), NO_3 (55 mg/L to 1680 mg/L), NO_2 (37.5 mg/L to 2730 mg/L), and PO_4^{3-} (50 mg/L to 1427 mg/L) were higher than the maximum permissible limits set by Department of Water Affairs and Forestry (DWAF) . For soil samples TDS (0.01g/L to 0.88 g/L), COD (40 mg/L to 304 mg/L), NO_3 (32.5 mg/L to 475 mg/L), and NO_2 (7.35 mg/L to 255 mg/L) and PO_4^{3-} (32.5 mg/L to 475 mg/L) were observed to be higher than recommended limits set by Federal Ministry for the Environmental (FME). The viable cells in soil samples 30cm depth ranged from 0 cfu/mL to 2.44 x 10^{10}cfu/mL, in soil 5cm depth ranged from 1.00 x 10^{1} cfu/mL to 1.91 x 10^{10} cfu/mL,

and in water samples viable cells ranged from 5.00 x 10^1 to 5.05 x 10^9. *Pseudomonas luteola (Ps. luteola), Escherichia vulneris (E. vulneris), Salmonella choleraesuis* spp *arizonae , Escherichia coli 1(E. coli 1),Enterobacter cloacae , Pseudomonas flourescens/putida (Ps. flourescens/putida), Enterobacter aerogenes , Serratia ordoriferal , Pasteurella pneumotropica , Ochrobactrum antropi , Proteus vulgaris group ,Proteus vulgaris , Salmonella* spp *, Aeromonas Hydrophila/caviae/sobria1 , Proteus Mirabillis , Vibrio fluvials , Rahnella aquatillis , Pseudomonas aeruginosa (Ps. aeruginosa), Burkholderia Cepacia , Stenotrophomonas maltophilia (St. maltophilia), Shwenella putrefaciens, Klebsiela pneumonia, Cedecea davisa, Serratia liquefaciens, Serratia plymuthica, Enterobacter sakaziki, Citrobacter braakii, Enterobacter amnigenus 2, Yersinia pestis, Serratia ficaria, Enterobacter gergoriae, Enterobacter amnigenus 1, Serratia marcescens, Raoutella terrigena, Hafnia alvei 1, Providencia rettgeri,* and *Pantoa* were isolated from soil and water samples from the pig farm. Isolates were highly resistant to Penicillin G, Sulphamethaxazole, Vancomycin, Tilmocozin, Oxytetracycline, Spectinomycin, Lincomycin, and Trimethoprim. The most resistance genes detected in most isolates were aa (*6')-le-aph (2")-la, aph (2")-lb, aph (3")-llla, Van A, Van B, Otr A* and *Otr B.* Pig farm seepage is causing bacterial pollution which is impacting negatively on the natural environment in the vicinity of pig farm by introducing bacterial pathogens that have an antibiotic resistance gene and is increasing the physicochemical parameters for soil and water in the natural environment at the pig farm.

It is therefore recommended that pig farms should consider the need to implement appropriate regulatory agencies that may include the regular monitoring of the qualities of final effluents from wastewater treatment facilities. In addition there is a need to limit soil pollution in order to safe guard the natural environment in the vicinity of pig farm from bacteriological pollution and introduction of antibiotic resistance gene. It is also recommended that more advanced technologies should be introduced that will assist pig farms to manages the seepage properly.

Chapter 1: Introduction

Agricultural activities overburdens the environment with organic substances from seepage mainly livestock droppings, heavy metals, fertilizers and pesticides (Mawdsley *et al.*, 1995). Mismanagement of seepage pollute the soil and water with nitrogen, phosphorus, bacteriological pathogens, and parasites, which may impact negatively on the environment (Ramı´rez *et al.*, 2004). Seepage is animal waste that may negatively impact on the environment and therefore may affect the health of plants and animals. Applying seepage to land is an effective way of disposing animal waste, and this solves the problem of animal waste disposal and also improves agricultural productivity. Unfortunately this can also introduce bacteria pollutants to the soil in the surrounding environment (Obasi *et al.*, 2008). Mass storage production of seepage may also be a serious hazard for biological balance of the environment (Mawdsley *et al.*, 1995). Bacteriological pollution of soil and water through agricultural practices usually has an overall effect on both animals and the natural environment (Toa *et al.*, 2010). Pollution caused by bacterial pathogens may cause numerous waterborne diseases, either as a result of ingestion or direct contact, or inhalation of contaminated aerosols (Tyrrel and Quinton, 2003).

Pollution is caused when a change in the physical, chemical, or biological condition in the environment is negatively affected by a contaminant (Forenshell, 2001). Potential sources of bacterial pollution include open feedlot pastures, treatment lagoons, manure storage, and also land application fields (Tyrrel and Quinton, 2003). Oxygen demanding substances such as ammonia, nutrients (particularly nitrogen and phosphorus), solids, pathogens, and odorous compounds are the pollutants most commonly associated with seepage (Zarnea, 1994). According to Madigan *et al.*, (2000), the physical and chemical seepage treatment process has

been developed to limit nitrogen and phosphorus pollution but these treatment processes do not eliminate microbial pollution. Pathogenic bacteria can be contained in treated seepage which can still pollute the soil and water systems through surface runoff, and leaching (Madigan *et al.,* 2000). These pathogens can reach drinking water sources through runoffs and they may have significant health risks to consumers by contaminating the environment and causing waterborne diseases (Madigan *et al.,* 2000).

Environmental contamination by seepage can be associated with heavy disease burden and the assessment of seepage is very important to safeguard the environment (Okoh *et al.,* 2007). Monitoring the physicochemical parameters of soil and water systems is important to safely assess the degree of environmental contamination (Morrison *et al.*, 2001). Seepage discharge or spillage is a major component of water pollution that contributes to oxygen demand, nutrient loading, promotes toxicity, algal blooms which leads to the destabilization of the environment (Morrison *et al.*, 2001).

Agricultural run-offs or accidental spillage of seepage are very important sources of bacterial pollution in waterways and soil (Tyrrel and Quinton, 2003). Runoff and seepage discharges can also contribute both organic and inorganic nutrients that are known to encourage the growth and proliferation of indigenous or introduced pathogens (Tyrrel and Quinton, 2003). As seepage application can increase a number of faecal bacterial pathogens in the soil, pig farm seepage may also represent a significant risk to the environment (Madigan *et al.*, 2000).

Faecal pathogens that are of environmental concern and that may be detected in seepage includes *Escherichia coli* O157:H7, *Salmonella* spp., *Campylobacter* spp., *Shigella* spp., *Giardia lamblia*, *Cryptosporidium parvum,* and *Vibrio Cholerae* (Obasi *et al.*, 2008). These pathogens can have a serious impact on the biosphere, the physicochemical parameters of soils and water and can also impact on the quality of the soil and water (Obasi *et al.*, 2008). Bacterial

pollution may also introduce a bacterial pathogen that possesses an antibiotic resistance gene (ARG).

Antibiotics are commonly used in pig farms to treat infections, diseases and to promote growth (Kumar *et al*., 2005). Unfortunately the use of this antibiotics continuously can lead to the development of resistance gene in bacteria species (Kumar *et al*., 2005). Antibiotics are poorly adsorbed into the gut of farm animals and therefore can be exposed to natural faunas and floras of soil through faeces and urine of pigs and in soil through surface runoff (Kumar *et al*., 2005).

Previous studies reported that seepage discharge or spillage can lead to the potential spread of bacteria that harbour antibiotic resistance gene in the environment (Ghosh and Lapara, 2007). This bacteria can transfer these resistance determinants to the natural microorganism in the soil by lateral gene transfer (Ghosh and Lapara, 2007). Moreover apart from lateral gene transfer, antibiotic resistance genes can be transferred between pathogens and non-pathogens, and distantly related organisms such as gram-positive and gram-negative bacteria by horizontal gene transfer where this is selective pressure in the environment (Kruse and Sorum, 1994).

The rapid growth of antimicrobial agents in the environment due to the widespread use in pig farms emphasizes the need for intervention (Roberts, 2005). Resistance to penicillin, emanating from pig farm soil and seepage, was reported to be as high as 79 % in China and the rise of antibiotic resistance is considered to be closely linked to the widespread use of antibiotic pharmaceuticals in animals and antibiotic residues that are excreted in an unaltered state (Roberts, 2005). Some of the antibiotic residues can be removed by treatment plants, given that the treatment plants are not designed for the treatment of micro pollutants, residual antibiotics can be released into the environment and they may exert selection pressure on natural soil and aquatic microorganisms (Kruse and Sorum, 1994)

Chen *et al*., (2010) and Zhu *et al*., (2013) reported that bacteria with lincomycin resistance gene (*InuA* and *InuE*) were mostly found in soil and water adjacent to pig farms in China. Despite this few investigations have searched for the lincomycin- resistance genes (especially *Inu* genes) in the environment of pig farms. Vancomycin resistance in the environment is a clinical concern and *VanA, VanB* and *VanC* resistance genes can be distinguished on the basis of the level and inducibility of resistance to vancomycin (Schwartz *et al*., 2002).

Tetracycline and sulphonamides are widely used in pig farms, and therefore tetracycline (*Tet*) and sulphonamide (*sul*) resistant bacteria have been detected in soil and water in pig farms because of their persistence in the environment (Luo *et al*., 2010; Gao *et al*., 2012). Zhu *et al*., (2013) assessed the concentration of antibiotics in pig farm seepage and detected 149 unique resistance genes and amongst the detected resistance genes *tet* and *sul* genes were observed to be the most prevailing in the environment.

In addition other studies designed to detect tetracycline genes detected eight genes i.e. *tetQ, tetO, tetW, tetM, tetB, tetS, tetT, and OtrA* in two pig farm seepage and subsurface soil (Chee-Sanford *et al.*, 2001). McKinney *et al*., (2010) also detected tetracycline *tetO* and *tetW* and *sul1* and *sul2* resistance genes in pig farm seepage. Smith *et al*., (2004) and Wu *et al*., (2010) also observed a significant correlation between antibiotic resistant bacteria on soil and water in pig farms. Few studies have however been conducted to evaluate the diversity of *tet* and *sul* resistance genes in pig farm seepage which facilitate the transfer of ARGs from faecal microorganisms to indigenous environmental bacteria through continuous seepage application (Wu *et al*., 2010). The pathogenic bacteria *Ps. aeruginosa, Stenotrophomonas maltophilia, Burkholderia cepacia, Acinetobacter* spp, *Achromobacter xylosoxidans, Serratia marcescens* and *Aeromonas* spp are intrinsically resistant to penicillin and the β-lactam antibiotics (Wu *et al*., 2010).

Water is an elixir of life and it governs the evolution and the function of the universe on earth (Muhibbu-din *et al.*, 2011). Pollution of water can seriously affect the biosphere, with negative impact on the aquatic life forms, ranging from microorganisms to insects, birds, fish, and at the same time, the health of the terrestrial animals and plants (Yates and Yates, 1988). In addition, bacteriological pollution also affects potential of using the soil and water by both humans and animals (Landry and Wolfe, 1999), and their physicochemical parameters might have been affected or drifted from acceptable standard reference range.

The physicochemical parameters of soil and water that may be affected by bacterial pollution includes pH, temperature, electrical conductivity (EC), salinity, turbidity, total dissolved solids (TDS), dissolved oxygen (DO), chemical oxygen demand (COD), nitrate (NO_3), nitrite (NO_2), and orthophosphate (PO_4^{3-}) levels (Morrison *et al.*, 2001). Surrounding environments in the vicinity of pig farms may be contaminated due to faecal residues, seepage runoff and mismanagement of pig farm seepage and this can cause a threat to rivers, lakes and land surrounding the farms, with a significantly high contamination potential for groundwater (Landry and Wolfe, 1999). Depending on the nature and intensity of bacteriological pollution of soil and water; soil and water availability for any purpose (e.g. physiologic, hygienic, industrial, or recreation) may be diminished or annulled (Madigan *et al.*, 2000).

The objective of this project was to determine and assess the level of bacteriological pollution emanating from the pig farm and their impact on the physicochemical parameters of soil and water as well as to identify the presence of antibiotic resistance genes in the bacteria species isolated

Chapter 2: Literature review

Wastewater is any water that has been adversely affected in quality by anthropogenic influence and may contain, pathogens, organic particles, soluble organic materials, inorganic particles, soluble inorganic materials, animals (protozoa, insects, arthropods, small fish, etc.), gases, toxins, and pharmaceuticals and other hormones etc. (Bohdziewicz and Sroka, 2005).Wastewater in agriculture is mainly used for irrigation which if done improperly can cause different degrees of environmental nuisance and cause hazard contamination due to their compromised chemical and microbiological characteristics (Bohdziewicz and Sroka, 2005). With the high concentration of bacterial pathogens that the seepage contains, if it accidentally flows into the environment it may cause bacteriological contamination (pollution) by introducing pathogens into the natural environment (Toa *et al.*, 2010).

Bacteriological pollution is defined as contamination of water, soil and air with bacterial pathogen (Toa *et al.*, 2010). Bacteria pollution may impact negatively on the environment and can have detrimental consequences both economically and with human health. Environmental impacts caused by bacterial pollution includes:

- Impair soil nutrient cycling
- Alters decomposition rates by competing with indigenous soil and aquatic bacteria
- Renders vegetation unsuitable for grazing
- Renders rivers and streams unsuitable for recreation
- Renders natural environment unsuitable as a habitat.

The treatment of seepage is very important to safe guard the environment. Many agricultural sectors have developed treatment systems for the treatment of seepage which includes the use of constructed wetland (González *et al.*, 2009). Agricultural sectors and most pig farms use

constructed wetlands to treat their seepage because they are cheap and easy to operate. Constructed wetlands are treatment systems that use natural processes involving wetland vegetation, soils, and their associated microbial assemblages to improve water quality (González *et al.*, 2009). A constructed wetland system (CWS) pre-treats wastewater by filtration, settling, and bacterial decomposition in a natural-looking lined marsh (González *et al.,* 2009). Even though the advantages of using constructed wetlands for seepage treatment is widely published, constructed wetland can have negative impacts on the environment.

2.1 Impacts of constructed wetlands on the environment

2.1.1 Poor Phosphorus Removal

The removal of phosphorus in most constructed wetland systems is not very effective because of the limited contact opportunities between the wastewater and the soil (Choundhary *et al.*, 2011). The reasons for this are not entirely clear, but may be related to reduce oxygenation of the root zone resulting in slow-moving waters (Choundhary *et al.*, 2011). Although constructed wetlands may be able to remove Biological Oxygen Demand (BOD), suspended solids, and nitrogen compounds with reasonable effectiveness, they may not remove phosphorus properly (Greenway and Woolley, 2001). Where phosphorus enrichment is a concern (because of the potential for eutrophication) only a small proportion of phosphate removal by constructed wetlands can be attributed to nutritional uptake by bacteria, fungi and alga (Mortsch, 1998). Choundhary *et al.,* (2011) in their study made a comparison of phosphorus removal efficiency of two large-scale, surface flow constructed wetland systems where phosphorus adsorption indicated that for the first two months of wetland operation, the mean phosphorus removal efficiency of system 1 and system 2 were 38 percent (%) and 22 percent (%), respectively. With constructed wetland removal efficiencies for phosphorus occurs during the first year of operation and during the second year of operation more phosphorus is realised than was put in.

Greenway and Woolley (2001) reported that there were indications of phosphorus reductions during the early stages of constructed wetland operation but over time export of phosphorus, particularly on a concentration basis occurred where there was an initial average reduction in concentration of 55% phosphorus removal during the first six months, reducing to 8 % for the next ten months after which concentration in the effluent always exceed the influent.

2.1.2 Climate change

Constructed wetlands do not work effectively in cold weathers and in cold seasons because bacteria and plants living in the constructed wetland's soil die back, thus slowing or stopping nutrients removal during hard cold weather (Mortsch, 1998). This can cause substantial release as the organism previously removing and storing this nutrients died due to cold weather, thus causing them to release their own nutrients back into the system and thus impacting on the nearby streams and soil (Mortsch, 1998). In spring and during summer thunderstorms and excessive rainfall can cause floods in constructed wetland thus decreasing its treatment effectiveness. Mortsch (1998) had made an analysis on the influence of climate change on the ecology of wetland and hydrological characteristics with the shoreline of the Great Lakes as the subject, while Dawson *et al.*, (2003) had carried out the water-balance analysis in application of climate change scenario. As a result of their research, it was analysed that there was a change in water-balance of wetland due to climate changes, and also, there was a change in diversity of species of the living things due to changes in environment of habitats because of the changes in water-balance. Erwin (2009) in his study also explained that the environment of wetland habitats is influenced by discharge and water quality, and such discharge and water quality are influenced by climate change, causing changes in the environment of wetland habitats. Pressures on wetlands are likely to be mediated through changes in hydrology, direct and indirect effects of changes in temperatures, as well as land use change (Ferrati *et al.*, 2005).

Impact of climate exerted on constructed wetland includes (Ferrati *et al.*, 2005) :

- change in base flows;
- altered hydrology (depth and hydro-period);
- extended range and activity of some pest and disease vectors;
- increased flooding,
- landslide, avalanche, and mudslide damage;
- increased soil erosion;
- increased flood runoff resulting in a decrease in recharge of some floodplain aquifers;
- decreased water resource quantity and quality and ;
- increased damage to coastal ecosystems such as coral reefs and mangroves

Under currently predicted future climate scenarios, the spread of exotics in constructed wetlands will probably be enhanced, which could increase pressure on watersheds and ecosystems (Root *et al.*, 2003). Other variables related to impacts of climate change on constructed wetlands which may play a very important roles in determining the regional and local impacts, including increased temperature and altered evapotranspiration, altered biogeochemistry, altered amounts and patterns of suspended sediment loadings, fire, oxidation of organic sediments and the physical effects of wave energy (Burkett and Kusler, 2000).

2.1.3 **Creation of Toxic Wetlands**

As constructed wetlands accumulate sediments in their system, they can also accumulate the many pollutants that have a natural affinity for solids (Moorhead and Reddy, 1988). These pollutants may include some forms of phosphorus, many heavy metals, some trace organic pollutants and microorganisms (Burkett and Kusler, 2000). Plants can oxidize the sediments in the root zone through the movement of oxygen downwards through parenchyma tissue and this oxidation can remobilize the metal contaminants, thus increasing the otherwise low availability

of metals in wetland sediment. When a constructed wetland is retired at the end of its useful life, it is not an inert or innocuous component of the environment but rather a hazardous waste disposal site and this can cause the pollutants to accumulate at a high concentration and can become toxic to surrounding soil, streams, surface water and ground water (Kadlec, 2000).

2.1.4. **Greenhouse gases emission**

The constructed wetlands produce greenhouse gases due to decomposition of harmful solids and compounds which severely harm the atmosphere (Kadlec, 2000). The wetlands containing nitrate contaminated water could release a significant amount of nitrite based on the quantity of nitrate (Kadlec, 2000). Gasses such as carbon dioxide ($CO_{2)}$, methane (CH_4), and nitrogen oxide (N_2O) are still released from constructed wetlands because of the activity of anaerobic microorganisms (Wright and Reddy, 2009)

Seepage from constructed wetlands may overflow partially untreated and thus expose pollutants such as organic matter, metals, and bacterial pathogens to the receiving environment (Ferguson *et al.,* 2003). The bacteria that is introduced to the environment can be transmitted to subsurface, groundwater and rivers and streams. When bacteria are introduced into the environment due to bacterial pollution caused by seepage they can be transmitted to the subsurface and groundwater thus causing even further pollution (Hurst *et al.,* 1980).

The presence of bacterial pollution in the environment is taken as a sign of faecal contamination due to seepage (Ferguson *et al.*, 2003). Bacteria can be transmitted in the environment through water (surface and ground water), soil, and air. Seepage can be deposited on land surfaces and can release pathogens into surface waters via storm water runoff or collected wastes can be discharged directly into a waterway (Ferguson *et al.,* 2003). The runoff or discharge may go directly into the growing area or indirectly as is the case with wastes transported by freshwater streams to estuarine or marine waters (Ferguson *et al.,* 2003). Pathogens can also enter the soil

through infiltration and surface run-off depending on the size of the soil particles (Ferguson *et al.*, 2003).

2.2 Transport of bacteria in soil and water

The major transport modes of pathogens in soils are through movement with infiltrating water and surface run-off and with the movement of sediments and waste particles (Hurst *et al.*, 1980). Suspended particles, including bacteria which become deposited at the soil surface, can act as a filter trapping more bacteria. In unsaturated soils, bacteria can travel with mobile water and their cells can interact with air or solid phase, which result in temporary or permanent immobilization. In such conditions bacteria can also be entrapped in stagnant pore water between gas bubbles (Hurst *et al.*, 1980). During rain events, connectivity between mobile and immobile water increases, allowing bacteria to migrate with the advancing wetting front. During rainy seasons the bacteria can reach the ground water through absorption and infiltration and thus contaminate the groundwater, they can also be transmitted through air by dust (Hurst *et al.*, 1980).

Another mode of transmission is through air by dust particles. The bacteria are also able to attach themselves to soil particles and thus can be carried away when wind occurs (Hurst *et al.*, 1980). Other transmission includes contact with live-stock animal waste; swimming in contaminated water impacted by animal faeces and exposure to potential vectors (such as flies, mosquitos, waterfowl, and rodents) (Hurst *et al.*, 1980). Once bacterial contamination of soil, surface and ground water has occurred the bacteria can have negative impacts on the environment and can also impact the indigenous fauna and flora of both the soil and water (Hurst *et al.*, 1980).

In general, the hydrologic transport of wastewater bacterial pathogens with runoff is influenced by several dynamic and interacting factors (Santamaria and Toranzos, 2003). Antecedent soil

water content or the extent of soil saturation at the time of a rain event, for example, will affect the quantity of runoff. Runoff from a dry soil will be less than runoff from the same soil when it is saturated (Santamaria and Toranzos, 2003). In addition, runoff is affected by soil type as defined by soil profile, textural class (sandy, loam, silt, or clay), hydraulic conductivity, and infiltration rate, as well as vegetative cover, rain intensity and distribution, management practices such as no-till versus conventional till, and topography (Santamaria and Toranzos, 2003).

Waste water pathogens in runoff are further affected by deposition and re-suspension. Both affect pathogen retention by soil and sediment as well as by release into surface water and ground water. Bacterial pathogens can be transported in soil and thus reaching the groundwater and it is dependent on factors influencing their transport. The major transport modes of pathogens in soils are through movement with infiltrating water and surface run-off and with the movement of sediments and waste particles (Reddy *et al.*, 1981). Suspended particles, including bacteria which become deposited at the soil surface, can act as a filter trapping more bacteria (Corapcioglu and Haridas, 1984). The rapid movement of microorganisms in soil is facilitated by root channels, earthworm channels, and naturally occurring cracks. Bacteria are also retained in saturated soils than unsaturated soils (Corapcioglu and Haridas, 1984).

In unsaturated soils, bacteria can travel with mobile water and their cells can interact with air or solid phase, which result in temporary or permanent immobilization. In such conditions bacteria can also be entrapped in stagnant pore water between gas bubbles (Hurst *et al.*, 1980). During rain events, connectivity between mobile and immobile water increases, allowing bacteria to migrate with the advancing wetting front. During rainy seasons the bacteria can reach the ground water through absorption and infiltration and thus contaminate the groundwater, and they can also be transmitted through air by dust (Hurst *et al.*, 1980).

Bacterial movement in soil is dependent on the water saturation state (Santamaria and Toranzos, 2003). Bacteria move rapidly under saturated conditions, but only for a few centimetres, because bacteria are in close contact with soil particles. When soil is saturated all pores are open and thus allow bacteria to pass through the soil.

The availability of bacterial pathogens at the soil surfaces during rainfall events is depended on several factors and processes (Reddy *et al.*, 1981). Those important processes include

- The die-off rate/ survival rate of pathogens on the soil system
- The soil adsorption or retention of bacterial pathogens

Bacterial contamination of soil and subsequent entry into a water supply is dependent on survival of the organisms during residence time in soil and likelihood of being washed out by storm water runoff (Santamaria and Toranzos, 2003). As soil pores become increasingly water filled, bacteria may find themselves in an anoxic or at least micro-aerophilic environment (Griffing, 1981) and for obliged aerobes this will probably result in decreased viability and survival. Studies have shown that the movement of bacterial pathogens is increased in saturated soils. Water is drained from pores as matric potential falls and hence water content together with pore size will determine the ability of bacteria pathogens to move through soil whether by active movement or by Brownian movement with result generally indicating increased movement in saturated soil (Mawdsley *et al.*, 1995). Ground water in most times is considered the most uncontaminated water source as it feeds rivers, streams, oceans, dams and wells with water. Once the bacterial pathogen is able to reach the groundwater, it will contaminate a large number of water systems soil (Mawdsley *et al.*, 1995).

2.3 Survival of bacteria in the environment

When pathogenic bacteria is introduced into the environment due to bacterial pollution caused by seepage, it adapts in order to survive (Hurst *et al.,* 1980). The ability of this bacteria to survive harsh environmental conditions is dependent on several factors (Table 2.1).The survival of bacterial pathogens in the environment differs from bacteria to bacteria and they can range from 30 minutes to several years (Reddy *et al.*, 1981). Once the bacterial is exposed to the environment, the factors that impact in their survivability includes pH, sunlight, temperature, moisture, salinity, predation, and sediment, antibiotics, toxic substances, competitive organism, available nutrients, and soil type.

2.3.1 pH

The viability of many bacterial pathogens depend on the pH of the environmental aerosol (Hurst *et al.*, 1980). Most natural environments have pH value in the range of 5 to 9 and few microorganisms can grow below pH 4 or above pH 10, but both high and low pH are known to decrease the survival of most bacterial pathogens (Hurst *et al.*, 1980; Reddy *et al.*, 1981). Thus pH affects the absorption of characteristic of cells, so inactivation in acidic environments is lower (Hurst *et al.,* 1980). Increase in cations concentration also result in increased absorption rates consequently affecting microbial survival (Reddy *et al.*, 1981). However both biological and physicochemical properties of environmental aerosols are affected by pH and in this turn will affect the survival and transport of bacterial pathogens, but the pH measurement of environmental aerosol reflects only for its bulk pH and not of those individual microenvironments (Reddy *et al.*, 1981). Within the environment spatial variation in pH will influence the survival and transport of bacterial pathogens.

2.3.2 Sunlight

Ultraviolet (UV) light is known to damage the nucleic acids of the bacterial cell (Sinton *et al.*, 1994). Even though UV light can damage the nucleic acids of the bacterial cell, some bacteria are capable of limited repair damaged nucleic acids (Sinton *et al.*, 1994). Bacterial pathogens are able to survive in the environment by avoiding direct exposure to UV light, either by remaining below the soil/ sediment surface or by remaining at lower depths in the water column, thus in doing that the bacteria increases its chance of survival (Sinton *et al.*, 1994).

2.3.3 Temperature

Temperature plays a big role in the growth of bacteria thus the survivability of bacterial pathogen it inversely related temperature, i.e. with every ten degrees Celsius (°C) rise in temperature the die-off rate (Survival rate) of bacterial pathogen doubles. The relationship between die-off rate and temperature has been studied by several researchers which also includes McFeters and Stuart (1972) and Crane *et al.*, (1980). Thus bacteria survive longer at lower temperatures (Sinton *et al.*, 1994).

2.3.4 Moisture

Bacteria are able to survive longer in moist environments than in dry environments (Sinton *et al.*, 1994). Moisture contents of soil is very important and it is known that bacteria are able to survive more in saturated soils that unsaturated soils (McFeters and Stuart, 1972)

2.3.5 Soil type

Other soils like clay soil favours the absorption of bacteria and thus reduces the die-off rate by protecting bacterial cells and by creating a barrier against microbial predators and parasites (Mawdsley *et al.*, 1995). Thus the survival rate of bacteria is low in sandy soil as it has low water-holding capacity.

2.3.6 Soluble organics

Increases survival and possible regrowth when sufficient amounts of organic matter are present (Mawdsley *et al.,* 1995) anaerobically on nitrate or other alternative electron acceptors. *Ps. aeruginosa* can catabolize a wide range of organic molecules, including organic compounds such as benzoate which makes *Ps. aeruginosa* a very ubiquitous microorganism, for it has been found in environments

Table 2.1: Factors influencing the survival of bacteria in the subsurface

Factors influencing the survival of bacteria in the subsurface		
Physiochemical Characteristics of Soil	**Atmospheric Conditions**	**Biological Interactions**
a) pH	a) sunlight	a) competition
b) porosity	b) moisture	b) antibiotics
c) organic matter	c) temperature	c) toxic substances
d) texture		
e) temperature		
f) moisture		
g) adsorption/ filtration		
I) nutrients		

Cited from Warnemuende and Kanwar (2002)

2.4 Bacteria associated with pollution seepage from pig farms

The bacterial that are associated with pig waste seepage includes *Salmonella, Campylobacter, E. coli, Proteus, Giardia, Klebsiella, Shigella* and *Chlamydia, Enterobacter aerogenes,* and *Ps. aeruginosa* etc. (Ryan and Ray, 2004).

Campylobacter

Campylobacter (meaning 'twisted bacteria') is a genus of bacteria that are Gram-negative, spiral, and micro-aerophilic. Motile, with either unipolar or bipolar flagella, the organisms have a characteristic spiral/corkscrew appearance and are oxidase-positive (Ryan and Ray, 2004). *Campylobacter jejuni* is now recognized as one of the main causes of bacterial foodborne disease in many developed countries (Moore *et al.*, 2005). *C. fetus* is a cause of spontaneous abortions in cattle and sheep, *C. jejuni* is routinely associated with diarrheal disease; however, *C. coli* , distinguished from *C. jejuni* on the basis of hippurate hydrolysis, is occasionally isolated from diarrheic animals and is routinely recovered from asymptomatic pigs (Ryan and Ray, 2004).

Campylobacteriosis is an infection that is caused by *Campylobacter* (Humphrey *et al.*, 2007). The common routes of transmission are faecal-oral, ingestion of contaminated food or water, and the eating of raw meat. It produces an inflammatory, sometimes bloody, diarrhoea, periodontitis (Humphrey *et al.*, 2007) or dysentery syndrome, mostly including cramps, fever and pain. The infection is usually self-limiting and in most cases, symptomatic treatment by liquid and electrolyte replacement is enough in human infections. The use of antibiotics, on the other hand, is controversial. Symptoms typically last for five to seven days (Humphrey *et al.*, 2007).

Salmonella

Salmonella is a genus of rod-shaped, Gram-negative, non-spore-forming, predominantly motile enterobacteria with diameters around 0.7 micro metre (µm) to 1.5 micro metre (µm), lengths from 2 µm to 5 µm, and flagella and they are chemoorganotrophs, obtaining their energy from oxidation and reduction reactions using organic sources, and are facultative anaerobes, (i.e. peritrichous) (Ryan and Ray, 2004). Most species produce hydrogen sulphide which can

readily be detected by growing them on media containing ferrous sulphate and most isolates exist in two phases: a motile phase and a nonmotile phase (Clark and Barret, 1987). Cultures that are nonmotile upon primary culture may be switched to the motile phase using a Craggier tube (Clark and Barret, 1987). *Salmonella* is closely related to the Escherichia genus and are found worldwide in cold- and warm-blooded animals (including humans), and in the environment. They cause illnesses like typhoid fever, paratyphoid fever, and foodborne illness (Ryan and Ray, 2004).

Shigella

Shigella is a genus of Gram-negative, nonspore forming, non-motile, rod-shaped bacteria closely related to *Escherichia coli* and *Salmonella* (Ryan and Ray, 2004). *Shigella* infection is typically via ingestion (faecal–oral contamination) and depending on age the conditions of the host, less than 100 bacterial cells can be enough to cause an infection (Levinson and Warren, 2006). *Shigella* causes dysentery that result in the destruction of the epithelial cells of the intestinal mucosa in the cecum and rectum. Some strains produce enterotoxin and *Shigella* toxins, similar to the verotoxin of *E. coli O157:H7* and other verotoxin-producing *Escherichia coli* (Hale *et al.*, 1996).

Klebsiella

The *Klebsiella* genus is part of the Enterobacteriaceae family and are nonmotile, rod-shaped, gram negative bacteria with a major polysaccharide capsule which cover the entire cell surface providing protection against most host defence mechanisms (Ryan and Ray, 2004). *Klebsiella* genus generally display two types of antigens on the surface of the cell, lipopolysaccharide (O antigen) and capsular polysaccharide (K antigen), both contributing to pathogenicity (Ryan and Ray, 2004).

Enterobacter aerogenes

Enterobacter aerogenes is a Gram-negative, oxidase negative, catalase positive, citrate positive, indole negative, rod-shaped bacterium that is a nosocomial and pathogenic bacterium that causes opportunistic infections including most types of infections (Ryan and Ray, 2004). The majority are sensitive to most antibiotics designed for this bacteria class which can be complicated by their inducible resistance mechanisms, particularly lactamase which means that they quickly become resistant to standard antibiotics during treatment, requiring change in antibiotic to avoid worsening of the sepsis (Ryan and Ray, 2004). *E. aerogenes* is generally found in the warm blooded animal's gastrointestinal tract and does not generally cause disease in healthy individuals and has also been found to live in various wastes (Ryan and Ray, 2004).

Proteus spp

Proteus spp. are part of the Enterobacteriaceae family of gram-negative Bacilli which are oxidase-negative, but catalase- and nitrate-positive (Ryan and Ray, 2004). *Proteus* organisms are implicated as serious causes of infections in animals and humans, along with *Escherichia, Klebsiella, Enterobacter,* and *Serratia* spp. *Proteus* spp are most commonly found in the intestinal tract as part of normal intestinal flora, along with *Escherichia coli* and *Klebsiella* spp, of which *E. coli* is the predominant resident (Ryan and Ray, 2004).

Ps. aeruginosa.

Ps. aeruginosa is a Gamma Proteobacteria class of bacteria which is a gram-negative, aerobic rod (about 1 µm -5 µm long and 0.5 µm -1.0 µm wide, asporogenous, and monoflagellated bacterium that has an incredible nutritional versatility (Ryan and Ray, 2004). *Ps. aeruginosa* is an obligate respire, using aerobic respiration (with oxygen) as its optimal metabolism although can also respire such as soil, water, animals, plants, sewage (Ryan and Ray, 2004).

Pseudomonas luteola (Ps. luteola)

Ps. luteola is formerly known as *Chryseomonas luteola*, is a motile, strictly aerobic, gram-negative rod, nonfermentative, oxidase negative, and catalase positive producing a distinct yellow-orange pigment (Anzai *et al.*, 1997). *Ps. luteola* has been isolated from many water, soil, and damp environments and is considered to be a saprophyte or commensal organism only rarely pathogenic to humans (Casalta *et al.*, 2005) Clinical infections due to *Ps. luteola* microorganism have rarely been reported and mostly includes septicaemia, meningitis, peritonitis, endocarditis, and ulcer infections (Casalta *et al.*, 2005) and has been shown to be susceptible to extended-spectrum cephalosporins, aminoglycosides, and fluoroquinolones (Hawkins *et al.*, 1991). When tested with a large panel of β-lactam antibiotics, resistance to original-spectrum and broad-spectrum cephalosporins was observed, whereas susceptibility to penicillins was variable (Hawkins *et al.*, 1991) thus suggesting that this microbe may produce a natural β-lactamase gene.

Escherichia vulneris

Escherichia vulneris is a species of gram-negative bacteria in the same genus as *E. coli*., *E. vulneris* is a gram negative, fermentative, oxidase negative, motile rod made motile by peritrichous flagella, which holds characteristics similar to that of the order Enterobacter and family Enterobacteriaceae (Hawkins *et al.*, 1991). *E. vulneris* is susceptible to 14 antibiotics, including third generation cephalosporins, aminoglycosides, trimethoprim, and Sulphamethaxazole-trimethoprim and they also have some type of resistance to the antibiotics such as penicillin and clindamycin, and were also marginally resistant to carbenicillin, erythromycin, tetracycline, chloramphenicol and nitrofurantoin (Hawkins *et al.*, 1991).

2.5 **Antibiotics and resistance gene in the environment**

The bacteria that is introduced into the environment may contain antimicrobial resistance gene due to the vast uses of antibiotics on pig farms (Cheng *et al.,* 2013). The use of antibiotics agents can also cause overgrowth of a bacterial strain that has a gene expressing a resistance to the antibiotic agent and this can result in assembly and evolution of complex genetic vectors encoding, expressing, linking, and spreading the bacteria and other resistance genes (Cheng *et al.,* 2013). The strain of a bacteria that is resistant to an antibiotic differs from that of a bacteria that comprises of susceptible strains by being able to make a specific protein that inactivates the agent or otherwise circumvents the agent's damaging effect on bacteria and that specific protein is expressed by a resistance gene (Cheng *et al.,* 2013).

In commercial livestock production, antibiotics are used for (Hawkins *et al.,* 1991):

- therapeutically to treat existing disease conditions,
- prophylactically at sub therapeutic doses to mitigate infection by bacterial pathogens of livestock animals undergoing high stress situations, and
- Sub-therapeutically to enhance growth. Antibiotics such as tetracycline in pig farms are not only used to treat and prevent diseases but also for growth promotion.

Many antibiotics are not completely absorbed in the gut, resulting in the excretion of the parent compound and its fragmented metabolites (Boxall *et al.,* 2004). Elmund *et al.,* (1971) in their study have estimated that as much as 75 % of the antibiotics administered to feedlot animals could be excreted into the environment.

Antibiotic exposed to the environment can cause a wide spread of pools of antibiotic resistance across a wide range of microorganism (Boxall *et al.,* 2004). The presence of a resistance gene in the environment can also be transferred to a natural soil microbial via genetic transfer. Antibiotics such as sulphamethaxazole can affect nitrification bacteria which may also affect

soil fertility (Cheng *et al.*, 2013). Presence of antibiotics and resistance genes may hold a threat to the natural environment and presents a hazard to the aquatic and soil organisms (Yang *et al.*, 2004). Persistence of antibiotics and resistance gene may occur for a long period. Persistence of antibiotics in the terrestrial environment is a key factor in determining their adverse environmental impact (Cheng *et al.*, 2013). Antibiotic persistence in the terrestrial environment depends not only on the antibiotic properties but also on the soil properties and weather conditions (Cheng *et al.*, 2013). With regards to their persistence, the important antibiotic properties are photo-stability, binding, and adsorption to soil solids, biodegradation, and water solubility. Accurate and meaningful information on the persistence and dissemination of antibiotic resistance genes in bacteria is of fundamental importance in assessing potential health risks and environmental quality (Boxall *et al.*, 2004).

Microbial resistance traits once the resistance gene has been acquired can be rapidly transferred vertically through division of the host cell, and/or horizontally between different bacteria (both commercial and pathogenic) via transduction (a bacteriophage- mediated process), conjugation/ mobilization (requiring contact between donor and recipient cell), or transformation (transfer of free DNA into competent recipient cell) (Kelly *et al.*, 1986). Figure 2.1 shows transfer of genetic material through transformation, translation, and conjugation. In the mixed bacteria population of animals, conjugation and mobilization are considered to be of primary importance for the spread of resistance gene (Schwartz and Chaslus- Dancla, 2001). Transduction only happen between similar species and genera as it is limited by host-specificity of bacteriophages, and therefore plays a lesser role in the spread of resistant traits in the milieus (Bennet, 1995).

In the horizontal transfer the primary genetic element involved include plasmids, transposons, and intergrons gene cassettes (Bennet, 1995). Aside from the antimicrobial resistance traits, plasmids and transposons may also carry genes (such as the trans -gene complex) which allow

them to move from one bacterial cell to another via conjugation or mobilization and plasmids may serve as vectors for transposons and intergrons/ genes cassettes facilitating their horizontal transfer to component cell (Bennet, 1995). Transposons and intergrons genes cassettes can be transferred via transduction when resistance gene are co-located with pro-phage genes that are not exercised precisely from chromosomal DNA prior to packing into phage cells (Bennet, 1995).

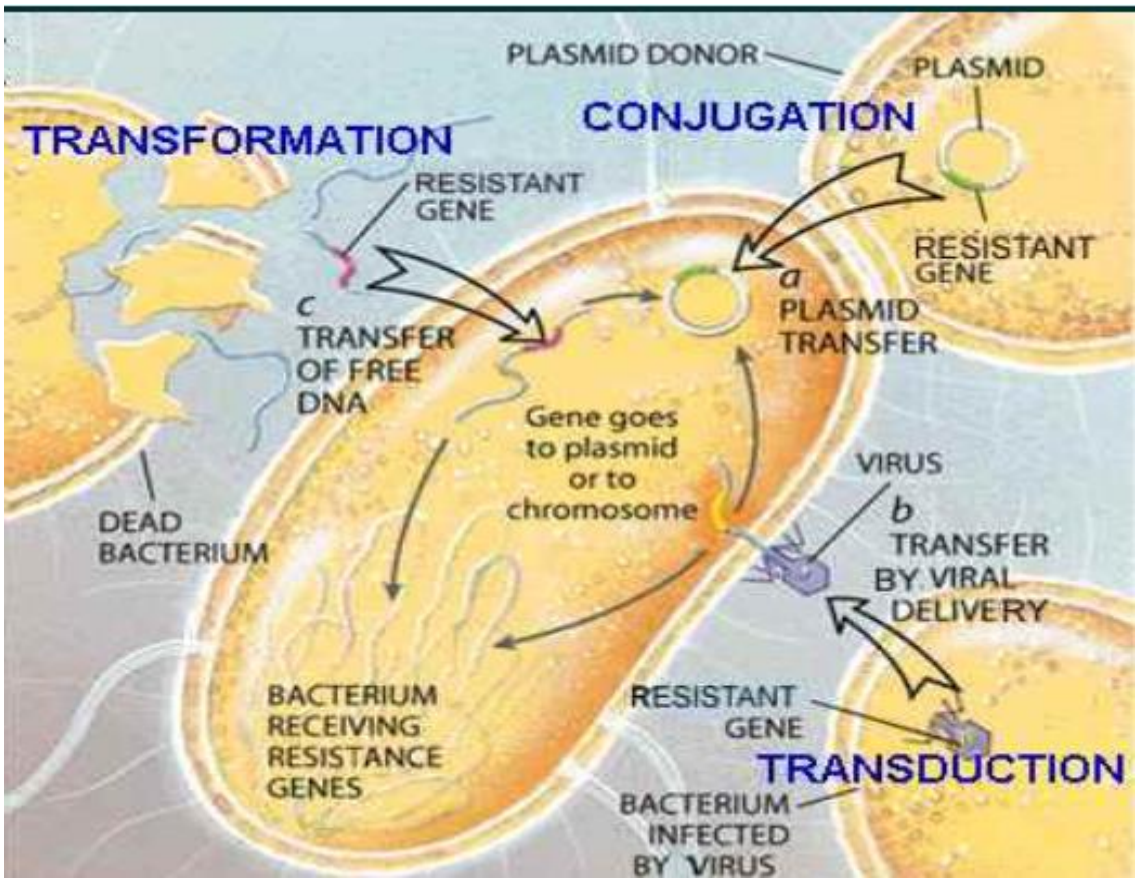

Figure 2.1: Transfer of Genetic Material through transformation, translation, and conjugation. Figure taken from Levy, 1998

Antibiotic resistance has become a serious concern and a threat worldwide and in many areas of the world there are no effective antibiotic therapies available for life-threatening infections, and the pace of development of novel antibiotics is now alarmingly low (Walsh, 2003). Increasing attention is being placed turned towards factors that potentially contribute to

antibiotic resistance and this has been declared by the World Health Organization (WHO) (WHO, 2012a) as a 'complex problem driven by many interconnected factors. Environmental pathways of antibiotic resistance has not yet been directly addressed by WHO (2012a) but recent research has highlighted soil and water environments as recipients, reservoirs and sources of ARGs of clinical concern (Martínez, 2009; Wright, 2010). Recent studies have found that antibiotic resistance genes develop in bacteria in the environment as a direct result of pig farm seepage in soil and groundwater in the vicinity of pig farm facilities maybe potential sources of antibiotic resistance in the environment (Elmund *et al.*, 1971; Donoho, 1984; Galvalchin and Katz, 1994; Chee-Sanford *et al.*, 2001). Soil and water environments receive inputs of antibiotics and antimicrobials, which can serve to amplify ARGs (Chee-Sandford *et al.*, 2009; Heuer *et al.*, 2011).

Table 2.2: Mechanisms of action of antibacterial agents

Mechanism of action	Class of antibiotic	antibiotic
Interference with cell wall synthesis	Lactams	penicillins, cephalosporins, carbapenems, monobactams
	Glycopeptides	vancomycin, teicoplanin
Protein synthesis inhibition	Lincosamide and Streptogramins (Anti-50S ribosomal subunit)	chloramphenicol, clindamycin, quinupristin-dalfopristin, linezolid
	Aminoglycosides and tetracyclines (Anti-30 S ribosomal subunit)	Gentamycin, Neomycin, Streptomycin
Interference with nucleic acid synthesis	Fluoroquinolones (Inhibits DNA)	Nalidixic acid, Norfloxacin
	Rifampin (Inhibits Ribonucleic acid (RNA))	Rifampin
Folic Acid synthesis inhibitors	Sulfonamides, folic acid analogues	Sulphametaxazole, Trimethoprim
Mycolic Acids Synthesis Inhibitors	Isoniazid	Isoniazidz

The Table shows mechanism of action different antimicrobial agents (Roberts, 1996).

Although reports of the percentage of viable, culturable antibiotic-resistant bacteria in swine effluent vary, it is clear that antibiotic resistance is a common phenomenon (Roberts, 1996). Since the isolation of the first tetracycline resistant (*tetR*) bacterium *Shigella dysenteriae*, tetracycline resistance has been detected in a wide variety of bacteria (Roberts, 1996). A study conducted in the 1984 by Hanzawa *et al.,* of coliforms in swine waste found that 97% of *E. coli* were resistant to at least one of the following antibiotics: ampicillin, furatrizine, chloramphenicol, kanamycin, streptomycin, sulphonamides or tetracycline. In their study Hack and Andrews (2000) found that 71 % of *Enterococcus faecalis* isolates from swine farrowing house effluent were resistant to tetracycline while Cotta *et al.*, (2003) found that between 4 % and 32 % of the bacteria in swine manure were resistant to tylosin. Therefore, antibiotic resistance could be considered as an environmental pollution problem, with resistance gene vectors as the target pollutants (Roberts, 1996).

Bacterial pollution emanating from pig farm seepage does not only exposes bacteria with antimicrobial resistance gene but may also impact on the physicochemical parameters of the receiving environment (Morrison *et al.*, 2001). The physicochemical parameters of soil and water that may be affected by bacterial pollution includes pH, temperature, electrical conductivity, salinity, turbidity, total dissolved solids (TDS), dissolved oxygen (DO), chemical oxygen demand (COD), nitrate, nitrite, and orthophosphate levels (Morrison *et al.*, 2001).

2.6 Physicochemical parameters impacted by seepage

2.6.1 Dissolved oxygen

Oxygen occurs naturally in the atmosphere as gas and is also produced via photosynthesis but is not readily soluble in water, and its solubility relies on temperature, salinity and atmospheric pressure (DWAF, 1996c). Dissolved oxygen (DO) is critical for sustenance of aquatic life in

order for aerobic species to be able to survive and carry out their ecological functions (Kartal *et al.,* 2006). Under natural freshwater conditions, DO concentrations are expected to be at the saturation point of 6 mg/l DO at 25 ºC (Palmer *et al.,* 2004b; 2005) and the standard for sustaining aquatic life is stipulated at 5 mg/l DO and concentration below this value adversely affects aquatic biological life, while concentration below 2mg/l may lead to death for most fishes (Chapman, 1996). Low DO concentrations lead to formation of anaerobic conditions and hence, reduced aerobic functions (Kartal *et al.,* 2006) and therefor lack of DO can lead to anaerobic decomposition of organic matter, resulting in unpleasant odours that are indicative of formation of hydrogen sulphide and ammonium (Schindler, 1981). Furthermore, anoxic conditions can result in changes in sediment chemistry due to hydrodynamic, geochemical and environmental conditions modification caused by low DO (Kartal *et al.*, 2006) and such modifications can result in desorption of heavy metals from sediment into the water column, hence becoming more bioavailable and therefore more toxic chemical forms, posing severe threats to aquatic species (Schindler, 1981; Eggleton and Thomas, 2004). The DO is a measure of the degree of pollution by organic matter, the destruction of organic substances as well as the self-purification capacity of the water body (Schindler, 1981).

2.6.2 pH

The pH value is a measure of the balance of positive hydrogen ions (H+) and negative hydroxide ions (OH-) in water and thus assesses its acidic or basic nature (Dallas and Day, 2004). At a specific pH, carbonate/bicarbonate ions can be formed from the dissociation of carbonic acid. The maximum carbonic acid production happens at pH 8 (Dallas and Day, 2004). Alkalinity is controlled by carbonate/bicarbonate species, and is represented as mg/l Calcium carbonate ($CaCO_3$) (Dallas and Day, 2004). The pH changes are controlled by temperature, the organic and inorganic ions and biological activity and also pH plays crucial roles in toxicity and availability of metals and non-metallic ions e.g. ammonium (Dallinger *et al.,* 1987). If not

buffered properly, low pH levels can allow for the formation of toxic substances, leading to species diversity and structure alterations (Kartal *et al.,* 2006). The buffering capacity of an ecosystem is important for sustenance of aquatic life and is measured through alkalinity/hardness (DWAF, 1996c)

2.6.3 Electrical conductivity and TDS

Electrical conductivity (EC), also called salinity, is the parameter that is used to estimate concentrations of total dissolved solids (TDS) (DWAF, 1996c). Dissolved salts or ions carry an electric charge while the concentration of TDS is proportional to the EC of the water (DWAF, 1996c). The EC in freshwater ecosystems is regulated by rocks' mineral composition, size of the watershed and other sources of ions (Hudson-Edwards *et al.,* 2003). A common example is limestone which is known to contribute to higher EC in water due to the dissolution of carbonate into river basins (Roelofs, 1991; O'Keeffe *et al.,* 1996). A larger watershed will allow more water drainage into the river basin which allows more salts extraction from the soils, hence contributing to higher EC levels (Vega *et al.,* 1998). Wastewaters from industries, sewage treatment works and septic tanks, and non-point sources from settlements and agriculture are other sources that contribute to in-stream EC (Roelofs, 1991; Nielsen *et al.,* 2003). The United States Department of Primary Industry and Fisheries (USDPIF) reported that atmospheric depositions, evaporation and microbial activities also contribute to increased EC levels in the aquatic systems (USDPIF, 1996). Determining EC is important as high TDS concentrations can have adverse effects on the aquatic life (DWAF, 1996c).

2.6.4 Turbidity and suspended solids

The American Public Health Association (APHA) (1998) explain turbidity as a representation of the optical property of water that causes light scattering or absorption. Light scattering results from the suspended matter (e.g. clay, silt, organic and inorganic matter, plankton and

other microorganisms (Dallas and Day, 2004). Primary production is reduced in turbid waters as a result of decreased photosynthesis due to light scattering. Turbidity > 5 NTU can cause reduction of primary production. Primary production decrease reduces food availability at multiple trophic levels in the aquatic ecosystems (Ryan, 1991). Turbidity is caused by runoffs from non-point (e.g. irrigation schemes) and point sources (e.g. seepage effluent). Higher turbidity can affect benthic, invertebrates and fish communities (Wood and Armitage, 1997).

2.6.5 Biochemical Oxygen Demand

Biochemical oxygen demand (BOD) is defined as the amount of oxygen required by bacteria while stabilizing decomposable organic matter under aerobic conditions (Sawyer and McCarty, 1978). It is a test applied to measure the amount of biologically oxidizable organic matter present and determining the rates at which oxidation will occur or BOD will be exerted (Sawyer and McCarty, 1978). In order to make the test quantitative, the samples must be placed in an airtight container and kept in a controlled environment for a preselected period of time. In the standard test, a 300-mL BOD bottle is used and the sample is incubated at 20 °C for five days (Peavy *et al.*, 1985). The BOD is then calculated from the initial and final dissolved oxygen (DO) concentration. The greater the decomposable matter present, the greater the oxygen demand and the greater the BOD values (Ademoroti, 1996; Standard methods, 1996).

2.6.6 Chemical Oxygen Demand

The chemical oxygen demand (COD) test is used to measure the total organic content of wastewaters. During the determination of COD, organic matter is converted to carbon dioxide and water using a strong chemical oxidizing agent (dichromate) in the presence of a catalyst and strong acid. The dichromate reflux method has been preferred over procedures using other oxidants because of superior oxidizing ability with a wide variety of samples, and ease of manipulation (Boyles, 1997a). In the COD test, organic materials are oxidized regardless of

the biological assimilability of the substances. As a result, COD values are greater than BOD values and may be much greater when significant amounts of biologically resistant organic matter are present (Sawyer and McCarty, 1978). While COD is the measure of amount of oxygen required by both potassium dichromate and concentrated sulphuric acid to breakdown both organic and inorganic matters (Boyles, 1997a).

CHAPTER 3: Methodology

The methodological procedure followed for the research is outlined below:

3.1 Study Area

The project was conducted at the Agricultural Research Council- Animal Production Institute (ARC- API) at Irene in Pretoria. The institution houses a dairy farm, pig farm, sheep farm and chicken farm. It deals mainly with research on domestic animals. The ARC-API has several satellite stations strategically positioned throughout the country, and is one of eleven research institutes of the Agricultural Research Council (ARC). The Institute carries out primary and secondary research, development and technology transfer with respect to Animal Breeding and Improvement, Rangelands and Nutrition, and Food Science and Technology to improve productivity and sustainable resource utilisation.

The ARC- API is situated about 25 km south of Pretoria (25°52′S 28°13′E / 25.867°S 28.217°E / -25.867; 28.217 in Gauteng) adjacent to the village of Irene in the suburb of Centurion. The campus houses the Head Office of the ARC-Animal Improvement Institute (ARC-AII), as well as the ARC-Animal Nutrition and Animal Products Institute (ARC-ANPI). The area has a typical Highveld climate (altitude 1 523 m), with hot days and cool nights in summer and moderate winter days with cold nights. ARC-API is located on the Highveld like Johannesburg and Pretoria and has a similar climate, with dry, sunny winters (max daytime temperature around 20 °C dropping to a crisp average minimum of 5 °C, and warm to hot summers (October to April) tempered by late-afternoon showers often accompanied by extreme thunder and lightning. Hailstorms are not uncommon, but a serious hailstorm has not happened for many years. Summer temperatures range from the mid-20s to the mid-30s (°C). Irene normally receives about 556 mm of rain per year, with most rainfall occurring during summer

Soil structure in ARC-API is mostly structured and coarse, with pores of 0.7 mm to 2 mm in size. Most of the soil in ARC-API is loam soil which mostly contains silt soil, which is richer in nutrients and is able to retain water for long periods. ARC-API receives their water supply from the municipality. Water quality that the ARC-API receives from the municipality is of good condition as it is odourless, colourless, tasteless, and free from harmful contaminants (bacteria, fungi, viruses, chemicals).

3.2 Sampling

Soil and water samples were collected at the pig farm, ARC-API. Samples were collected monthly from March to August 2013 between 07h00 and 09h00 in the morning. Soil samples were collected from pig enclosures, soil 20 m and 100 m away from the pig farm enclosures, soil 20 m and 100 m from constructed wetland. Soil samples we collected in sterile polythene bags at depths of 5 cm (Surface soil) and 30 cm.

Water samples were collected in glass bottles cleaned with dilute Nitric acid (HNO_3) and detergent followed by distilled water from eight different sites on the pig farm i.e. pig enclosures, pig influent 2 m from the constructed wetland, 2 dams at pig farm constructed wetland for wastewater treatment, and effluent 2 m from the constructed wetland.

3.3 Physicochemical analyses

Water and soil samples were collected to determine their physicochemical characteristics namely, BOD, COD, Salinity, pH, Temperature, EC, TDS, Turbidity, DO, concentrations of NO_3, NO_2, and PO_4^{3-}. The method adopted from Igbinosa and Okoh, (2009) and Standard Methods (2001) were used to collect water samples. Water samples (1 L) were collected in triplicates in 1 L glass bottles cleaned with dilute Nitric acid (HNO_3) and detergent, then followed by deionised water. The method adopted from Bhat *et al.*, (2011) and Standard Methods (2001) was adopted for soil sampling. Approximately 2 Kg of soil samples were

collected in sterile polythene bags at a depth of 30 cm. Water and soil samples were place on ice in a cooler box immediately after sampling and transported to the lab for analysis.

Before collecting water samples, the bottles were rinsed three times with sample water before being filled with the sample. The actual water samples were collected midstream by dipping each sample bottle at approximately 20-30 cm below the water surface, projecting the mouth of the container against the flow direction (Igbinosa and Okoh, 2009). The critical parameters such as BOD, salinity, pH, temperature, EC, TDS, Turbidity, DO, concentrations of NO_3, NO_2, and PO_4^{3-}, were tested on the same day while the COD parameter was tested within its time limit. Samples for analyses of COD were collected separately in 1 L bottles and preserved with 0.2 mL of concentrated sulphuric acid on point of sampling and was analysed within 28 days.

All field meters and equipment were checked and calibrated according to the manufacture's specification. Parameters such as pH, temperature, EC, TDS, salinity, DO, turbidity and BOD parameters of water samples were determined onsite using a multi-parameter ion specific meter (Hanna instruments, version HI9828) that was checked for malfunctioning by passing standard solutions of all the parameters to be measured. Physicochemical analyses were performed in triplicates. Blank samples (deionized water) were passed between every three measurements of samples to check for any eventual contamination or abnormal response of equipment.

The pH analyses method was adopted from Singh *et al.*, (2012). The pH was read using PHC101 probe and the pH meter was calibrated using buffers of pH 4.0, 7.0 and 10.0. The pH reading was taken in triplicates and the PHC101 probe was rinsed with deionized water in between the readings. Temperature was measured onsite, using a sterile mercury thermometer for both water and soil samples.

Initial analysis of BOD and DO were performed onsite, and again in the laboratory. BOD and DO were measured using BOD LDO Probe (Model LBOD 10101). The probe was calibrated

according to manufacturer's requirements. The BOD and DO determination of the water samples was carried out using standard methods described by APHA (1998). A 300mL BOD bottle was used to add 297 mL of BOD nutrient pillow and 3 mL of sample. The probe was placed into the sample and the stir paddle was switched on to stir the solution. The results for BOD were recorded when the probe had stabilized. The dissolved oxygen content was determined before and after incubation. Sample incubation was for five days in the dark at 20 °C in BOD bottle. The following formula was used to calculate BOD_5:

$BOD_5 = (D_1 - D_2)/P$

Where:

BOD_5 = BOD value from the five day test

D_1 = DO of diluted sample immediately after preparation

D_2 = DO of diluted sample after five days incubation at 20 ± 1 °C, in mg/L

P = Decimal volumetric fraction of sample used

For measuring DO in samples, 300 mL of sample was poured into 300 mL BOD bottles and the probe was placed into the sample and the stir paddle was switched on to stir the sample. The results for DO were recorded when the probe had stabilized.

Analyses of TDS, EC NO_3, NO_2, PO_4^{3-}, and salinity were also adopted from Singh *et al.*, (2012) (with amendments) and Standard Methods (2001) were followed in determining the above variables. Salinity, TDS, and EC were measured using HACH CDC401 probe. Aliquots of 250 mL of the sample was poured into a 300 mL beaker and placed on a magnetic stirrer plate, the probe was placed in the sample and the results were taken in triplicates. The probe was rinsed in deionized water after each test.

Concentrations of nitrate nitrite, orthophosphate and COD were read using spectrophotometer HACH DR 500. Blank determinations were performed for COD, nitrate, nitrite and orthophosphate. The presence of orthophosphate was determined using the Molybdovanadate method (HACH Method 8114) (HACH, 2008). Orthophosphate was measured by adding 20 mL of the sample into a 25 mL graduated mixing cylinder and 1 content of Molybdate Reagent Powder Pillow was added to each sample. The cylinder was stoppered and shaken to dissolve reagents. Then 10 mL of the prepared sample was added to a 10 mL square sample cell and 0.5 mL of molybdenum 2 Reagent was added and the cell was swirled and left for 2 minutes for reaction to complete and results were taken immediately upon completion.

Nitrate was analysed using the cadmium reduction method (HACH Method 8039) (HACH, 2008). Nitrate was then measured by adding 10 mL of sample into a square sample cell and NitraVer 5 Nitrate Reagent powder pillow (HACH) was added to the sample. The reaction was left to stand for 1 minute, shaken vigorously and left for another 5 minutes for the reaction to complete. The results were read immediately.

Nitrite was analysed using the ferrous sulphate method (HACH Method 8153) (HACH, 2008). Nitrite was measured by adding 10 mL of sample into a square sample cell and 1 content of NitriVer 2 Nitrite Reagent Powder pillow (HACH). The cell was stoppered and shaken to dissolve the contents. When completely dissolved the solution was left for 10 minutes for the reaction to complete and results were taken immediately.

Standard Methods (2001) was followed for analyses of COD, where 100 mL of sample was homogenized in a blender for 30 seconds and 250 mL of sample was poured into a beaker and gently stirred on a magnetic stir plate. About 2 mL of the homogenized sample was pippeted from the beaker into a vial containing potassium dichromate. The vial was inverted several

times and then placed into a 150 °C preheated DRB200 reactor for 2 hours. Results were read when vials were completely cooled.

Standard Methods (2001) was followed for analyses of turbidity and was measured using DR/4000 1-inch cell adapter. For turbidity 1.5 mL of sample was pipetted in 2 mL cuvettes and placed in the DR/4000 1-inch cell adapter and results were read at 860 nm wavelength.

For soil samples, 100 g of soil sample was mixed with 1 L of deionized water in a 1 L bottle previously cleaned with dilute Nitric acid (HNO_3) and detergent, then followed by deionised water. These soil solution was mixed for 3 hours using a magnetic stir plate. The solution was then removed and placed on the bench and left for 30 minutes for soil to settle completely at the bottom. Soil samples were analysed for pH, temperature, salinity, EC, DO, TDS, NO_2, COD, NO_3, and PO_4^{3-}. Similar procedure for analysing the physicochemical parameter for water samples were also adopted for analysing physicochemical parameters of soil samples.

3.4 Bacteriological analyses

3.4.1 Bacteria Isolation

Water samples (100 mL) were concentrated to 20 mL by centrifuging. The method from Bezuidenhout *et al.*, (2002) was used. Serial dilution method was adapted and 1 mL of the concentrated sample was used in performing the serial dilution of 10^{-1} up to 10^{-8} using sterile 0.9% (w/v) saline solution. The media that were used were, Nutrient agar, MacConkey Agar, Xylose Lysine Deoxycholate agar (XLD agar), and Eosin Methylene Blue (EMB). Pour plate method was used in which 1 mL of each dilution was placed in aliquots of 15 mL of agar and mixed to contents of the tubes. Agars were left to solidify and incubated at 37 °C for 48 hours. Isolates were streaked three times on nutrient agar for pure colony.

An aliquot of 100 mL of distilled water was measured in a graduated cylinder and added to the sterile bottle (100 mL). Approximately 10 g of the soil sample was weighed and added to the

bottle of distilled water. The bottle was tightly capped and mixed thoroughly for 30 minutes using magnetic stirrer plate. Dilutions were done using 0.9 % (w/v) of saline solution and 1 mL of each dilution was added to 15 mL of agar in test tube. The contents in the tube were poured into a petri dish, allowed to solidify and incubate at 37 °C for 2 days. Similar agar media that were used for water samples were also used for soil samples. Isolates were streaked three times on nutrient agar for pure colony.

3.5 Identification of Isolates

Isolates were identified using API 20E identification kit (bioMérieux South Africa (Pty) Ltd). Pure isolates were streaked on nutrient agar and incubated at 37 °C for 24 hours. The overnight grown cultures were then inoculated in 5 mL of 0.85 % (w/v) saline solution and the turbidity of the resulting solution was adjusted to 0.5 McFarland Standard. The manufactures procedure was followed in inoculating the isolates on the API 20E test strips.

All 20 micro-wells were inoculated by filling the tube section and also the cupule sections of the citrate, VP, and gelatin tests. The cupule sections of the arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, and urease tests were filled with sterile mineral oil. A plastic lid was placed over the strip, and the strip was incubated at 37 °C for 20 to 24 h. The cover was then removed, and one drop of 40 % KOH and then one drop of 6 % a-naphthol were added to the VP test; one drop of ferric chloride was added to the tryptophan deaminase test; and one drop of Kovac reagent was added to the Indole test. All reactions were then read according to the recommendations of the manufacturer. The seven-digit octal number was calculated, and the organism identity was determined using the apiweb.

3.6 Susceptibility Analyses

Antibiotic resistance was determined by the Kirby-Bauer disk diffusion method using the standard procedure of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2007). The pure colonies were isolated and cultured and subjected to an antibiotic susceptibility test. The isolates were screened for susceptibility to a panel of antibiotics (Table 3.1) using Mueller Hinton agar (Oxoid, UK).

Method for susceptibility test was adapted from Kumar *et al.*, (2013). The inoculum for antibiotic resistance susceptibility testing was prepared in 0.9 % (w/v) saline solution by dispensing a single colony picked up with a sterile cotton swab into a 9 ml saline solution and incubated at 37 °C for 24 hours. The turbidity of the resulting solution was adjusted to 0.5 McFarland Standard. An aliquot of 100 µL of solution was spread plated onto Mueller Hinton agar plates (OXOID) and each sample was analysed in triplicate. The antibiotic discs were placed 30 mm apart on the plates using a disc dispensing apparatus. Fifteen minutes after applying the discs, the plates were inverted and incubated at 37 °C for 20 hours. The inhibition zone diameters were measured in millimetre and recorded. Each bacterial isolate was classified as susceptible (S), intermediate (I), or resistant (R) to the antibiotics according standard reference values (CLSI, 2007). The experiments were performed in triplicates and the average values were considered for patterns of antibiotic resistance or sensitivity.

The multiple drug resistances index (MDRI) for isolates was calculated according to Krumperman (1983). The frequency of antibiotic-resistant of isolates was calculated by the equation: A/B x 100%, where A is the number of isolates resistant to an antibiotic and B is the total number of isolates from the sample. The MDRI of each sample was estimated by the equation: a/ (b x c), where a represents the aggregate antibiotic resistance score of all isolates

from the sample, b represents the number of antibiotics, and c represents the number of isolates from the sample.

TABLE 3.1: Antibiotics used for susceptibility Analyses

Antibiotics	**Concentrations**
Penicillin G (P),	10 µg
Sulphamethaxazole (RL),	25 µg
Vancomycin (VA),	30 µg
Ampicillin (AML),	10 µg
Amoxicillin (APR),	25 µg
Apramycin (AMP),	15 µg
Neomycin (N),	30 µg
Tilmocosin (TIL),	15 µg
Oxytetracyclin (OT),	30 µg
Spectinomycin (SH),	25 µg
Lincomycin (MY),	15 µg
Trimethoprim (TM).	2.5 µg
Nalidixic Acid (NA)	30 µg
Gentamycin (CAZ)	10 µg
Tetracycline (TE)	30 µg
Ceftadizime (CN)	10 µg
Norflaxacin (NOR)	10 µg
Nitrofurantoin (NI)	300 µg
Penicillin G (P),	10 µg

The Table shows the 19 different antibiotics that were used on isolates for determining their sensitivity for antibiotics.

3.7 Detection of resistance gene in identified isolates

3.7.1 DNA Isolation

The isolates were cultured in nutrient broth and incubated for 24 hours at 37 °C. NucleoSpin Tissue Genomic DNA purification kit (Machery-Nagel) was used to isolate genomic DNA from the identified isolates. The manufactures procedure was followed for isolation of the genomic DNA (support protocol for bacteria). In a sterile 1.5 ml Eppendorf 1 mL was pipetted and centrifuged at 8000 rpm for five minutes, the supernatant was discarded and the pellet was used for isolating genomic DNA. For pre-lysing, the pellet was re-suspended in 180 µL of Buffer T1 and mixed by pipetting up and down. After mixing 25 µL of Proteinase K (mixed with 1.35 mL of Proteinase Buffer) was added. The mixture was vortexed vigorously and incubated at 56 °C for three hours for complete lyses to be obtained. For Gram positive isolates pre-incubation with a lytic enzyme was used instead of Buffer T1 whereby the pellets were re-suspended in 20 mM Tris/HCl; 2 mM EDTA; 1% Triton X-100; pH 8 and supplemented with 20 mg/mL of lysozyme and incubated for one hour at 37 °C, after incubation 25 µL of Proteinase K was added and incubated at 56 °C for three hours for complete lyses. After incubation the samples were vortexed and 200 µL of Buffer B3 was added and the mixture was vortexed vigorously and incubated at 70 °C for ten minutes. After incubation 210 µL ethanol (96 % to 100 %) was added to bind DNA and samples were vortexed vigorously. For each sample one NucleoSpin Tissue Column was placed into a Collection Tube and centrifuged for one minute at 11000 rpm. The flow-through was discarded and the column was placed back into the collection tube. The silica membrane was washed with 500 µL of Buffer BW and centrifuged for one minute at 11000 rpm. The flow-through was discarded and the column was placed back again into the collection tube. The silica membrane was washed again with 600 µL of Buffer B5 [mixed with 28 mL of ethanol (96 % to 100)] and centrifuged for one minute at 11000 rpm. The flow was discarded and the column was placed back into the collection tube.

The silica membrane was dried by centrifuging the column at 11000 rpm for one minute. The DNA was eluted using 100 µL Buffer BE (5 mM Tris/HCL, pH 8) prewarmed at 70 °C, and centrifuged for one minute at 11000 rpm. The aqueous phase containing purified DNA and was directly used for the subsequent experiments and also stored at -20 °C. The purity and yield of the DNA was assessed spectrophotometrically by calculating the A_{260}/A_{280} ratios and the A_{260} values to determine protein impurities and DNA concentrations. The concentration and quality of the DNA were determined by agarose gel electrophoresis and spectrophotometer analysis (NanoDrop ND-2000c, Thermo).

3.7.2 PCR for detection Resistance Genes

PCR assays were performed to determine the presence of antimicrobial resistance genes in identified bacterial isolates. The method adopted was that outlined by Hsu *et al*., (2007) but with amendments. The sequences of primers used for PCR amplification of antibiotic resistance genes are listed in Table 3.2. Amplification of the DNA was performed in a PCR apparatus with iProof High Fidelity DNA Polymerase (BIO-RAD). The 20 µL reaction mixture contained 0.02U/ µL iProof DNA Polymerase; 1X iProof HF Buffer; 3% DMSO; 700 µM $MgCl_2$; 200 µM dNTPs; 0.5 µM Forward Primer; 0.5 µM Reverse Primer; 1 µg DNA Template; and 11.4 µL of nuclease free water. The PCR was initiated by incubating the reaction mixture at 98 °C for 30 seconds, followed by 35 cycles of 10 seconds at 98 °C, 30 seconds at the annealing temperature of Primer, and extension for 30 seconds at 72°C. The reaction was terminated after a final extension step for ten min at 72 °C. All PCR experiments contained a positive control (*E. coli* ATCC 25922, *Ps. aeruginosa* ATCC 19429, *S. marscensce* ATCC 14041), and a blank control (reaction mixture with no DNA template). Amplified DNA from each sample (10 µL) was mixed with 1 µL of 6x loading buffer dye and loaded on a 1% horizontal agarose gel containing 0.5 mg/mL of ethidium bromide. A 100bp DNA ladder ranging from 100bp to 3000 bp (Thermo Scientific) was also added on each gel to confirm the size of amplified DNA bands.

All gels were ran in 1 X TAE buffer at 5 V/cm for 30 min, and visualized by UV trans-illumination.

Table 3.2: Primers for detection of antibiotic resistance genes

Primers	Sequence (5' to 3')	Annealing Temperature	Reference
aadA	F- 5'TGATTTGCTGGTTACGGTCAG'3 R- 5'CGCTATGTTCTCTTGCTTTTG'3	53 °C	Vakulenko *et al.*, (2003)
aa(6')-le-aph(2")-la	F-5'CAGGAATTTATCGAAAATGGTAGAAAAG'3 R- 5'CACAATCGACTAAAGAGTACCAATC'3	55 °C	Vakulenko *et al.*, (2003)
aph(2")-lb	F- 5'CTTGGACGCTGAGATATATGAGCAC'3 R- 5'GTTTGTACGCAATTCAGAAACACCCTT'3	58 °C	Vakulenko *et al.*, (2003)
aph(2")-lc	F- 5'CCACAATGATAATGACTCAGTTCCC'3 R- 5'CCACAGCTTCCGATAGCAAGAG'3	58 °C	Vakulenko *et al.*, (2003)
aph(2")-ld	F- 5'GTGGTTTTTACAGGAATGCCATC'3 R- 5'CCCTCTTCATACCAATCCATATAACC'3	56 °C	Vakulenko *et al.*, (2003)
aph(3")-llla	F- 5'GGCTAAAATGAGAATATCACCGG'3 R- 5'CTTTAAAAAATCATACAGCTCGCG'3	54 °C	Vakulenko *et al.*, (2003)
ant(4')-la	F- 5'CAAACTGCTAAATCGGTAGAAGCC'3 R- 5'GGAAAGTTGACCAGACATTACGAAACT'3	58 °C	Vakulenko *et al.*, (2003)
aac(3')-lv	F- 5'GTCGTCCAATACGAATGGCG'3 R- 5'CAGCAATCAGCGACCTTG'3	55 °C	Vakulenko *et al.*, (2003)
VanA	(F)CAT GAA TAG AAT AAA AGT TGC AAT A (R) CCC CTT TAA CGC TAA TAC GAT CAA	55 °C	Jánošková and Kmeť, (2004)
VanB	(F)GTG ACA AAC CGG AGG CGA GGA (R)CCG CCA TCC TCC TGC AAA AAA	58 °C	Jánošková and Kmeť, (2004)
VanC1	(F)GGT ATC AAG GAA ACC TC (R)CTT CCG CCA TCA TAG CT	54 °C	Jánošková and Kmeť, (2004)
VanC2/C3	(F) CGG GGA AGA TGG CAG TAT (R) CGC AGG GAC GGT GAT TTT	55 °C	Jánošková and Kmeť, (2004)
OtrA	(F) GAACACGTACTGACCGAGAAG (R) CAGAAGTAGTTGTGCGTCCG	57 °C	Nikolakopoulou *et al.*, (2005)
OtrB	(F) CCGACATCTACGGGCGCAAGC (R) GGTGATGACGGTCTGGGACAG	61 °C	Nikolakopoulou *et al.*, (2005)

bla$_{SHV}$	(F) ATGCGTTATATTCGCCTGTG (R) TTAGCGTTGCCAGTGCTCGA	53 °C	Jiang *et al.,* (2006)
bla$_{TEM}$	(F) ATGAGTATTCAACATTTTCG (R) TTACCAATGCTTAATCAGTG	47°C	Strateva *et al.,* (2007)
bla$_{OXA}$	(F) CGAGCGCCAGTGCATCAAC (R) CCGCATCAAATGCCATAAGTG	56 °C	Strateva *et al.,* (2007)
bla$_{VEB}$	(F) CGACTTCCATTTCCCGATGC (R) GGACTCTGCAACAAATACGC	55 °C	Strateva *et al.,* (2007)
bla$_{PER}$	(F) AATTTGGGCTTAGGGCAGAA (R) ATGAATGTCATTATAAAAGC	45 °C	Strateva *et al.,* (2007)
Sul1	F- 5' GGATCAGACGTCGTGGATGT'3 R- 5' GTCTAAGAGCGGCGCAATAC'3	62 °C	Faldynova *et al.,* (2013)
Sul2	F'- 5' CGCAATGTGATCCATGATGT'3 R'- 5' GCGAAATCATCTGCCAAACT'3	60 °C	Faldynova *et al.,* (2013)
Inu(A)	(F) GGTGGCTGGGGGGTAGATGTATTAACTGG (R) GCTTCTTTTGAAATACATGGTATTTTTCGA	56 °C	Li *et al.*, (2013)
Inu(B)	(F) CCTACCTATTGTTTGTGGAA (R) ATAACGTTACTCTCCTATTTC	50 °C	Li *et al.*, (2013)
Inu(C)	(F) AATTTGCAATAGATGCGGAGA (R) TCATGTGCATTTTCATCA	52°C	Li *et al.*, (2013)
Inu(D)	(F) ACGGAGGGATCACATGGTAA (R) TCTCTCGCATAATAACCTTACGTC	55 °C	Li *et al.*, (2013)
Inu(F)	(F) CACCATGCTTCAGCAGAAAATGATC (R) TTACTTGTTGTGCGGCGTC	55 °C	Li *et al.*, (2013)

The Table shows 29 different primers used for the Polymerase Chain Reaction (PCR) detection of resistance genes.

Chapter 4: Results

4.1 Results for physicochemical results of water and soil samples.

The mean and standard deviation (SD) values for the physicochemical parameter analyses of the water samples are given in Table 4.1 and their P-values including the F-values and their significance are given in Table 4.2

The pH values ranged from 6.5 to 9 (Table 4.1) and the values varied significantly with months ($p<0.05$) and sampling points ($p<0.05$). In addition the interaction effect of both month and sampling point was also significant ($p<0.05$) for pH (Table 4.2). The combined effect for pH on month and sampling point also varied significantly ($p<0.05$) (Table4.2). The temperatures of the water samples (Table4.1) ranged from 8 °C to 28 °C. Temperature values for water sample as shown on Table 4. 2 varied significantly with months and sampling points ($p<0.05$), and the interaction effects of both month and sampling point were also significant ($p<0.05$) .The results for electrical conductivity in water samples (Table 4.1) ranged from 1.25 mS/cm to 5.58 mS/cm and their variation were insignificant (Table 4.2). Results for the BOD for water samples (Table 4.1) ranged from 163 mg/L to 3550 mg/L and these results varied significantly ($p<0.05$) in months and sampling points(Table 4.2). The combined effect of months and sampling points for BOD for water samples (Table 4.2) also varied significant ($p<0.05$).

The results for TDS for water samples (Table4.1) ranged from 0.77 g/L to 6.48 g/L. The results for TDS did not vary significantly (Table 4.2). Salinity in this study ranged from 0.83 psu to 6.35 psu for water samples as shown in Table 4.1 and the results varied significantly ($p<0.05$) with months and with sampling points. Table 4.2 also shows that the combined effect of months and sampling points on water samples salinity parameter also varied significantly varied significantly ($p<0.05$). Turbidity values for water samples (Table 4.1) ranged from 0.21 NTU to 3.65 NTU. The results for water samples turbidity (Table 4.2) did not vary significantly. The

results for COD for water samples (Table 4.1) ranged from 210 mg/L to 9400 mg/L and the results varied significantly ($p<0.05$) monthly and in sampling points (Table 4.2) and the combined effect of months and sampling points varied significantly ($p<0.05$).

The results for DO range from 4.14 mg/L to 7.64 mg/L for water samples (Table 4.1). The DO results for water samples (Table 4.2) varied significantly ($p<0.05$) monthly and in sampling points. The combined effect of months and sampling points for DO (Table 4.2) also varied significantly ($p<0.05$). Results for concentration of nitrate for water samples is shown in Table 4.1 and ranged from 55 mg/L to 1680 mg/L. Nitrate concentrations for water samples (Table 4.2) varied significantly ($p<0.05$) monthly and in sampling points. The combined effect of months and sampling points for nitrate in water samples (Table 4.2) also varied significantly ($p<0.05$). Results for nitrite concentrations in water samples are also given in Table 4.2 and values ranged from 37.5 mg/L to 2730 mg/L. This nitrite values varied significantly ($p<0.05$) monthly and in sampling points (Table 4.2) and the combined effect of months and sampling points on nitrite also varied significantly ($p<0.05$). Orthophosphate (as P) results for water samples (Table 4.1) ranged from 50 mg/L to 1427 mg/L and this results varied significantly ($p<0.05$) monthly and in sampling points (Table 4.2). The combined effect of months and sampling points varied significantly ($p<0.05$).

TABLE 4.1: Results for the physicochemical analyses of water samples

Sampling period	Sampling point	parameters											
		pH	Temp.	Salinity	EC	BOD	TDS	Turbidity	COD	DO	Ortho-P	NO_2	NO_3
March	EC H_2O	7.25±0.5	22.4±0.95	1.18±0.07	3.10±0.13	413.54±15.94	6.19±0.17	1.23±0.13	3122.8±22	7.35±0.66	246.89±7.60	308.78±12	430.51±6.6
	Influent	9±0.00	25±0.00	2.08±0.06	3.50±003	694.5±31.25	6.48±0.10	1.79±0.31	4050±78.25	5.18±0.09	324.5±0.45	498.5±0.05	517.50±2.3
	CW	8.5±0.71	28±1.41	0.99±0.06	2.11±0.31	289.2±95.05	6.10±0.23	1.01±0.20	1025.3±704	6.14±0.93	331.04±40	209.13±93	438.1±232
	Effluent	8±0.00	26±0.00	1.08±0.01	1.58±0.04	163±5.23	4.07±0.01	0.41±0.11	521±13.50	6.51±0.25	55.9±0.35	75±0.13	550±0.31
April	EC H_2O	7.5±0.8	15.8±1.05	2.90±0.49	2.64±0.19	767.25±5.91	2.28±0.48	2.57±0.20	5087.5±246	7.64±0.09	825.13±53	66.88±7.12	146±27.53
	Influent	8±0.00	20±0.00	3.74±0.10	4.17±0.05	770±49.35	3.66±0.11	3.10±0.71	9400±99.1	5.64±0.47	980.5±0.4	202.5±1.1	625±3.41
	CW	8.±0.00	19.5±0.71	1.21±0.27	2.02±0.39	645.5±160.5	1.16±0.02	1.41±0.45	843±357.80	7.25±0.70	833.96±42.2	109.9±55.3	169.5±85.6
	Effluent	8±0.00	18±0.00	0.83±0.04	1.25±0.06	623±11.31	1.03±0.04	0.58±0.52	512±96.07	6.19±0.05	992.32±0.08	52±0.71	70.83±0.72
may	EC H_2O	8.25±0.5	8.75±1.5	4.58±0.58	3.49±0.35	1247.95±292	3.73±1.14	2.43±0.17	6545±456.9	5.94±0.52	893.88±69.9	169.75±13	1306±134
	Influent	8±0.00	15±0.00	6.35±0.16	5.58±0.13	2562.5±25	6.32±0.26	2.85±0.27	7065±87	5.23±0.11	1427±0.2	233.5±0.4	1407±48.4
	CW	8±0.00	18±1.41	3.42±1.76	2.75±0.63	1758.95±320	3.91±1.05	1.93±0.74	3288±1403	5.39±0.20	170.5±112.4	170.5±112	464.3±89.4
	Effluent	8±0.00	18±0.00	1.13±0.05	2.24±0.08	1263.2±83	2.12±0.05	0.41±0.36	760±99.6	5,43±0.02	53.19±0.1	37.5±1.37	637.5±2.0
June	EC H_2O	7.5±0.58	9.0±4.08	1.42±0.16	2.72±0.28	2168.5±244.3	1.61±0.29	2.26±0.18	5832.5±541	5.87±0.79	829±44.80	1425±132	471.3±22.9
	Influent	8±0.00	14±0.00	2.04±0.07	3.04±0.07	3350±209	2.03±0.05	2.60±0.39	7500±74.	4.14±0.05	925±0.28	2458±1.0	693±0.99
	CW	8±0.00	14±1.41	1.04±0.18	2.03±0.34	1745±625.80	1.02±0.17	1.33±0.34	6210±622.3	4.54±0.11	373.16±229	1131±149	325±176.8
	Effluent	8±0.00	15±0.00	0.93±0.07	2.24±0.04	1010±99.0	0.77±0.06	0.74±0.29	4560±94	4.87±0.08	99.31±0.3	653±0.17	145±0.93
July	EC H_2O	7.75±0.5	8.13±1.65	1.66±0.37	3.23±0.61	2066.38±607	1.67±0.33	2.26±0.25	6464±373.9	5.22±0.31	175.75±14	1625±64.5	581.3±41.5
	Influent	8±0.00	12,5±0.00	3.64±0.09	4.29±0.02	3152±68.3	2.64±0.09	3.65±0.46	7295±89.9	4.71±0.06	235±0.31	2730±1.21	1680±1.80
	CW	7.5±0.71	13.25±2.48	1.06±0.18	2.07±0.34	1020±38.89	1.05±0.18	1.01±0.57	1792.5±894	5.05±0.55	170±21.21	1125±460	1060±283
	Effluent	7±0.00	13±0.00	0.84±0.06	1.67±0.04	402.5±34.5	0.83±0.04	0.21±0.19	740±79.83	5.80±0.08	125±0.32	350±0.07	530±0.61
August	EC H_2O	7.5±0.58	8.0 ±0.82	1.46±0.43	2.20±0.82	1583.63±317	1.53±0.33	1.49±0.17	1718.5±132	5.73±0.45	173.75±14.9	1173.5±33.	178.8±11.8
	Influent	6±0.00	11±0.00	2.64±0.13	4.01±0.06	3550±480.8	3.35±0.06	2.24±0.51	3580±90.91	4.46±0.21	240±0.27	1850±0.86	490±1.31
	CW	8±0.00	11.5±1.41	1.15±0.15	2.54±0.71	1405±134.35	1.13±0.14	0.92±0.25	1100±608.1	4.97±0.35	107.5±38.89	425±35.36	105±35.36
	Effluent	8±0.00	16±0.00	0.93±0.07	1.84±0.06	1170±10.61	0.93±0.01	0.52±0.22	210±127.28	5.75±0,08	50±0.10	250±0.01	55±0.20
Standards		6-9	<25 °C	33-35psu	70 mS/cm	<40 mg/L	450 mg/L	<5 NTU	≤1000 mg/L	≥5 mg/L	≤30 mg/L	≤0.5 mg/L	≤20 mg/L

Temp: Temperature; EC: Electrical Conductivity; BOD: Biological Oxygen Demand; TDS: Total Dissolved Solids; COD: Chemical Oxygen Demand; DO: Dissolved Solids; Ortho-P: Orthophosphate; NO_2: Nitrite; NO_3: Nitrate.

The Table shows results for physicochemical parameters of water samples where values are expressed in milligrams per litre except for pH, temperature (in degrees Celsius), turbidity (in nephelometric turbidity unit), salinity (in practical salinity unit), and EC (in micro-Siemens per centimetre), and TDS (grams per litre). The standards were adopted from WHO (2004), DWAF (1996c), FAO (1992), and Government Gazette (1984). Results highlighted in red were those that exceeded the recommended standards.

TABLE 4.2: The P-value and F-value for physicochemical results of water samples.

P and F values	Parameters											
	pH	Temp.	Salinity	EC	BOD	TDS	Turbidity	COD	DO	Ortho-P	NO_2	NO_3
F values [a]	2.91	71.59	32.21	1.01	28.62	0.86	0.74	3.79	32.58	13.95	39.53	7.22
P values [b]	0.02*	0.00*	0.00*	0.42	0.00*	0.51	0.60	0.00*	0.00*	0.00*	0.00*	0.00*
F values [c]	6.07	4.75	7.85	1.01	7.23	1.21	1.25	2.45	6.37	9.90	4.99	2.08
P values [d]	0.00*	0.00*	0.00*	0.43	0.00*	0.30	0.28	0.02*	0.00*	0.00*	0.00*	0.05*
F values [e]	3.55	10.22	11.52	0.93	10.28	0.98	0.98	2.87	4.95	9.14	12.58	4.00
P values [f]	0.00*	0.00*	0.00*	0.52	0.00*	0.47	0.47	0.00*	0.00*	0.00*	0.00*	0.00*

Temp: Temperature; EC: Electrical Conductivity; BOD: Biological Oxygen Demand; TDS: Total Dissolved Solids; COD: Chemical Oxygen Demand; DO: Dissolved Solids; Ortho-P: Orthophosphate; NO_2: Nitrite; NO_3: Nitrate.

*= $P<0.05$ significant variation

Values are expressed in milligrams per litre except in pH, temperature (in degrees Celsius), salinity (in practical salinity unit), and EC (in micro-Siemens per centimetre), TDS (grams per litre).

[a] F values for parameters and month

[b] P values for parameters and month

[c] F values for parameters and sampling point

[d] P values for parameter and sampling point

[e] F values for combined effect of month and sampling point on parameters

[f] P values for combined effect of month and sampling point on parameters

The correlation of the physicochemical parameter of water samples are shown in Table 4.3. The pH for water samples had a positive correlation with conductivity, salinity, turbidity, COD, temperature and a negative correlation with BOD, TDS, DO, orthophosphate, nitrite, and nitrate(Table 4.3). The correlation of pH with temperature, TDS, DO, and nitrite for water samples (Figure 4.4) was significant ($p<0.05$). BOD for water samples had a positive correlation with conductivity, salinity, TDS, turbidity, orthophosphate, COD, nitrite, and nitrate (Table 4.3) and a negative correlation DO and temperature. Table 4.3 also shows that the correlation between BOD with TDS, and turbidity in water samples was insignificant, and the correlation of BOD in water sample was significant ($p<0.05$) with temperature, conductivity, salinity, DO, orthophosphate, COD, nitrate and nitrite.

Temperature of water samples (Table 4.3) had a positive correlation with conductivity, DO, nitrite, and a negative correlation with salinity, TDS, turbidity, orthophosphate, COD, and nitrate. These correlations were significant ($p<0.05$) only for temperature with salinity, DO, turbidity, orthophosphate, COD, and nitrite (Table 4.3). Conductivity for water samples (Table 4.3) had a positive correlation with salinity, TDS, turbidity, orthophosphate, COD, and nitrate and a negative correlation with DO and nitrite and the correlation of conductivity was significant ($p<0.05$) with salinity and orthophosphate (Table 4.3) also shows that the correlation of conductivity for water samples was insignificant with TDS, DO, turbidity, COD, nitrite and nitrate.

Salinity for water samples (Table 4.3) had a positive correlation with TDS, DO, turbidity, orthophosphate, COD and nitrate, and a negative correlation with nitrite. These correlations with salinity in water samples were significant ($p<0.05$) with orthophosphate, nitrate, COD, and was insignificant with TDS, DO, turbidity, and nitrite. There are several reports in the literature suggesting that EC and TDS are good and easy indicators of salinity (Oluyemi *et al.*,

2006; Akan *et al.*, 2008). The positive correlation between conductivity and salinity in the present study showed that the higher the conductivity the more the salinity in seepage.

Table 4.3 shows that TDS for water samples had a positive correlation with turbidity, COD, nitrite, and negative correlation with DO, orthophosphate, and nitrate and the correlations were insignificant. The DO for water samples (Table 4.3) had a positive correlation with orthophosphate and a negative correlation with turbidity, COD, nitrate and nitrite. The correlation was significant ($p<0.05$) with orthophosphate, COD, nitrite.

Turbidity for water samples (Table 4.3) had a positive correlation with orthophosphate, COD, and nitrate and a negative correlation with nitrite and all correlation were insignificant. Orthophosphate had a positive correlation with COD and a negative correlation with nitrate and nitrite. In addition the correlation was significant ($p<0.05$) with COD and insignificant with nitrate and nitrite. COD for water samples (Table 4.3) had a positive correlation with nitrate and a negative correlation with nitrite. The correlation for both nitrite and nitrate with COD were insignificant. Nitrate had a positive correlation with nitrite and the correlation was insignificant.

TABLE 4.3: Correlation matrix of physicochemical parameters for water samples

	pH	BOD	Temperature	EC	Salinity	TDS	DO	Turbidity	Ortho-P	COD	Nitrate	Nitrite
pH	1											
BOD	-0,0503	1										
Temperature	0,2075*	-0,5933*	1									
Conductivity	0,0415	0,1173*	0,0064	1								
Salinity	0,0379	0,2685*	-0,1843*	0,2731*	1							
TDS	-0,1011*	0,0184	-0,0418	0,021	0,0359	1						
DO	-0,2956*	-0,582*	0,3268*	-0,0486	0,0017	-0,0219	1					
Turbidity	0,0266	0,0786	-0,1212*	0,0178	0,0687	0,0213	-0,088	1				
Ortho-P	-0,0914	0,1827*	-0,1343*	0,2563*	0,6206*	-0,0091	0,1874*	0,0561	1			
COD	0,0512	0,3591*	-0,1856*	0,0269	0,3199*	0,0434	-0,1467*	0,0335	0,1472*	1		
Nitrate	-0,1561*	0,3184*	-0,218*	0,0423	0,176*	-0,0003	-0,2076*	0,0468	-0,0085	-0,0247	1	
Nitrite	-0,0543	0,171*	0,0905	-0,0489	-0,0961	0,055	-0,1921*	-0,012	-0,0475	0,0922	0,0647	1

TABLE 4.3: Correlation coefficient of water sample physicochemical parameters. COD: Chemical Oxygen Demand; BOD: Biological Oxygen Demand; TDS: Total Dissolved Oxygen; DO: Dissolved Oxygen; EC: Conductivity; Ortho-P: orthophosphate.

*= $P<0.05$ significant variation

The Mean and standard deviation (SD) results for the physicochemical parameter analyses of the soil samples are given in Table 4.4 and their P-value including the F-value and their significance are given in Table 4.5. Soil pH results for soil samples (Table 4.4) ranged from 6.28 to 8.43. The results varied significantly ($p<0.05$) monthly and the results also varied significantly ($p<0.05$) in sampling points (Table 4.5). The combined effect of sampling point and sampling month on pH varied significantly ($p<0.05$). Temperature results for soil samples (Table 4.4) ranged from 12 °C to 25.5 °C. The values obtained varied significantly ($p<0.05$) monthly and results did not vary significantly in sampling (Table 4.5). Table 4.5 also shows that the combined effect of sampling month and sampling point for temperature varied significantly ($p<0.05$).

Results for soil electrical conductivity (Table 4.4) ranged from 0.11mS/cm to 1.37mS/cm. The results soil electrical conductivity (Table 4.5) varied significantly ($p<0.05$) monthly and with sampling points ($p<0.1$) and the results for the combined effect of sampling month and sampling point for electrical conductivity also varied significantly ($p<0.05$). Salinity results for soil samples (Table 4.5) ranged from 0.01 psu to 0.13 psu. The results varied significantly ($p<0.05$) monthly and in sampling points. The results for the combined effect on sampling points and sampling months also varied significantly ($p<0.05$). TDS of soil samples ranged from 0.01 g/L to 0.88 g/ L. The results varied significantly ($p<0.05$) monthly and the variation in results varied significantly in sampling points. The results for the combined effect also varied significantly ($p<0.05$) (Table 4.5).

COD results for soil samples ranged from 40 mg/L to 304 mg/L (Table 4.4). The results varied significantly ($p<0.05$) monthly and the results also varied significantly ($p<0.05$) in sampling points (Table 4.5). The combined effect of sampling points and sampling month varied significantly ($p<0.05$). Dissolved oxygen for soil samples ranged from 5.31 mg/L to 8.45 mg/L (Table 4.4). The results varied significantly ($p<0.1$) monthly and also in sampling points

($p<0.05$) and the combined effects of sampling point and sampling month for DO also varied significantly ($p<0.05$) (Table 4.5).

Results for soil orthophosphate (Table 4.4) ranged from 7.35 mg/L to 255 mg/L and the results varied significantly ($p<0.05$) every sampling month and also varied significantly ($p<0.05$) in sampling points (Table 4.5). The combined effect of sampling point and sampling month on orthophosphate varied significantly ($p<0.05$). Results for nitrite ranged from 9 mg/L to 142 mg/L (Table 4.4) and the results varied significantly ($p<0.05$) every sampling month and also varied significantly ($p<0.05$) in sampling points (Table 4.5). The combined effect of sampling point and sampling month on nitrite varied significantly ($p<0.05$). Results for nitrate for soil samples (Table 4.4) ranged from 32.5 mg/L to 475 mg/L and the results varied significantly ($p<0.05$) every sampling month and also varied significantly ($p<0.05$) in sampling points (Table 4.5). The combined effect of sampling point and sampling month on nitrate varied significantly ($p<0.05$).

TABLE 4.4: Results for physicochemical parameters of soil samples

Period	Sampling point	Parameters									
		pH	Temp.	Sal.	EC	TDS	COD	DO	Ortho-P	NO_2	NO_3
March	**Enc-S**	6.75±0.37	25.00±1.0	0.06±0.03	0.54±0.01	0.66±0.10	242.67±4.73	7.68±0.21	62.27±6.72	56.23±3.15	152.7±46.89
	Enc S-20m	7.2±0.03	13±0.0	0.03±0.01	0.48±0.0	0.49±0.02	258±2.15	7.91±0.1	53.61±0.07	46.37±0.67	79.5±0.57
	Enc S-100m	6.67±0.01	23±0.0	0.01±0.00	0.39±0.0	0.37±0.00	159±2.89	7.69±0.3	27.19±0.9	39.26±0.1	51±2.01
	CW S-20m	6.54±00	25.5±0.0	0.05±0.01	0.45±0.0	0.29±0.01	217±3.05	8.03±0.2	33.38±0.54	12.67±0.10	273.5±1.15
	CW S-100m	6.91±0.01	22±0.0	0.02±0.01	0.33±0.0	0.13±0.00	152±1.89	8.45±0.1	19.01±0.31	11.05±1.22	117.5±1.09
April	**Enc-S**	6.95±0.23	20.17±0.76	0.05±0.01	0.28±0.04	0.13±0.02	141.13±2.52	7.54±0.10	52.40±3.15	49.39±6.57	162.17±35.3
	Enc S-20m	7.4±0.03	13±0.0	0.02±0.00	0.22±0.0	0.16±0.01	108±3.75	7.61±0.1	40.50±0.1	29.77±0.2	87±2.06
	Enc S-100m	7±0.02	20±0.0	0.07±0.01	0.12±0.0	0.10±0.01	91±3.12	7.81±0.2	21.37±0.3	10.84±0.4	64.5±1.45
	CW S-20m	6.28±0.01	18.5±0	0.03±0.01	0.14±0.0	0.14±0.01	117±2.56	7.55±0.1	28.50±0.3	41.91±0.1	280.5±0.7
	CW S-100m	7.03±0.01	16±0.0	0.01±0.01	0.11±0.0	0.05±0.01	86±4.35	8.01±0.0	14.02±0.29	12.35±1.31	110.5±0.37
May	**Enc-S**	7.14±0.48	17.67±1.53	0.07±0.03	0.75±0.48	0.24±0.02	169.33±8.50	6.69±0.60	37.83±2.70	48.33±5.75	135±28.83
	Enc S-20m	7.07±0.01	13±00	0.06±0.00	0.43±0.0	0.16±0.00	118±4.32	7.34±0.2	26.8±0.08	31.00±0.1	77.5±0.72
	Enc S-100m	6.98±0.01	17±00	0.09±0.02	0.32±0.0	0.09±0.02	82±3.65	7.56±0.1	12.05±0.29	15.50±0.03	32.5±1.74
	CW S-20m	7.98±0.02	15±00	0.01±0.00	0.34±0.0	0.07±0.00	152±2.50	7.36±0.3	21.7±0.35	52.50±0.1	217.5±1.3
	CW S-100m	7.11±0.03	14.5±00	0.02±0.01	0.18±00	0.05±0.0	92±1.55	7.71±0.3	10.17±0.49	10.25±0.07	122.5±1.75
June	**Enc-S**	6.91±0.52	14.00±1.00	0.04±0.02	0.65±0.41	0.22±0.04	122.67±4.73	5.64±0.55	24.37±0.96	29±7.72	272.83±19.8
	Enc S-20m	6.97±0.01	13±00	0.01±0.00	0.33±0.0	0.14±0.01	98±2.56	7.14±0.3	13.1±0.36	13.9±0.10	135±1.10
	Enc S-100m	6.87±0.02	12±00	0.01±0.00	0.28±0.0	0.08±0.01	41±1.25	7.48±0.4	9.2±0.30	9.6±0.37	50±0.42
	CW S-20m	7.73±0.02	16±00	0.03±0.01	0.30±0.0	0.15±0.02	107±2.55	7.44±0.1	15±0.14	21±0.29	225±1.81
	CW S-100m	6.93±0.01	14±00	0.00±0.00	0.19±0.0	0.06±0.00	72±3.01	7.75±0.1	7.35±0.27	9.23±0.34	112.5±2.00
July	**Enc-S**	8.01±0.60	13.33±2.08	0.05±0.01	0.73±0.08	0.29±0.15	260.17±9.57	5.31±0.40	41.82±2.46	79.67±95.62	241.67±27.5
	Enc S-20m	7.41±0.02	13±00	0.01±0.00	0.42±0.0	0.11±0.01	140±2.43	6.02±0.1	21.7±0.58	17±0.13	130±1.15
	Enc S-100m	7.67±0.02	12±00	0.01±0.00	0.31±0.0	0.03±0.01	124±1.46	6.25±0.0	10±0.26	9.01±0.39	52.2±0.96
	CW S-20m	8.15±0.01	15±00	0.04±0.02	0.48±0.0	0.54±0.01	260±2.03	7.10±0.3	64±0.17	71±0.32	301.5±2.78
	CW S-100m	7.99±0.02	13±00	0.01±0.00	0.33±00	0.01±0.01	108±2.99	7.89±0.2	24±0.20	11±0.19	90±1.42
August	**Enc-S**	7.82±0.34	13.17±0.29	0.02±0.01	0.28±0.02	0.15±0.03	266±4.58	6.12±0.13	137.67±6.43	17.33±2.52	146.67±12.6
	Enc S-20m	7.76±0.01	13±00	0.01±0.00	0.21±0.0	0.09±0.01	140±3.45	6.69±0.2	45±0.09	9±0.51	80±1.66
	Enc S-100m	6.95±0.01	12.5±00	0.01±00	0.23±0.4	0.011±00	40±2.18	7.01±0.1	19±0.21	8±0.21	75±1.38
	CW S-20m	8.43±0.01	16±00	0.13±0.02	1.37±0.0	0.88±0.01	304±2.79	6.52±0.4	255±0.68	142±0.15	475±1.19
	CW S-100m	7.75±0.02	14±00	0.02±0.01	0.54±0.0	0.03±0.01	152±4.04	6.85±0.2	48±0.31	12±0.31	100±1.74
Standards		6.5-8	<40 °C	0.1 psu	2 mS/cm	≤500 mg/L	≤ 200mg/L	≥ 5mg/L	≤ 5 mg/L	≤ 13 mg/L	≤ 120 mg/L

Temp: Temperature; EC: Electrical Conductivity; TDS: Total Dissolved Solids; COD: Chemical Oxygen Demand; DO: Dissolved Solids; Ortho-P: Orthophosphate; NO_2: Nitrite; NO_3: Nitrate. All parameters are expressed in mg/L except for temperature (in degrees Celsius), salinity (in practical salinity unit), and EC (in micro-Siemens per centimetre), and TDS (grams per litre). Standards were adopted from FME, Government Gazette and DWAF (1998). Results highlighted in red were those that exceeded the recommended standards.

TABLE 4.5 The P-value and F-value for physicochemical results of soil samples.

and F values	Parameters									
	pH	Temp.	Salinity	EC	TDS	COD	DO	Ortho-P	NO_2	NO_3
F values [a]	26.03	20.68	15.96	7.62	17.69	20.58	17.37	20.57	48.21	2.80
P values [b]	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*	0.07**	0.00*	0.00*	0.02*
F values [c]	4.88	0.86	8.80	10.32	5.88	9.74	7.64	4.74	13.63	38.77
P values [d]	0.00*	0.53	0.00*	0.06**	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*
F values [e]	2.09	6.88	8.50	6.56	9.34	8.62	3.32	9.13	5.38	8.36
P values [f]	0.03*	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*

Temp: Temperature; EC: Electrical Conductivity; BOD: Biological Oxygen Demand; TDS: Total Dissolved Solids; COD: Chemical Oxygen Demand; DO: Dissolved Solids; Ortho-P: Orthophosphate; NO_2: Nitrite; NO_3: Nitrate.

*P<0.05, **P<0.1: significant variation

Values are expressed in milligrams per litre except in pH, temperature (in degrees Celsius), salinity (in practical salinity unit), and EC (in micro-Siemens per centimetre), TDS (grams per litre).

[a] F values for parameters and month

[b] P values for parameters and month

[c] F values for parameters and sampling point

[d] P values for parameter and sampling point

[e] F values for combined effect of month and sampling point on parameters

[f] P values for combined effect of month and sampling point on parameter

Results for correlation coefficient physicochemical parameters for soil samples are presented in Table 4.6. pH for soil samples had a positive correlation with EC, orthophosphate, COD, nitrate, DO and a negative correlation with salinity, temperature, TDS, and nitrite. The correlation for pH for soil samples with temperature, EC, TDS, DO, orthophosphate, COD, and nitrate were significant at $p<0.05$ (Table 4.6). Temperature for soil samples had a positive correlation with EC, TDS, DO, COD, nitrite and a negative correlation with orthophosphate and nitrate. The correlation of soil temperature was significant ($p<0.05$) with EC, salinity, TDS, DO, COD, nitrite

EC for soil samples had a positive correlation (Table 4.6) with salinity, TDS, COD, nitrate, nitrite and a negative correlation with DO and nitrate. The correlation for soil EC was significant with salinity, TDS, DO, COD, nitrate and nitrite. Salinity had a positive correlation with TDS, orthophosphate, COD, and nitrate and nitrite and a negative correlation with DO. The correlations of soil salinity with TDS, DO, COD, nitrate and nitrite were significant at $p<0.05$ (Table 4.6). TDS for soil samples had a positive correlation with orthophosphate, COD, nitrate, nitrite and a negative correlation with DO. The correlations of soil TDS were significant ($p<0.05$) with orthophosphate, COD, nitrate and nitrite (Table4.6).

DO for soil samples (Table 4.6) had a positive correlation with nitrite and a negative correlation with orthophosphate, COD, and nitrate. Soil DO had a significant ($P<0.05$) correlation with orthophosphate, COD, and nitrate. Orthophosphate had a positive correlation with COD, nitrate and nitrite and all the correlations were significant at $p<0.05$ (Table 4.6). COD had positive correlation with nitrate and nitrite (Table 4.6) and the correlation were all significant at $p<0.05$. Nitrate and a positive correlation with nitrite and the correlation was significant ($p<0.05$)

TABLE 4.6: Correlation matrix of physicochemical parameters for soil samples

	pH	Temperature	EC	Salinity	TDS	DO	0rtho-P	COD	Nitrate	Nitrite
pH	1									
Temperature	-0,54*	1								
EC	0,1404*	0,1068*	1							
Salinity	-0,007	0,2909*	0,6358*	1						
TDS	-0,113*	0,5796*	0,3488*	0,5266*	1					
DO	-0,453*	0,4721*	-0,5026*	-0,2149*	-0,0658	1				
Ortho-P	0,2924*	-0,0650	-0,0889	0,0661	0,3472*	-0,1805*	1			
COD	0,3655*	0,1083*	0,3338*	0,2920*	0,5927*	-0,3851*	0,6897*	1		
Nitrate	0,1551*	-0,0994	0,1666*	0,5571*	0,1975*	-0,3276*	0,4373*	0,3679*	1	
Nitrite	-0,004	0,3241*	0,1625*	0,4737*	0,5120*	0,0577	0,6111*	0,4238*	0,5935*	1

TABLE 4.6: Correlation coefficient of soil sample physicochemical parameters. COD: Chemical Oxygen Demand; BOD: Biological Oxygen Demand; TDS: Total Dissolved Oxygen; DO: Dissolved Oxygen; EC: Conductivity; Ortho-P: orthophosphate.

*= $P<0.05$ significant variation

4.2 Results for bacteriological analyses

Results for viable cell counts of pig farm water samples are shown In Figures 4.1.1 to Figure 4.1.4. The viable cell count ranged from 1.30 x 10^3 cfu/mL to 1.89 x 10^8 cfu/mL in XLD agar (Figure 4.1.3) and the results did not vary significantly from sampling points but had a significant variation ($p<0.1$) monthly. In MacConkey agar the viable cell counts ranged between 3.90 x 10^2 cfu/mL to 7.90 x 10^8 cfu/mL (Figure 4.1.4), the variation of viable cell counts were insignificant across sampling months and sampling points. In nutrient agar (Figure 4.1.1) the viable cell counts ranged from 1.29 x 10^4 cfu/mL to 5.05 x 10^9 cfu/mL and the viable cell counts on nutrient agar had insignificant variation across sampling points and months. In EMB agar (Figure 4.1.2) the viable cell counts ranged from 5.00 x 10^1 cfu/mL to 1.24 x 10^8 cfu/mL and the viable cell counts variation was not significant with regards to sampling points and months.

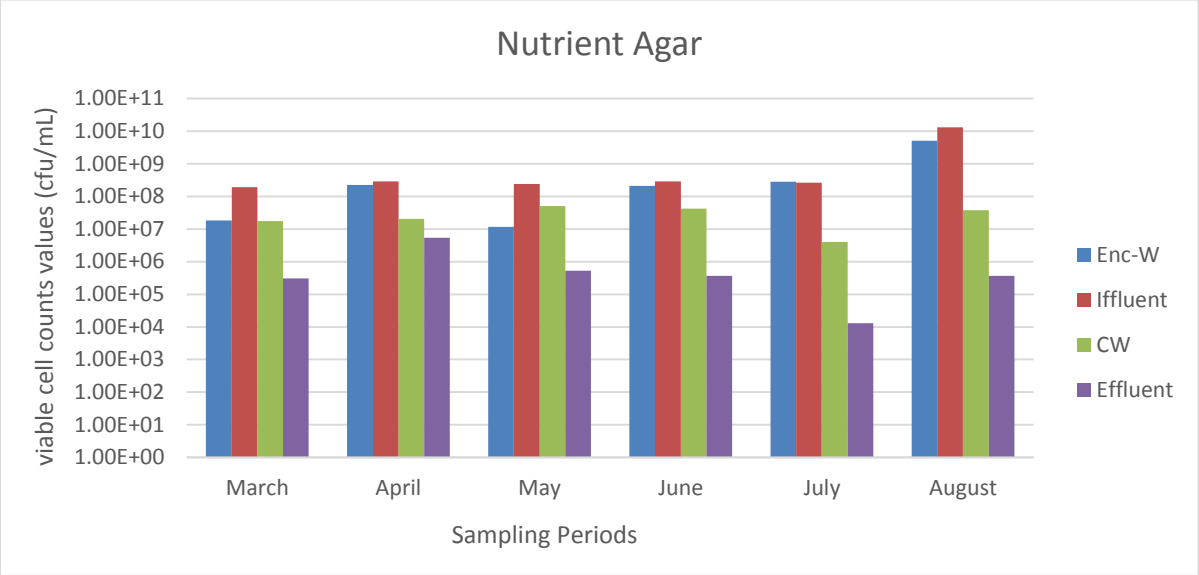

Figure 4.1.1: Results for bacteriological analyses of pig farm water samples on nutrient agar.
Key: Enc –W= Enclosure water; Influent = influent 2 m away from constructed wetland; CW = Constructed wetland; Effluent = Effluent 2 m away from constructed wetland.

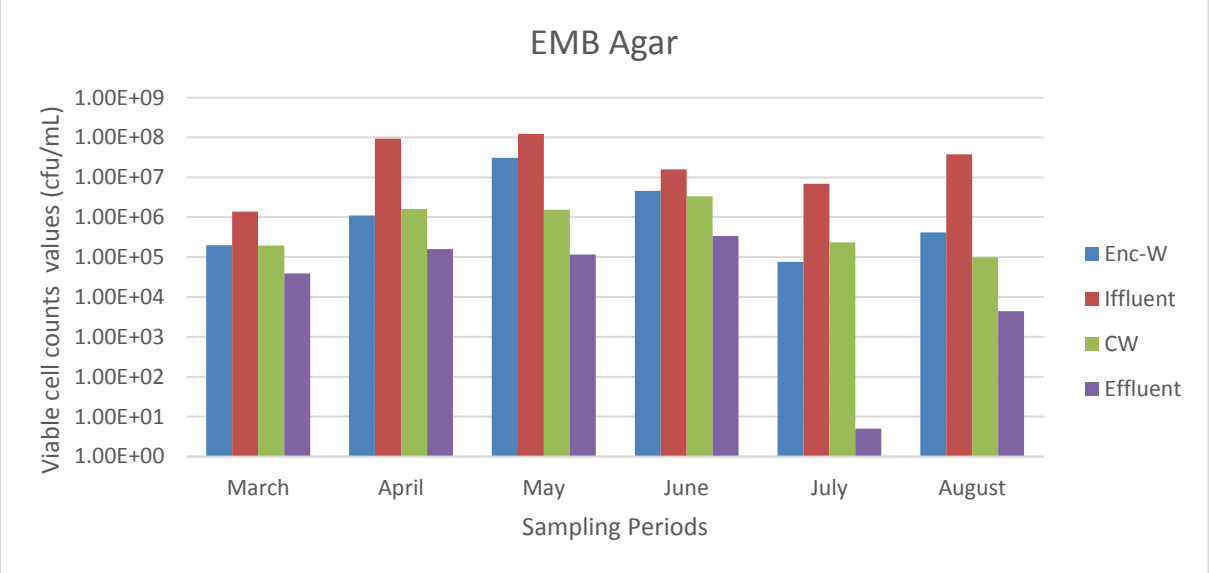

Figure 4.1.2: Results for Bacteriological analyses of pig farm water samples on EMB agar.
Key: Enc –W: Enclosure water; Influent = influent 2 m away from constructed wetland; CW = Constructed wetland; Effluent = Effluent 2 m away from constructed wetland.

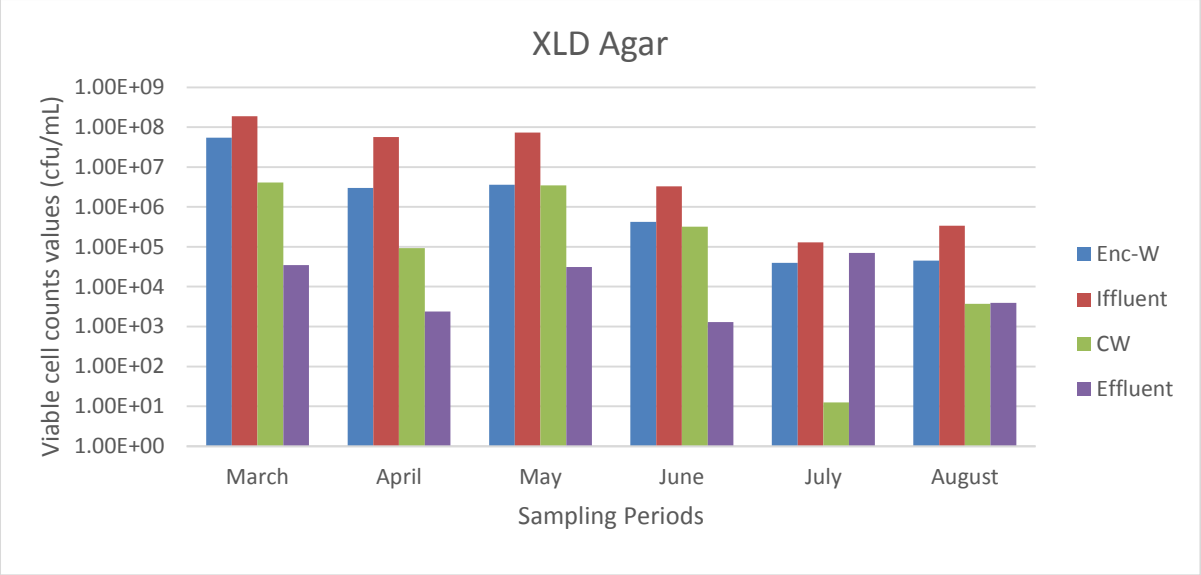

Figure 4.1.3: Results for Bacteriological analyses of pig farm water samples on XLD agar.
Key: Enc-W: Enclosure water; Influent = influent 2 m away from constructed wetland; CW = Constructed wetland; Effluent = Effluent 2 m away from constructed wetland.

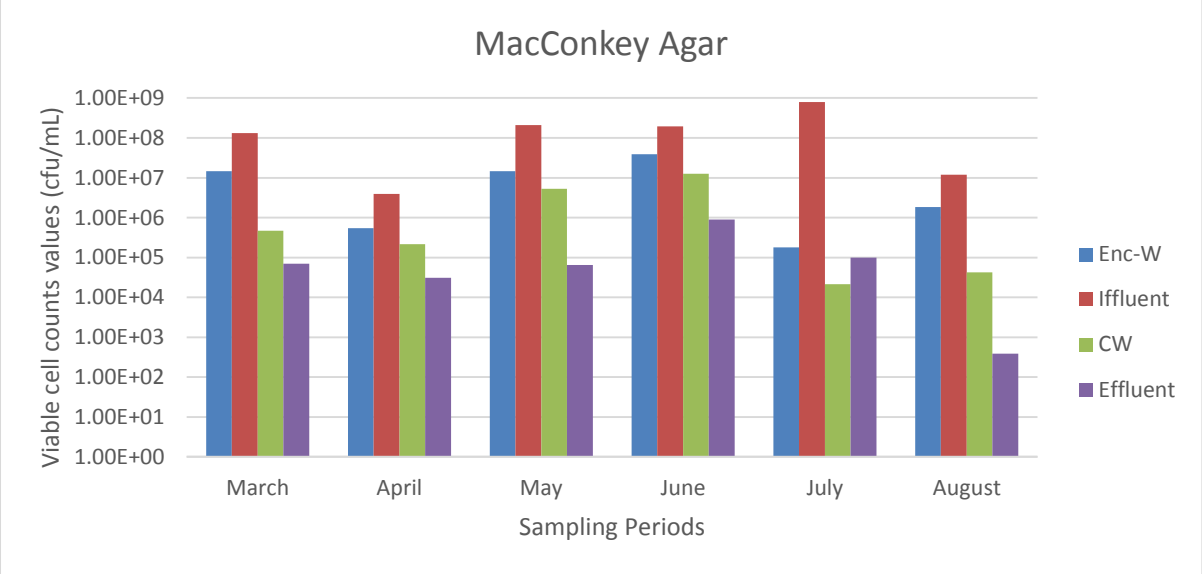

Figure 4.1.4: Results for bacteriological analyses of pig farm water samples on MacConkey agar. **Key: Enc – W: Enclosure water; Influent = influent 2 m away from constructed wetland; CW = Constructed wetland; Effluent = Effluent 2 m away from constructed wetland.**

Results for viable cell count of pig farm surface soil samples are shown in Figures 4.2.1 to Figure 4.2.4. The viable cells ranged from 1.9×10^1 cfu/mL to 1.22×10^8 cfu/mL in MacConkey agar (Figure 4.2.4), and results for viable cell counts varied significantly ($p<0.1$) monthly and also varied significantly ($p<0.1$) at sampling points. In XLD agar (Figure 4.2.3) viable cells ranged between 1.00×10^1 cfu/mL to 7.90×10^7 cfu/mL, and the viable cell counts on XLD agar did vary significantly monthly but varied significantly ($p<0.05$) at sampling points. In EMB agar (Figure 4.2.2) the viable cells ranged from 3.10×10^2 cfu/mL to 7.80×10^7 cfu/mL. The viable cell counts on EMB agar varied significantly ($p<0.05$) monthly and the variation in sampling points was insignificant. In nutrient agar (Figure 4.2.1) the viable cells ranged from 3.10×10^4 cfu/mL to 1.91×10^{10} cfu/mL. The viable cells counts on Nutrient agar varied significantly ($p<0.05$) monthly and the variation between sampling points was insignificant.

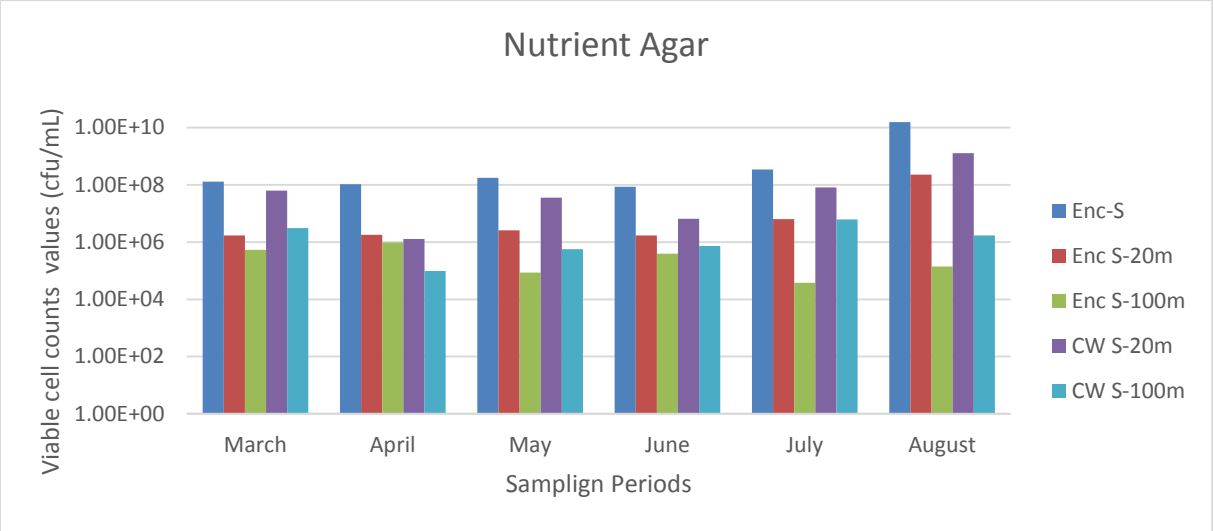

Figure 4.2.1: Results for bacteriological analyses of pig farm surface soil samples on Nutrient agar. **Key: Enc-S = Enclosure soil; Enc S-20m = Soil 20 m away from enclosures; Enc S-100m = Soil 100 m away from enclosures; CW S-20m = Soil 20 m away from constructed wetlands; CW S-100m = Soil 100 m away from constructed wetlands.**

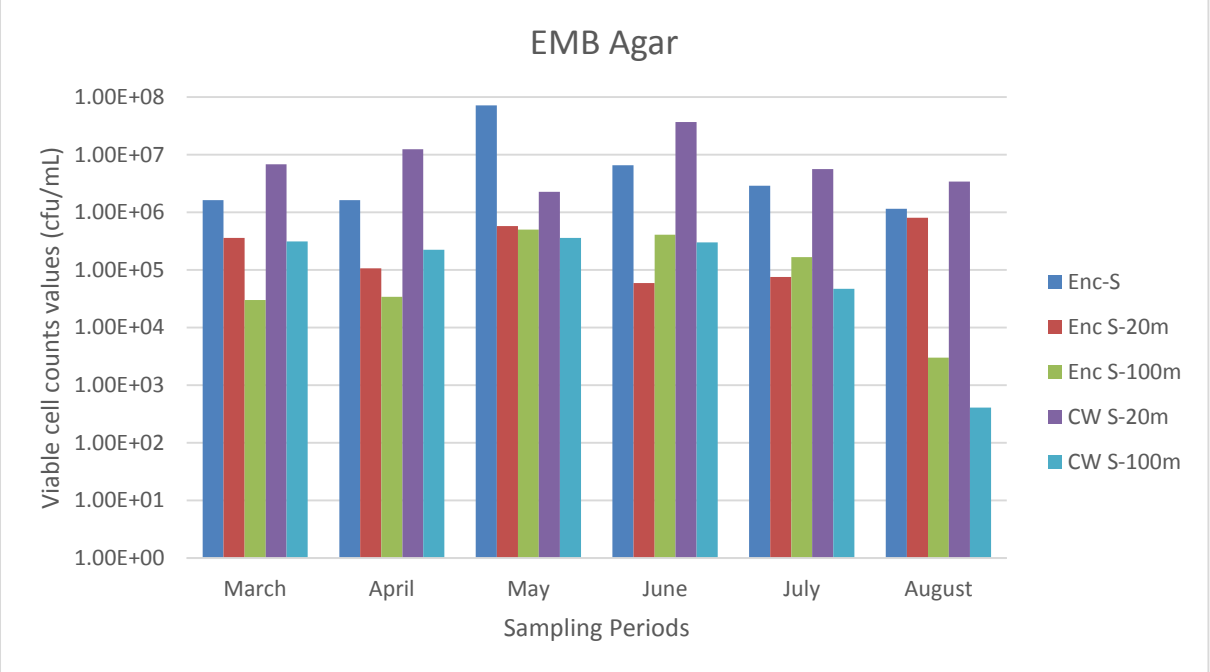

Figure 4.2.2: Results for bacteriological analyses of pig farm surface soil samples on EMB agar. **Key: Enc-S = Enclosure soil; Enc S-20m = Soil 20 m away from enclosures; Enc S-100m = Soil 100 m away from enclosures; CW S-20m = Soil 20 m away from constructed wetlands; CW S-100m = Soil 100 m away from constructed wetlands.**

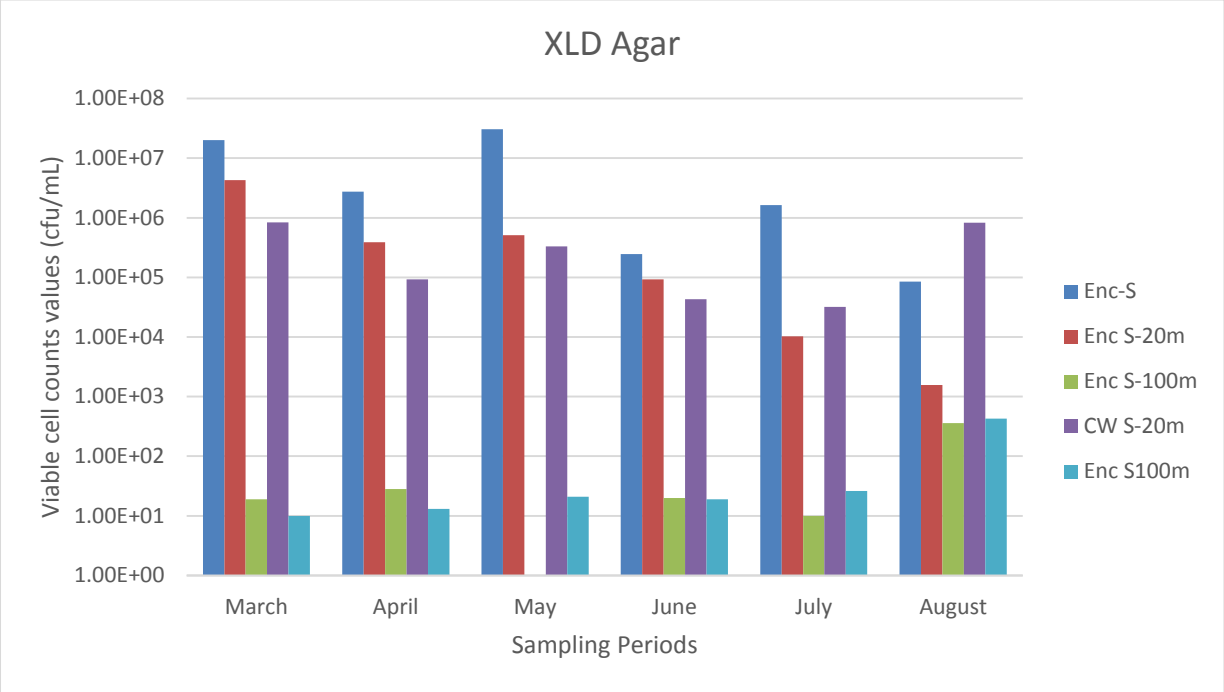

Figure 4.2.3: Results for bacteriological analyses of pig farm surface soil samples on XLD agar. **Key: Enc-S = Enclosure soil; Enc S-20m = Soil 20 m away from enclosures; Enc S-100m = Soil 100 m away from enclosures; CW S-20m = Soil 20 m away from constructed wetlands; CW S-100m = Soil 100 m away from constructed wetlands.**

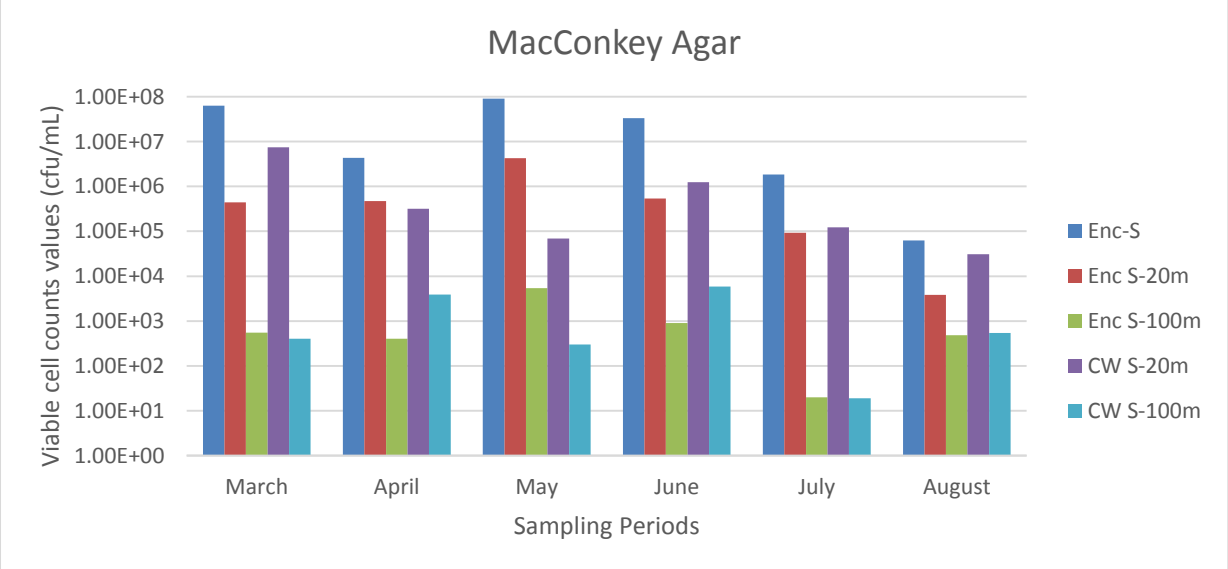

Figure 4.2.4: Results for bacteriological analyses of pig farm surface soil samples on MacConkey agar. **Key: Enc-S = Enclosure soil; Enc S-20m = Soil 20 m away from enclosures; Enc S-100m = Soil 100 m away from enclosures; CW S-20m = Soil 20 m away from constructed wetlands; CW S-100m = Soil 100 m away from constructed wetlands.**

Results for viable cell counts of pig farm soil 30 cm deep are shown in Figures 4.3.1 to Figure 4.3.4. The viable cells ranged from 3.50 x 10^2 cfu/mL to 4.80 x 10^7 cfu/mL in MacConkey agar (Figure 4.3.4). The results varied significantly ($p<0.05$) monthly and insignificantly at sampling points. The viable cell counts on MacConkey agar ranged between 0 cfu/mL to 8.90 x 10^6 cfu/mL in XLD agar (Figure 4.3.3). The results for viable cell counts on XLD agar varied significantly ($p<0.05$) monthly and insignificantly at sampling points. In EMB agar (Figure 4.3.2) the viable cell counts ranged from 2.40 x 10^1 cfu/mL to 8.50 x 10^7cfu/mL. The results for viable cell on EMB agar had insignificant variation monthly and also at sampling points. In Nutrient agar (Figure 4.3.1) the viable cells ranged from 3.10 x 10^4 cfu/mL to 2.44 x 10^{10} cfu/mL, and the results had insignificant variation monthly and also in sampling points.

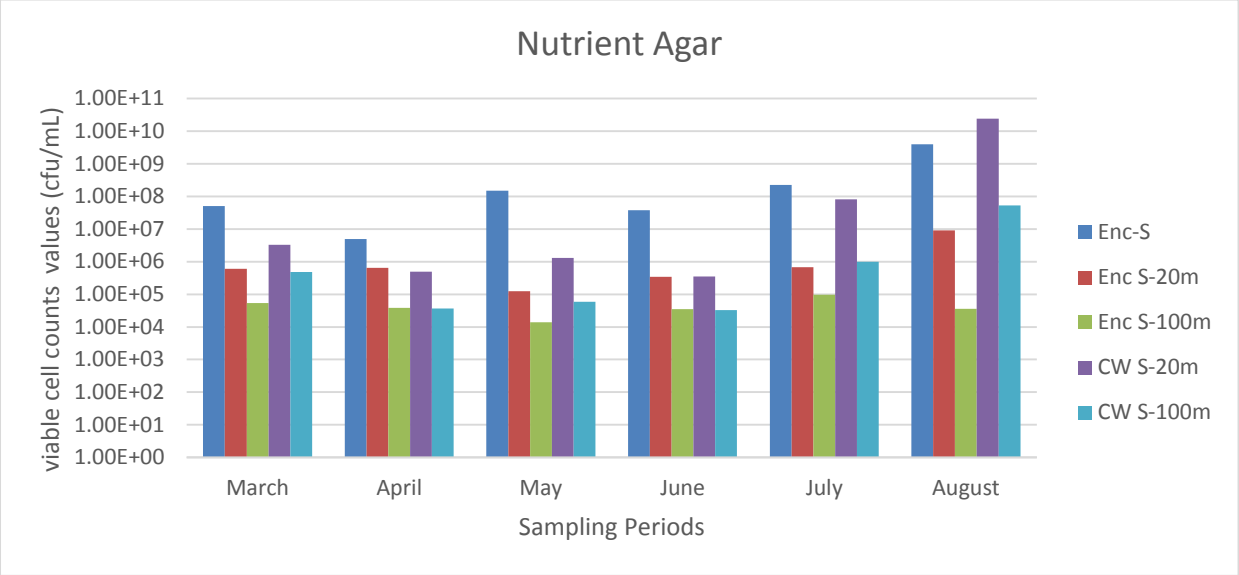

Figure 4.3.1: Results for bacteriological analyses of pig farm soil 30cm deep samples on Nutrient agar. **Key: Enc-S = Enclosure soil; Enc S-20m = Soil 20 m away from enclosures; Enc S-100m = Soil 100 m away from enclosures; CW S-20m = Soil 20 m away from constructed wetlands; CW S-100m = Soil 100 m away from constructed wetlands.**

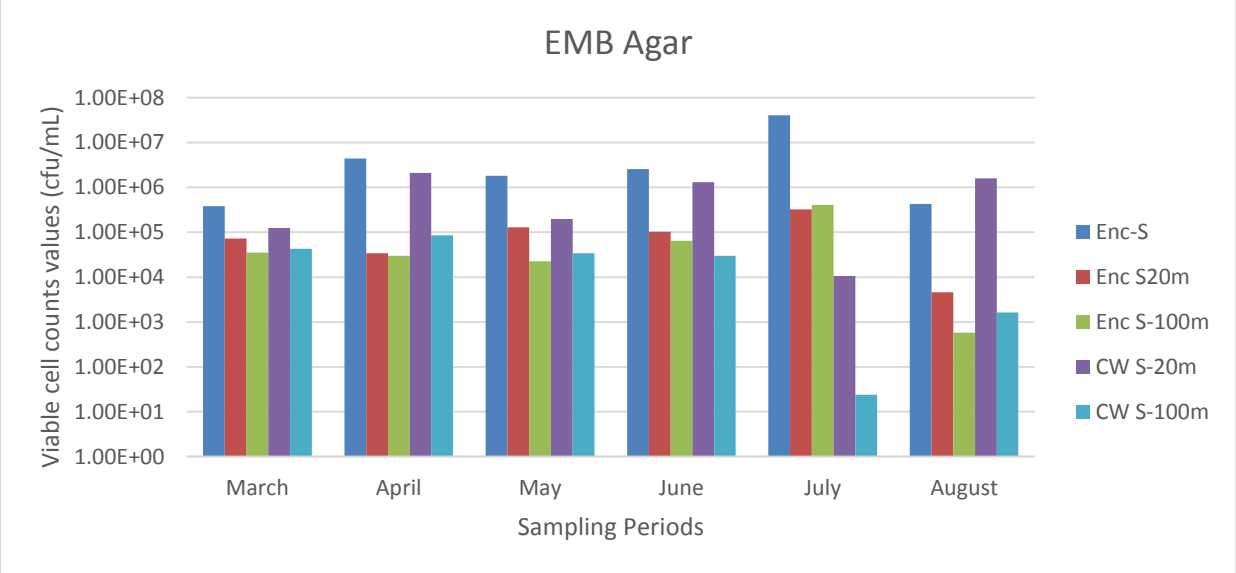

Figure 4.3.2: Results for bacteriological analyses of pig farm soil 30cm deep samples on EMB agar. **Key: Enc-S = Enclosure soil; Enc S-20m = Soil 20 m away from enclosures; Enc S-100m = Soil 100 m away from enclosures; CW S-20m = Soil 20 m away from constructed wetlands; CW S-100m = Soil 100 m away from constructed wetlands.**

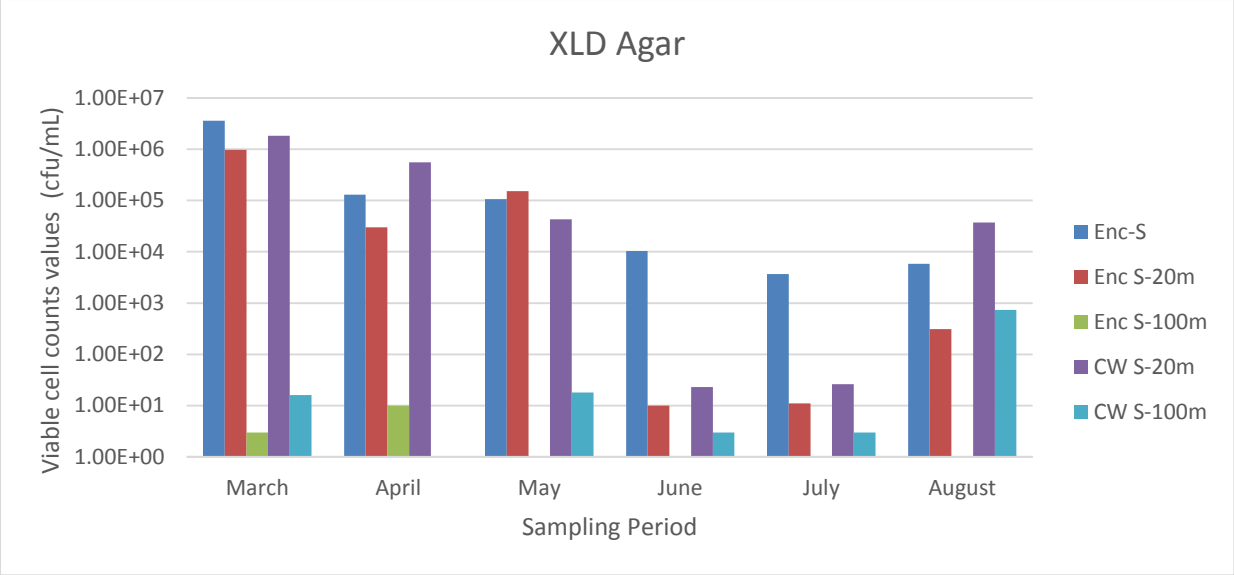

Figure 4.3.3: Results for bacteriological analyses of pig farm soil 30cm deep samples on XLD agar. **Key: Enc-S = Enclosure soil; Enc S-20m = Soil 20 m away from enclosures; Enc S-100m = Soil 100 m away from enclosures; CW S-20m = Soil 20 m away from constructed wetlands; CW S-100m = Soil 100 m away from constructed wetlands.**

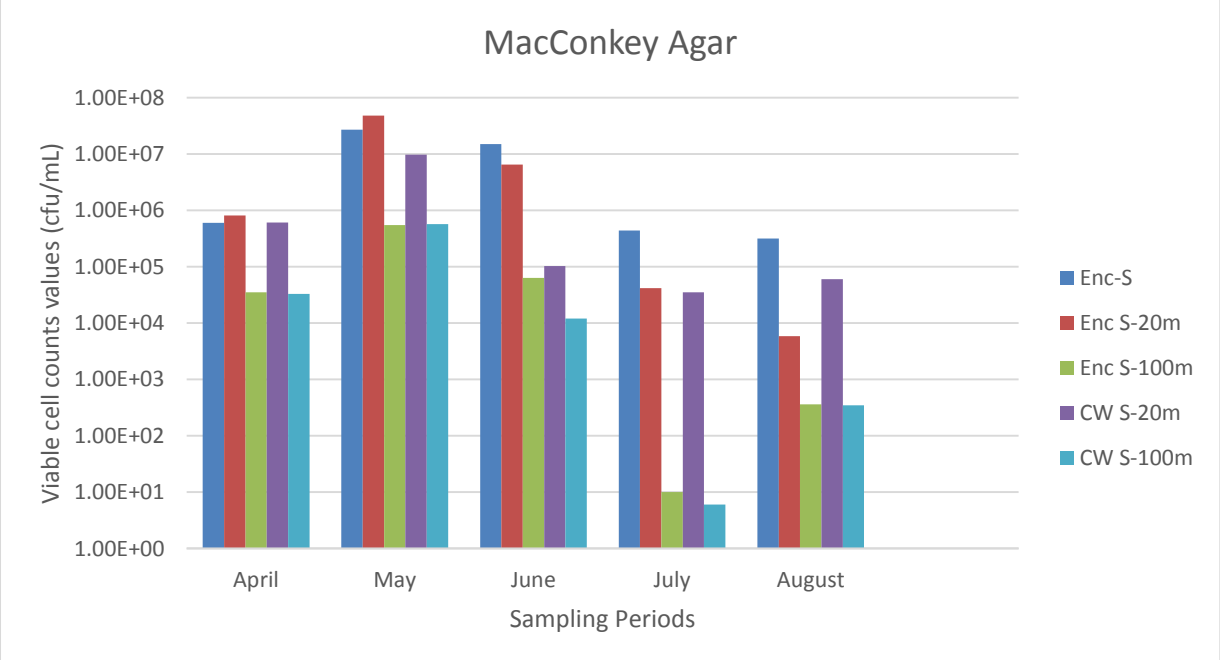

Figure 4.3.4: Results for Bacteriological analyses of pig farm soil 30cm deep samples on MacConkey agar. **Key: Enc-S = Enclosure soil; Enc S-20m = Soil 20 m away from enclosures; Enc S-100m = Soil 100 m away from enclosures; CW S-20m = Soil 20 m away from constructed wetlands; CW S-100m = Soil 100 m away from constructed wetlands.**

4.3 Results for identification of isolates using API 20E

The results for the Identification of isolates are shown in Table 4.7 and Figure 4.4.1 and Figure 4.4.2 shows API 20E test strips that showed positive identification of *E.coli 1* and *Salmonella* spp. Isolates were identified using API20E kit and the identification code was used to identify the microorganism on apiweb software. The identified isolates were *Pseudomonas luteola (Ps. luteola), Escherichia vulneris (E. vulneris), Salmonella choleraesuis* spp *arizonae , Escherichia coli 1(E. coli 1), Enterobacter cloacae, Pseudomonas flourescens/putida (Ps. flourescens/putida), Enterobacter aerogenes, Serratia ordoriferal, Pasteurella pneumotropica, Ochrobactrum antropi , Proteus vulgaris group, Proteus vulgaris, Salmonella* spp, *Aeromonas Hydrophila/caviae/sobria1, Proteus Mirabillis, Vibrio fluvials, Rahnella aquatillis, Pseudomonas aeruginosa (Ps. aeruginosa), Burkholderia Cepacia, Stenotrophomonas maltophilia (St. maltophilia), Shwenella putrefaciens, Klebsiela pneumonia, Cedecea davisa, Serratia liquefaciens, Serratia plymuthica, Enterobacter sakaziki, Citrobacter braakii, Enterobacter amnigenus 2, Yersinia pestis, Serratia ficaria, Enterobacter gergoriae, Enterobacter amnigenus 1, Serratia marcescens, Raoutella terrigena, Hafnia alvei 1, Providencia rettgeri, Pantoa*

TABLE 4.7: Results of identified of Isolates

Identified Bacteria	**Source (Soil or water sample)**
Ps. luteola	Soil and water
Escherichia vulneris	water
Salmonella choleraesuis spp *arizonae*	Soil and water
Escherichia coli 1	Soil and water
Enterobacter cloacae	water
Ps. flourescens/putida	water
Enterobacter aerogenes	Soil and water
Serratia ordoriferal	water
Pasteurella pneumotropica	Soil and water
Ochrobactrum antropi	Water
Proteus vulgaris group	Water and soil
Proteus vulgaris	Water and soil
Salmonella spp	water
Aeromonas Hydrophila/caviae/sobria1	water
Proteus mirabillis	water
Vibrio fluvials	water
Rahnella aquatillis	water
Ps. aeruginosa	Soil and water
Burkholderia cepacia	Soil
Stenotrophomonas maltophilia	Water and soil
Shwenella putrefaciens	soil
Klebsiela pneumoniae	Soil and water
Cedecea davisae	water
Serratia liquefaciens	Soil and water
Serratia plymuthica	water
Enterobacter sakaziki	Soil and water
Citrobacter braakii	Soil and water
Enterobacter amnigenus 2	Soil and water
Yersinia pestis	water
Serratia ficaria	water
Enterobacter gergoriae	water
Enterobacter amnigenus 1	Soil and water
Serratia marcescens	Soil and water
Raoutella terrigena	water
Hafnia alvei 1	water
Providencia rettgeri	water
Pantoa	water

Table shows results of bacteria isolated from water and soil samples identified with API 20E kit. Some Isolates were only detected in soil samples and the other were mostly detected in water samples, while some were detected in soil and water samples in the vicinity of pig farm

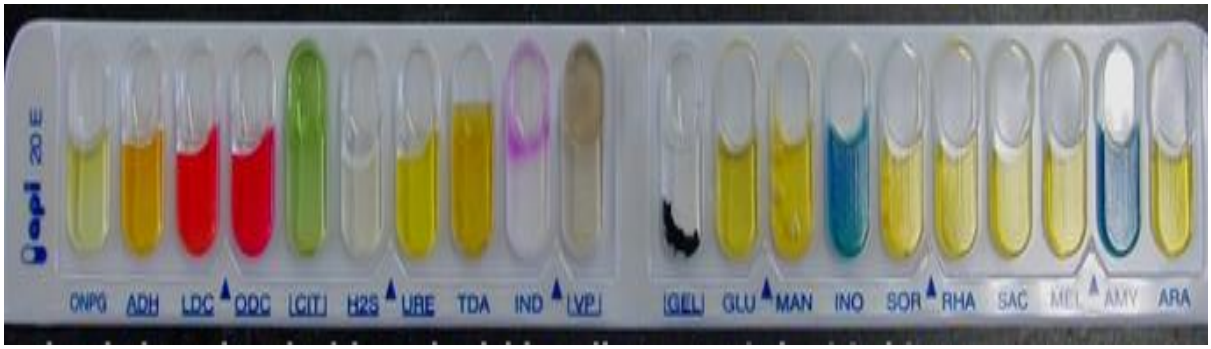

Figure 4.4.1: Results of API 20E for *Escherichia coli 1*

Figure 4.4.2: Results for API 20E for *Salmonella* spp

4.4 Results for susceptibility analyses

The results for susceptibility analyses using 19 different antibiotics are given in Figure 4. 5. The Figure shows the resistance (R), susceptibility (S) and intermediate (I) levels of isolates to tested antibiotics. The results showed that, 75 % of isolates were resistant to penicillin G, 63 % to sulphamethaxazole, 71 % to vancomycin, 62 % to tilmocozin, 80 % to oxytetracycline and spectinomycin, 79 % to lincomycin, and 54 % to trimethoprim. A large proportion of isolates were susceptible to norflaxacin (84 %) and ceftadizime (82 %). In addition the isolates were highly susceptible to tetracycline (63 %) and nalidixic acid (53 %) and with respect to ampicillin and apramycin. the percentage of susceptible isolates (47 % and 43 % respectively) compared to those that were resistant (46 % for both ampicillin and apramycin) did not vary too much.

Results for antibiotic Susceptibility test of 18 different antibiotics tested on Isolates

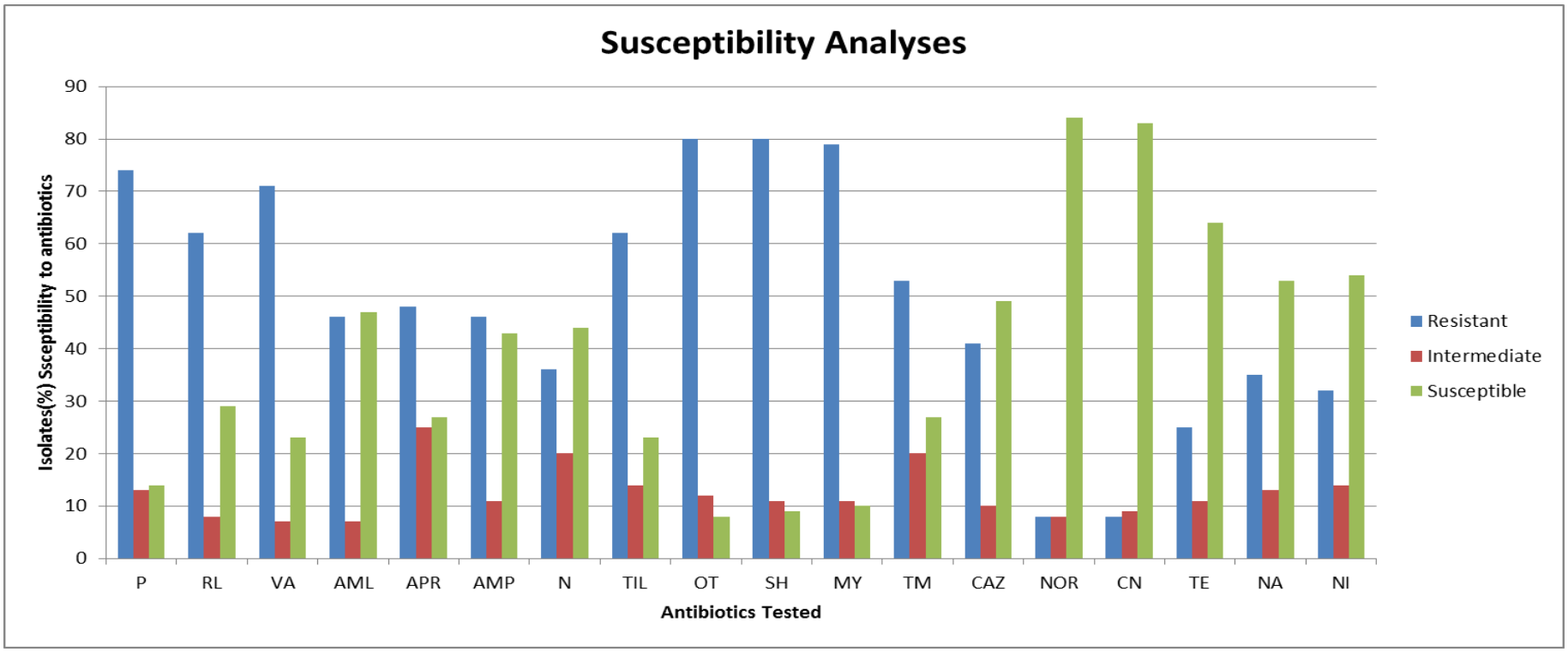

FIGURE 4.5: Results for susceptibility analyses of 18 different antibiotics used to test antibiotic sensitivity in isolates. Penicillin G (P), Sulphamethaxazole (RL), Vancomycin (VA), Ampicillin (AML), Amoxicillin (APR), Apramycin (AMP), Neomycin (N), Tilmocosin (TIL), Oxytetracyclin (OT), Spectinomycin (SH), Lincomycin (MY), Trimethoprim (TM). Nitrofurantoin (NI), Nalidixic Acid (NA), Norflaxacin (NOR), Oxytetracyclin (OT), Tetracycline (TE), Gentamycin (CAZ), Ceftadizime (CN)

This Figure shows sensitivity of isolates, where nearly 70% to 80% sensitivity were observed in five antibiotics tested. The graph also shows that most isolates were highly resistant to Penicillin G, Vancomycin, Oxytetracyclin, Spectinomycin, and were also highly susceptible to Norflaxacin and Ceftadizime.

TABLE 4.8: The predominant multiple antibiotic resistance phenotype and Multidrug Resistant Index of isolates

Phenotype antibiotics resistance			**Multidrug resistant index (MDRI)**			
Phenotype	Number(s) of Isolates	Percentage (%)	Isolates	MDRI (%)	Isolates	MDRI (%)
VA-SH-TM	2	10.50	EFF4a	100	CW1-3	83
SH-MY-TM	2	10.50	EFF6	100	IFF4	25
RL-APR-TIL-SH-MY-TM	2	10.50	EW8	100	IFF5	25
P-VA-TIL-OT-SH-MY	2	10.50	EW1	100	IFF6	83
P-RL-VA-TIL-OT-SH-MY	2	10.50	EW1	75	IFF7	25
P-RL-VA-APR-TIL-OT-MY	2	10.50	EW10	58	IFF9	92
P-RL-VA-APR-N-TIL-OT-SH-MY	6	31.60	EFF3	100	IFF8	58
P-RL-VA-APR-AMP-N-TIL-OT-SH-MY-TM	3	17.80	EW14	75	IFF1	75
P-RL-VA-AML-APR-AMP-TIL-OT-SH-MY-TM	3	17.80	EW11	75	IFF20	83
P-RL-VA-AML-APR-AMP-TIL-OT-SH-MY	4	21.05	EW12	67	IFF3	42
P-RL-VA-AML-APR-AMP-OT-SH-MY-TM	2	10.50	EW9	75		
P-RL-VA-AML-APR-AMP-N-TIL-OT-SH-MY-TM	15	78.95	EW7	100		
P-RL-VA-AML-APR-AMP-N-TIL-OT-SH-MY	4	21.05	EFF2	100		
P-RL-VA-AML-AMP-TIL-OT-SH-MY-TM	2	10.50	EFF5	100		
P-RL-VA-AML-AMP-SH-MY-TM	2	10.5	EFF15	100		
P-RL-VA-AML-AMP-N-TIL-OT-SH-MY	2	10.50	EFF1	92		
P-AML-AMP-OT-SH-TM	2	10.50	EW3	75		
OT	2	10.50	EFF4	100		
MY	2	10.50	EW2	83		

The Table shows the most occurring phenotype antibiotic resistant patterns and shows isolates with the highest MDIR where 10 isolates showed 100% MDRI. Isolate had up to 19 phenotype multiple resistance. Most isolate had predominant P-RL-VA-AML-APR-AMP-N-TIL-OT-SH-MY-TM (78.95%), and P-RL-VA-APR-N-TIL-OT-SH-MY (31.60%) phenotype multiple resistance. About 55 isolates had more than five phenotype antibiotic resistance patterns where Penicillin G (P), Sulphamethaxazole (RL), Vancomycin (VA), Ampicillin (AML), Tilmocosin (TIL), Oxytetracyclin (OT), Spectinomycin (SH), Lincomycin (MY) were the most predominant.

The results for phenotypic antibiotic resistance and Multidrug Resistance Index are shown on Table 4.8. Among 91 phenotype patterns observed resistant. The phenotypes that was mostly observed were P-RL-VA-AML-APR-AMP-N-TIL-OT-SH-MY-TM in 15 isolates, and P-RL-VA-APR-N-TIL-OT-SH-MY in 6 isolates. Some of resistance patterns were not frequently detected, and the isolates were found to be resistant to only 1 antimicrobial agent. Multidrug Resistance Index was also observed to be high with 10 isolates having an MDRI of 100 % and 12 isolates had MDRI ranging from 75 % to 92 %. The MDRI ranged from 25 % to 100 % with the mean of 78.14 %.

4.5 Results for PCR detection of resistance gene

Results for the detection of resistance gene are shown in Table 4.9.1, 4.9.2 and 4.9.3 and the gel electrophoresis results are also shown in Figure 4.6.1 and Figure 4.6.2. The results show that most isolates showed to possess *aa (6')-le-aph (2")-la gene, aph (2")-lb gene, aph (3")-llla* genes for aminoglycosides resistance, *Sul1* gene and *Sul2* gene for Sulphamethaxazole resistance, *VanA, VanB* and *VanC2/C3* resistance genes for vancomycin, *Inu A* and *Inu C* resistance genes for lincomycin, *OtrA* and *OtrB* resistance genes for oxytetracyclines and bla_{TEM} and bla_{PE} resistance gene for beta-lactamase resistance. Only 3 isolates namely *E. vulneris, Salmonella* spp, and *Cedecea davisa* were observed to have *aadA* resistance gene. *E. vulneris, Enterobacter cloacae, Ochrobactrum antropi, Ochrobactrum antropi, Enterobacter gergoriae, Enterobacter amnigenus 1, Pantoa* had *aph (2")-lc* resistance gene. Eleven Isolates had *aac (3')-lv,* four Isolates had *VanC* and *InuB,* 7 isolates had *InuF,* 6 isolate had bla_{SHV} and 8 isoaltes had $bla_{OXA.}$ Only *E.coli* had *aph (2")-ld* resistance gene and only a *Salmonella choleraesuis* spp *arizonae* had *VanD* resistance gene. *Proteus mirabilis* and *Enterobacter amnigenus* were the only isolates that had *InuD* gene.

TABLE 4.9.1: Results for detection of resistance genes in isolate

Isolates	Resistance genes						
	aadA	*aa(6')-le-aph(2")-la*	*aph(2")-lb*	*aph(2")-lc*	*aph(2")-ld*	*aph(3")-llla*	*ant(4')-la*
Ps. luteola	-	-	-	-	-	-	-
E. vulneris	+	+	+	+	-	-	-
Salmonella choleraesuis spp *arizonae*	-	+	-	-	-	+	-
E. coli 1	-	-	-	-	+	-	-
Enterobacter cloacae	-	-	+	+	-	+	-
Ps. flourescens/putida	-	-	-	-	-	-	-
Enterobacter aerogenes	-	-	+	-	-	+	-
Serratia ordoriferal	-	-	+	-	-	-	-
Pasteurella pneumotropica	-	+	-	-	-	-	-
Ochrobactrum antropi	-	+	+	+	-	+	-
Proteus vulgaris group	-	-	-	-	-	+	-
Proteus vulgaris	-	+	-	-	-	+	-
Salmonella spp	+	-	-	-	-	+	-
Aeromonas hydrophila/caviae/sobria1	-	+	+	-	-	+	-
Proteus mirabillis	-	-	-	-	-	+	-
Vibrio fluvials	-	+	-	-	-	+	-
Rahnella aquatillis	-	-	+	-	-	+	-
Ps. aeruginosa	-	-	-	-	-	-	-
Burkholderia cepacia	-	+	+	+	-	+	-
St. maltophilia	-	+	-	-	-	+	-
Shwenella putrefaciens	-	+	-	-	-	-	-
Klebsiela pneumoniae	-	-	-	-	-	-	-
Cedecea davisa	+	+	+	-	-	+	-
Serratia liquefaciens	-	-	+	-	-	+	-
Serratia plymuthica	-	-	+	-	-	+	-
Enterobacter sakaziki	-	-	+	-	-	+	-
Citrobacter braakii	-	+	-	-	-	+	-
Enterobacter amnigenus 2	-	-	-	-	-	+	-
Yersinia pestis	-	-	-	-	-	+	-
Serratia ficaria	-	+	+	-	-	+	-
Enterobacter gergoriae	-	-	+	+	-	-	-
Enterobacter amnigenus 1	-	+	-	+	-	-	-
Serratia marcescens	-	-	-	-	-	+	-
Raoutella terrigena	-	-	-	-	-	+	-
Hafnia alvei 1	-	-	-	-	-	+	-
Providencia rettgeri	-	+	+	-	-	+	-
Pantoa	-	+	+	+	-	+	-
Total	3	16	16	7	1	26	0

The Table shows that most isolates had *aa (6')-le-aph (2")-la, aph (2")-lb, aph (3')-IIIa* resistance genes. *ant (4')-la* resistant gene was not detected in all isolates and less than ten isolates had *aadA, aph (2")-lc* and *aph (2")-ld* resistance genes respectively. Only *Escherichia coli 1* had *aph (2")-ld* resistance gene

TABLE 4.9.2 Results for detection of resistance genes in isolate (Continues)

Isolates	Resistance genes									
	VanA	*VanB*	*VanC*	*VanC2/C3*	*VanD*	*InuA*	*InuB*	*InuC*	*InuD*	*InuF*
Ps. luteola	+	+	-	+	-	-	-	+	-	-
E. vulneris	+	+	-	-	-	+	-	-	-	-
Salmonella choleraesuis spp *arizonae*	+	+	-	+	+	-	-	-	-	-
E. 1	+	+	-	-	-	-	-	-	-	-
Enterobacter cloacae	+	+	+	+	-	+	-	-	-	-
Ps. flourescens/putida	+	+	-	+	-	-	-	-	-	-
Enterobacter aerogenes	+	+	-	+	-	+	-	+	-	-
Serratia ordoriferal	-	+	-	+	-	-	-	-	-	-
Pasteurella pneumotropica	+	+	-	+	-	+	-	+	-	-
Ochrobactrum antropi	-	-	-	-	-	+	-	+	-	-
Proteus vulgaris group	-	+	-	+	-	-	-	-	-	-
Proteus vulgaris	+	+	-	+	-	+	-	+	-	+
Salmonella spp	+	+	-	-	-	+	-	-	-	+
Aeromonas Hydrophila/caviae/sobria1	+	+	-	+	-	+	-	+	-	+
Proteus mirabillis	+	+	+	-	-	-	-	+	+	-
Vibrio fluvials	+	+	-	+	-	+	-	-	-	-
Rahnella aquatillis	-	-	-	-	-	-	-	-	-	-
Ps. aeruginosa	+	+	-	+	-	+	-	+	-	-
Burkholderia cepacia	-	-	-	-	-	+	-	-	-	-
St. maltophilia	-	-	-	-	-	-	-	+	-	+
Shwenella putrefaciens	-	-	-	-	-	+	-	-	-	-
Klebsiela pneumoniae	+	+	-	+	-	+	-	-	-	-
Cedecea davisa	-	-	-	-	-	+	-	+	-	
Serratia liquefaciens	-	+	+	-	-	+	-	-	-	+
Serratia plymuthica	-	-	-	-	-	+	+	-	-	-
Enterobacter sakaziki	+	+	-	-	-	+	-	-	-	-
Citrobacter braakii	-	+	-	+	-	-	-	+	-	-
Enterobacter amnigenus 2	+	+	-	-	-	-	+	+	-	+
Yersinia pestis	+	+	+	+	-	+	-	-	-	-
Serratia ficaria	+	+	-	-	-	-	+	+	-	-
Enterobacter gergoriae	+	+	-	+	-	-	-	-	-	-
Enterobacter amnigenus 1	+	+	-	+	-	-	-	-	+	+
Serratia marcescens	+	+	-	-	-	+	-	-	-	-
Raoutella terrigena	+	+	-	+	-	+	+	-	-	-
Hafnia alvei 1	-	-	-	-	-	-	-	-	-	-
Providencia rettgeri	-	-	-	-	-	-	-	+	-	-
Pantoa	-	-	-	-	-	+	-	+	-	-
Total	23	27	4	18	1	21	4	15	2	7

This Table shows that most isolates had *VanA, VanB, VanC2/C3, Inu A* and *Inu C* resistance genes. *VanD* resistance gene was detected in only *Salmonella choleraesuis* spp *arizonae* and less than 10 isolates had *VanC, InuB, InuD* and *InuF* resistance genes respectively.

TABLE 4.9.3: Results for detection of resistance genes in isolate (Continues)

Isolates	Resistance gene									
	bla$_{TEM}$	*bla$_{SHV}$*	*bla$_{OXA}$*	*bla$_{VEB}$*	*bla$_{PER}$*	*OtrA*	*OtrB*	*aac(3')-lv*	*Sul1*	*Sul2*
Ps. luteola	+	-	-	-	+	-	+	-	-	-
E. vulneris	+	-	+	-	+	+	+	-	-	-
Salmonella choleraesuis spp *arizonae*	-	-	+	-	-	+	+	+	+	+
E.coli 1	-	+	+	-	+	+	+	-	-	+
Enterobacter cloacae	-	-	+	-	-	+	+	+	-	-
Ps. flourescens/putida	+	-	-	-	+	+	+	-	-	-
Enterobacter aerogenes	+	-	-	-	-	+	+	+	+	+
Serratia ordoriferal	-	+	-	-	+	+	+	-	-	+
Pasteurella pneumotropica	+	-	+	-	-	+	-	-	-	+
Ochrobactrum antropi	-	-	-	-	+	+	+	-	+	-
Proteus vulgaris group	-	-	+	-	-	+	-	-	-	+
Proteus vulgaris	-	-	-	-	+	-	+	+	-	+
Salmonella spp	+	-	-	-	-	+	+	-	-	-
Aeromonas hydrophila/caviae/sobria1	+	+	-	-	-	+	+	-	+	+
Proteus mirabillis	+	-	-	-	+	+	+	-	-	+
Vibrio fluvials	-	-	-	-	-	+	+	-	-	-
Rahnella aquatillis	-	-	-	-	-	-	-	-	-	-
Ps. aeruginosa	-	-	+	-	+	+	+	-	-	+
Burkholderia Cepacia	-	-	-	-	-	+	+	+	+	-
St. maltophilia	+	-		-	-	-	-	-	+	+
Shwenella putrefaciens	-	-	-	-	+	+	+	-	+	-
Klebsiela pneumoniae	+	-	-	-	-	+	-	-	-	+
Cedecea davisa	+	-		-	-	-	-	-	-	+
Serratia liquefaciens	+	-		-	-	+	-	-	+	-
Serratia plymuthica	-	-	-	-	-	+	+	+	-	-
Enterobacter sakaziki	+	+	-	-	-	+	+	-	+	+
Citrobacter braakii	-	-	-	-	-	-	-	+	+	-
Enterobacter amnigenus 2	+	-		-	+	+	+	-	+	-
Yersinia pestis	-	+	-	-	-	+	+	-	+	+
Serratia ficaria	+	+	-	-	-	+	+	+	+	+
Enterobacter gergoriae	-	-		-	+	+	+	+	-	-
Enterobacter amnigenus 1	+	-		-	+	+	+	-	-	-
Serratia marcescens	+	-	-	-	-	+	+	+	-	+
Raoutella terrigena	+	-	-	-	-	+	-	-	+	-
Hafnia alvei 1	-	-		-	-	-	-	-	+	-
Providencia rettgeri	-	-	+	-	-	+	-	-	-	-
Pantoa	-	-	-	-	+	-	+	+	-	-
Total	18	6	8	0	14	29	27	11	15	17

Table shows that most isolates had *OtrA, OtrB, Sul1, Sul2, bla$_{TEM}$* and *bla$_{PER}$* resistance genes. *bla$_{VEB}$* was not detected in all isolates. Less than 10 isolates had *bla$_{SHV}$* and *bla$_{OXA}$* respectively and only 11 isolates had *aac (3')-lv* resistance genes

Gel electrophoresis results for *bla$_{TEM}$* resistance gene

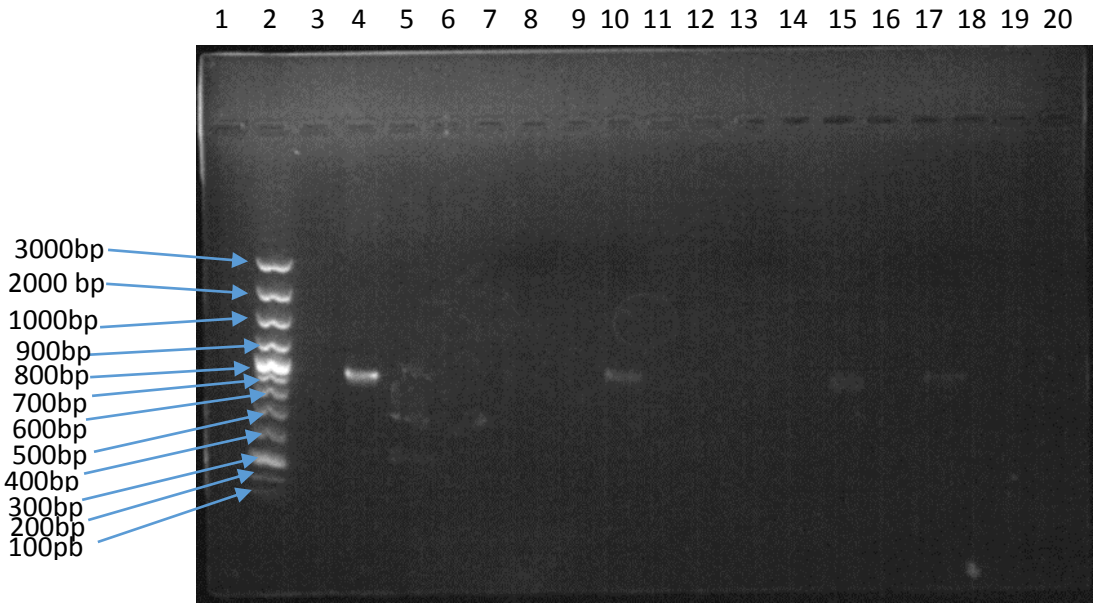

FIGURE 4.6.1: Results for PCR detection of *ble$_{TEM}$* resistance gene from identified isolates. Lane2: 100 bp DNA Ladder; Lane 3: Blank; Lane 4*: S. marcescens* ATCC14041 (control); Lane 5: *S. marcescens*; Lane 6: *S. maltophila*; Lane 15: *Salmonella* spp; Lane 17: *E. vulneris*

Gel electrophoresis results for *Inu A* resistance gene

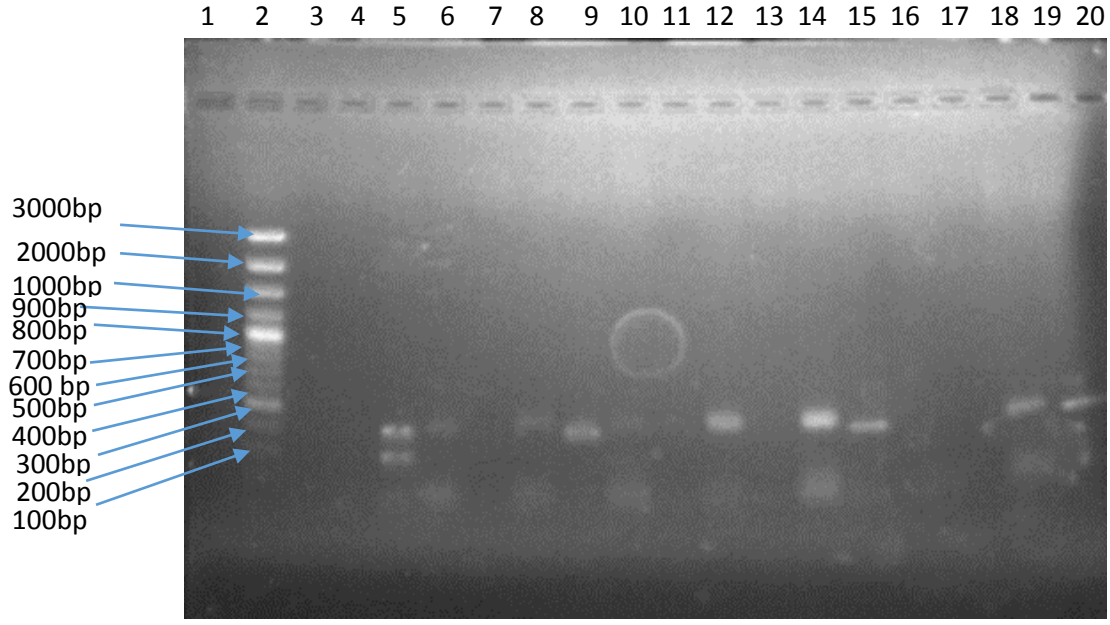

FIGURE 4.6.2: Results for PCR detection of *Inu A* resistance gene from identified isolates. Lane2: 100 bp DNA Ladder; Lane 12: *Ps. aeruginosa* ATCC 19429; Lane 5: *Ochrobactrum antropi*; Lane 6: *Salmonella* spp; Lane8: *Enterobacter cloacae*; Lane9: *Proteus vulgaris*; Lane10: *Escherichia vulneris*, lane14: *Ps. aeruginosa*; Lane15: *Serratia marcescens*, Lane19: *Vibrio fluvials* Lane20: *Enterobacter sakaziki*

Chapter 5: Discussion, Conclusion and Recommendations

5.1 Discussion

The results for the physicochemical analyses of soil and water samples were given in section 4.1 in chapter 4 (Table 4.1 and Table 4.4). The variation of results in pH values for water samples may likely be caused by the significant difference in pH values observed in March and April ($p<0.05$) and between May and June ($p<0.05$) and between July and August ($p<0.01$), while the variation in pH among sampling points must have been a function of the significant ($p<0.05$) lower as compared to other sampling points.

The composition of seepage varied from sampling point to sampling point depending on the level of treatments (Environment Canada, 2001) and this could be an important contributory factor to the observed differences in pH. The pH level of seepage determines its usefulness for a variety of purpose. Very high or low pH values has been reported by Morisson *et al.*, (2001) and DWAF, (1996c), to be toxic to aquatic life and alter solubility of other chemical pollutants as well as some essential elements in the water system (DWAF,1996c), thereby causing adverse effects on the ecosystem and those dependant on it. The standard for pH according to South Africa (DWAF,1996c) is 6-9 and the tolerance limit for pH in water for support of fisheries and aquatic life is also set at 6-9 pH units (Chapman,1996). According to Kunte *et al.*, (1998), pH values ranging from 3 to 10.5 could favour both indicator and pathogenic micro-organism growth. The pH values observed in this study (Table 4.1) across all sampled points fell within the recommended standards as set by DWAF (1996c). Results in this study were similar to those of González *et al.*, (2009) where the seepage pH ranged from 7.1 to 7.4. The results were also similar to those of Knight *et al.*, (2000) where a pH range of 6 to 8.4 was observed. Results were also similar to those observed by Aguilar *et al.*, (2011) (pH values of 6 to 8). The results observed in this study indicates that the seepage will not have any impact on

receiving environment with reference to pH standards. Physical parameters, such as pH, temperature and turbidity have a major influence on bacterial population growth (Nübel *et al.*, 1999; Byamukama *et al.*, 2000; Goñi-urriza *et al.*, 2000).

Temperature is an important parameter due to its influence on other parameters as it affects the solubility and also consequently the availability of oxygen in water (Akan *et al.*, 2008). Temperature also affects the toxicity of some chemicals in the water systems as well as the sensitivity of living organisms to toxic substances. . According to the South African standard for seepage temperature, the limit was set at ≤25 °C (DWARF, WRC, 1995). The temperature results observed in this study water samples (Table 4.1) across all sampling points were within the recommended standards except for Constructed wetland and effluent in March where temperature were observed to be as high as 28 °C and 26 °C respectively. With regards to the results observed, the seepage may not significantly offset the homeostatic balance of the receiving environment except in warm weathers observed in summer months (March) where temperature of seepage can reach as high as 28 °C. These relatively high temperatures in constructed wetland and effluent could be attributed to the fact that the water in these reservoirs were stagnant. Constructed wetlands in pig farm are constantly exposed to sunlight and there is no water flow, the water temperature remained high, especially with warm weather. Results in this study were lower than those observed by Singh *et al.*, (2012) where temperature ranged from 31.11 °C to 36.34 °C.

EC is a measure of dissolved ions in water systems, it has also been reported to be a useful and easy indicator of salinity or total salt content of water systems (Morrison *et al.*, 2001; Oluyemi *et al.*, 2006).The South African guideline for electrical conductivity in seepage and effluent that could be discharged into the receiving water bodies is 70 mS/cm (Government Gazette, 1984) and based on this guideline, the water samples from pig farm (Table4.1) does appear to be compliant with the regulation for electrical conductivity. Despite the low EC observed for

water samples in this study as shown in Table 4.1, the decrease in EC from sampling point to sampling point as observed in constructed wetland and effluent, may be explained by uptake of micro and macro elements and ions by plants and bacteria, and their removal through adsorption to plant roots, and settleable suspended particles (IWA, 2000). Results for EC observed in this study were also similar to those observed by Aguilar *et al.*, (2011). Results observed in this study were lower than those reported by Vanotti *et al.*, (2002) where EC values ranged from 5.1 mS/cm to 16 mS/cm.

The levels for BOD for water samples (Table 4.1) exceeded the recommended limit of 40 mg/L set by FAO (1992) for agricultural purposes. BOD levels in some sampling points were observed to be higher than those of COD, this may be attributed to the presence of ammonia which has been reported to significantly increase BOD levels as a result of microbial oxidation of ammonia ultimately to NO_3. Results observed in this study were similar to those reported by Aguilar *et al.*, (2011). BOD observed in this study were lower than those reported by Vanotti *et al.*, (2002) where BOD values ranged from 700 mg/L to 10580 mg/L. The results obtained in this study were also higher than those obtained by González *et al.*, (2009) where BOD were observed to range from 662 mg/L to 1823 mg/L. High BOD values can be attributed to the high decomposable organic matter by microorganism which can result to greater oxygen demand (Ademoroti, 1996).

TDS results for water samples in this study as were given in chapter 4 Table 4.1 and were observed to be higher than the recommended standards set by DWAF, (1998) where a limit of 450 mg/L of no risk to aquatic life was set for seepage released into the receiving environment (Akan *et al.*, 2008). WHO (2004) also set the limit of TDS for no risk to aquatic life at < 2000 mg/L. TDS values obtained in this study for water sample (Table 4.1) also did not meet the required WHO (2004) standards in all sampling points in March and May, influent, enclosures in April. Elevated TDS can be toxic to freshwater animals by causing osmotic stress and

affecting the osmoregulatory capability of the organisms (McCulloch *et al.*, 1993). Results observed in this study were lower than those reported by Vanotti *et al.*, (2002) where TDS values ranged from 4.30 g/L to 24.8 g/L.

Although there are no set standard for salinity level for effluent discharge into the aquatic ecosystems in South Africa. The salinity results in this study for water samples (Table 4.1) fell within the acceptable limit of 33 psu to 35 psu of no risk for all biological activities in the marine ecosystem (SANCOR, 1984, Whitefield and Bate, 2007). Some of the impacts of excess salinization on water resources include reduced crop yield, and increased requirements for pre-treatment of water for selected seepage (DEAT, 2000). Salinity is the saltiness of a water body and high salt content in effluents discharged into a receiving watershed could cause serious ecological disturbance that may result in adverse effects on the aquatic biota (Morrison *et al.*, 2001; Oluyemi *et al.*, 2006).

Turbidity values for water samples (Table 4.1) in this study fell within acceptable limits by WHO (WHO, 2004), standard (≤5 NTU). Excessive turbidity in seepage can cause problem with water purification processes such as flocculation and filtration, which may increase treatment cost (DWAF, 1998). High turbid waters are often associated with the possibility of microbiological contamination, as high turbidity makes it difficult to disinfect water properly (DWAF, 1998).

COD is a measure of the amount of oxygen required by a strong oxidant (e.g. sulphuric acid) to breakdown both organic and inorganic matters in a water system (Akan *et al.*, 2008). Elevated levels of COD in water systems leads to drastic oxygen depletion which adversely affects the aquatic life (Fatoki *et al.*, 2003). High COD causes soil fixation, resulting in lower availability of nutrients for plants (Chukwu 2005). The COD results for water samples (Table 4.1) in this study fell short of the acceptable limit (30 mg/L) recommended by the South African

government (Government Gazette, 1984) and suggest that the seepage may negatively impact on the receiving environment. The COD results (table 4.1) also exceed the acceptable limit (≤1000 mg/L) of no risk by WHO (2004), except in effluent. The higher COD values in this study could be attributed to the lesser rate of organic matter breakdown (occasioned by lower microbial activity) during the cold (winter) months compared to the warmer seasons (Tomida *et al.,* 1999). Results obtained in this study were higher than those reported by Knight *et al.*, (2000) where COD values of 405 mg/L to 1004 mg/L were observed. Similar results were also reported by Aguilar *et al.*, (2011) where COD from pig farm seepage was recorded to be as high as 9960.83 mg/L. Results observed in this study were lower than those reported by Vanotti *et al.*, (2002) where COD values ranged from 5360 mg/L to 31310 mg/L. The results obtained in this study were also higher than those obtained by González *et al.*, (2009) where COD were observed to range from 958 mg/L to 2833 mg/L.

The results for DO for water samples (Table 4.1) in this study fell short of the acceptable limit (≥5 mg/L) of no risk for the support of aquatic life (DWAF, 1998). Dissolved oxygen is essential in maintaining the oxygen balance in the environment especially aquatic ecosystem (Fatoki *et al.,* 2003). Low DO can negatively impact on aquatic life by increasing their susceptibility to disease, allergy, feeding migration, reproduction etc., and thus leading to loss of life (Environmental Canada). The DO content in influent and CW which was observed to deplete faster than DO from other sampling points could be attributed to the presence of degradable organic matter by microorganism which resulted in a tendency to be more oxygen demanding.

Nitrate concentration of water samples (Table 4.1) exceeded the acceptable limit of 20 mg/L set by WHO (2004). Nitrates are inorganic source of nitrogen that support the growth and development of living organisms at appropriate concentrations. However, high nitrate levels may have negative impacts on the receiving environment as it may result in excessive nutrient

enrichment in water systems (eutrophication) leading to loss of diversity in the aquatic biota and overall ecosystem degradation through algal blooms, excessive plant growth, oxygen depletion and reduced sunlight penetration (CCME, 2006). Results for nitrate concentration observed in this study were higher than those observed by knight *et al.*, (2000) where nitrate values ranged from 147.5 mg/L to 254.1 mg/L. Results observed in this study for nitrate concentration were also higher than those reported by Aguilar *et al.*, (2011) where nitrate levels for pig farm seepage was recorded to be as high as 806.4 mg/L. The results obtained in this study were also higher than those obtained by González *et al.*, (2009) where nitrates were observed to range from 56 mg/L to 77 mg/L.

Nitrite like nitrate is also a source of nutrition that could have adverse effects on aquatic ecosystems at elevated concentrations. The nitrite levels in this study for water samples (table 4.1) fell short of the South African standard (<0.5 mg/L NO_2) for preservation of aquatic ecosystem (DWAF, 1996c) and thus will impact negatively on the receiving environment as it has high risk of eutrophication. Results were higher than those observed by knight *et al.*, (2000) where nitrite values ranged from 98.9 mg/L to 273.6 mg/L.

Orthophosphate concentration levels for water samples (Table4.1) observed in this study exceeded the WHO (2004) standard of 30 mg/L. High orthophosphate concentration will have a negative impact on the receiving environment as it will increase algae and plant growth in aquatic systems and if the seepage is released or accidentally spilled into water systems it will cause pollution. Only a small proportion (<20%) of phosphate removal by constructed wetlands can be attributed to nutritional uptake by bacteria, fungi and algae. The lack of seasonal fluctuation in phosphorus removal rates suggests that the primary mechanism is bacterial and alga fixation Phosphates are reported to be the most important growth limiting factor in eutrophication and results in a number of undesirable effects in the water system (CCME, 2006). Results were higher than those observed by Knight *et al.*, (2000) where they observed

a range of 0.6 to 92 mg/L of orthophosphate. Results observed in this study were higher than those reported by Vanotti *et al.*, (2002) where orthophosphate values ranged from 63 mg/L to 513 mg/L. The results obtained in this study were also higher than those obtained by González *et al.*, (2009) where orthophosphate was observed to range from 44 mg/L to 88 mg/L.

Soil pH affects several characteristics such as soil weathering, soil structure, humification, biotic activity, mobilization of nutrients and ion exchange (Singh and Agrawal, 2012).The results for soil pH (Table 4.6) in this study fell within the limit set by Government Gazette (1984) for the protection of ground water and Federal Ministry for the Environmental (FME, 2011) where pH standard was set at 6 to 8.5. Low pH in soil can increase the availability of metals since hydrogen ions have the affinity for competing with metals ions and releasing the in soil solution for uptake (Singh and Agrawal, 2012). Results observed in this study were also similar to those observed by Aguilar *et al.*, (2011) where pH of soil in a pig farm ranged from 6.2 to 8.6. Pan *et al.*, (2012) have observed that pH values of 5.5 to 8 were suitable for microbial decomposition of organic matter in soil. Pan *et al.*, (2012) observed that in very acidic or alkaline environments, microbial activity is compromised and even inhibited. The increase in pH can be attributed to the volatilization and microbial decomposition of the organic acid and subsequent release of ammonia through mineralization of organic nitrogen source (McKinley and Vestal, 1985).

The temperature of soil greatly affects the physical, biological and chemical processes occurring in soils. Chemical and biological rates are slow in low soil temperature. Temperature plays a very important role in soil characteristics and seed germination. Temperature regenerates absorption and transport of water and nutrients ions in higher plants. The temperature value for soil samples (Table 4.6) obtained in this study indicated that there was no great temperature fluctuation within the analysed soils. The results in this study were within

the recommended limit of 40 °C as set by FME (2011). The results were lower than those observed by Roth *et al.,* (2012) where temperatures ranged from 28.3 °C to 30.8 °C.

The measurement of electrical conductivity is for measure the current that gives a clear idea of soluble salt present in the soil. Conductivity depends upon the dilution of soil suspension. Soil electrical conductivity (EC) is a measure of the amount of salts in soil (salinity of soil) and it is an important indicator of soil health. Soil EC affects crop yields, crop suitability, plant nutrient availability, and activity of soil microorganisms which influence key soil processes including the emission of greenhouse gases such as nitrogen oxides, methane, and carbon dioxide. The results for soil conductivity (Table 4.6) in this study fell within the required limit of 2 mS/cm which was set by Government Gazette (1984) for the protection of plants and ground water. Results observed in this study were higher than those reported by Aguilar *et al.,* (2011) where soil EC for pig farm seepage was recorded to be 0.003 mS/cm to 0.06 mS/cm.

Soil salinity is generally characterized by determining TDS or EC of soil solution. High salinity in soil hinder plant growth by affecting the soil-water balance in soil. Results for soil salinity (Table 4.6) observed in this study fell within the acceptable limit of 0.1 psu as set by Government Gazette (1984) except for results obtained in Soil 20 m away from Constructed wetland where a value of 0.13 psu in August was observed.

The results obtained in this study for soil TDS (Table 4.6) were within the limits of ≤500 mg/L for the protection of ground water as set by FME (2011). High accumulation of TDS in soil due to continuous exposure of seepage may enhance soil EC. The results obtained in this study for soil COD (Table 4.6) met the requirement limit of ≤ 300 mg/L for protection of ground water and aquatic life due to surface run-off (Government Gazette, 1984). The results obtained in this study for soil DO (Table 4.6) were within the required limit of ≥5 mg/L for the protection

of ground water and aquatic life due to surface run-off (Government Gazette, 1984) (DWAF, 1996c).

Concentrations of soil Nutrients i.e. phosphorus as orthophosphate, nitrate and nitrites are very crucial as this parameters are very crucial to soil health for support of soil biota and plant growth. Excess of this nutrients in the soil can lead to leaching to the ground water thus causing growth of algae and causing eutrophication. FME (2011) set the limit for Nitrate, Nitrite and Orthophosphate at ≤ 13 mg/L for nitrites and nitrates and ≤ 5 mg/L for orthophosphate. The results for concentration orthophosphate, nitrate and nitrites in soil samples did not fall within the required limit and may cause eutrophication of ground water and aquatic environments due to surface runoff. Results obtained in this study for Nitrate, Nitrites and Orthophosphate were higher than those obtained by Roth *et al.*, (2012), where nitrate ranged from 6.4 µg/g to 1013 µg/g, Nitrite ranged from 0.2 µg/g to 23.8 µg/g and orthophosphate ranged from 3.69 µg/g to 9.5 µg/g in soil at and near pig farm's enclosure.

The insignificant correlation between pH and salinity in soil and water samples (Table 4.3 and Table 4.6) may have been caused by the almost neutral pH concentration observed in this samples. The insignificant correlation between DO and salinity in both soil and water samples as shown in Table 4.3 and Table 4.6 indicates that DO concentration decrease with an increase in salinity levels as observed in this study. This may be due to a high microbial activity observed in this samples.

Several studies have reported that EC and TDS are a good indicators of salinity (Oluyemi *et al.*, 2006; Akan *et al*., 2008). In this study EC and TDS in both soil and water samples (Table 4.3 and Table 4.6) were significantly higher ($p<0.05$) as compared to salinity due to the high microbial activity observed in this samples. It is expected that seepage high in EC and TDS levels to promote microbial growth and this was the case in this study as high viable cell counts

were observed in samples with high TDS. This further implies that the type of dissolved solid present in the seepage may to a large extend promote microbial growth and activity.

The significant correlation ($p<0.05$) of salinity and nitrates in water samples (Table 4.3) indicates that the less saline seepage is the source of this nutrient and this can be attributed to the consistent high concentration of nitrate in pig enclosures and influent, as compared to other sampling points. The insignificant correlation of salinity with nitrates and nitrites in soil samples (Table 4.6) may be due to the high concentration of nitrate and nitrite observed in enclosures, soil 20 m away from enclosures and soul 20 m away from constructed wetland treating wastewater from pig farm. This is an indicative that the pig farm is a source of this nutrients in the environment. The positive correlation between TDS with nitrate and nitrate may be due to the microbial breakdown of organic and inorganic matter in the soil (Table 4.6).

The insignificant correlation between salinity and TDS with turbidity in water samples (Table 4.3) shows that effluents released from the pig farm cannot be a source of turbidity in the receiving environment. However as observed in this study, salinity and TDS in soil correlated significantly which may be due to the increase in microbial activity in the pig farm seepage. The insignificant correlation of COD with EC, TDS, salinity, pH, DO, in waters samples and the significant correlation($p<0.05$) of COD with salinity in soil samples was due to the high COD levels caused by high rate of organic breakdown due to microbial activity in seepage

The viable cells (shown in chapter 4 Figure 4.1.1 to 4.3.4) were observed to be high in enclosures for both soil and water, influent, soil 20 m from enclosures and soil 20 m from constructed wetland treating pig farm seepage, as compared to other sampling points.

Cold temperatures in the environment was reported by Kadlec and Reddy (2001) to promote bacterial growth as cold weather was reported to be favourable in preserving bacteria in

contaminated environment. It was observed that viable cell were higher in enclosures and soil 20 m away from enclosures and soil 20 m away from constructed wetland which is used to treat pig farm seepage(chapter 4 Figure 4.2.1 to Figure 4.3.4). In soil 100 m away from enclosures and constructed wetland the viable cell were within the required limits($<10^3$ cfu/mL) set by Government Gazette for bacteria that should be in soil for the prevention of contaminating ground water. This may be an indication of the bacterial pollution caused by pig farms on their natural environment. The viable cell counts observed in this study were higher than those recommended by DWAF and Government Gazette in which the viable cells are not supposed to exceed 1000 cfu/mL (DWAF, 1998).

Bacterial contamination refers to the non-intended or accidental introduction of infectious bacteria into the natural environment (Tymczyna *et al.,* 2000). Bacterial contamination has negative impacts on the environment as bacterial pathogens can spread waterborne and airborne disease, and they can compete with indigenous soil microorganism for nutrients, they may transfer antimicrobial resistance genes to indigenous soil microorganism important for soil remediation and they may cause a high level of aquatic life die-offs (Sasáková, *et al.*, 2007). Results were lower as compared to those observed by Cooks *et al.,* (2010) where 2.58 x 10^{10} cfu/mL to 1.49 x 10^{11} cfu/mL were obtained from farrowing facilities at pig farm. Sasáková, (2007) in their study observed viable cell counts ranging from 9.8 X 10^6 cfu/mL to 9.2 X 10^8 cfu/mL in pig farm seepage, results were similar to those observed in this study. Results were also similar to those observed by Knight *et al.,* (2000) where viable cells ranged from 1.34 x 10^3 cfu/mL to 1.03 x 10^8 cfu/mL. The results in this study were also higher than those observed by Tymczyna *et al.,* (2000) where viable cell from environmental samples in the vicinity of pig farm ranged from 1.00 x 10^4 cfu/mL to 3.00 x 10^4 cfu/mL

The identities of isolates are shown in Table 4 *Salmonella* genus and *E. coli* were detected in the soil and water samples in the environment of the pig farm. The soil bacteriological

examinations revealed that the environment indicated the presence of these rod shaped bacteria (Enclosures and soil 20m from constructed wetland and all water samples). Although *Salmonella* presence confirmed in the soil samples examined was occasional, still even their small count should be alarming because they can easily spread under favourable conditions and make a serious source of environmental pollution. In addition the identities of the isolates especially *E. coli* was not performed to strain level and therefore the isolate could belong to the serotype *E. coli* 0157:H4 and *E. coli* 0104:H4 that have been reported to cause disease in humans.

Detection of bacterial pathogens in soil samples may be attributable to the high load of animal excreta in the seepage (Ezeronye and Ubalua, 2005) and serves as a pointer for possible pollution and may have an effect on the soil ecological balance and aquatic life. Dubinský *et al.,* (2000) also identified *Salmonella* spp., *E. coli, Yersinia* spp in pig farm seepage. The results obtained in this study was also similar to that observed by Tymczyna *et al.,* (2000) where bacteria such as Salmonella spp, *Klebsiella* spp, *Pseudomonas* spp, *Proteus* spp, *enterobacter aerogenes, citrobacter* spp. etc. were isolated from soil and water samples from the environment in the vicinity of pig farm. In the works by Saba *et al.,* (1993) and Slawon *et al.*, (1994) the attention was drawn to the presence of bacterial pathogens in fur bearing animal farms. The authors state that the environment has been in danger of being polluted with bacterial pathogens coming from breeding farms due to a great concentration of animals at confined space which can be conducive for pathogenic factor transfer among animals and their natural environment.

The phenotype resistance patterns (Table 4.8) observed in this study showed that the isolates were highly resistant to more than three antibiotics, where 15 isolates were observed to be resistant to all antibiotics tested. The multidrug resistance index (MDRI) of isolates was also observed to be high (Table 4.8). Kotzamanidis *et al.,* (2009) observed an AML-CAZ-VA-TE

as the most occurring phenotype pattern in isolates isolated from pig farm environment. Results in this study for phenotype pattern were also similar to those observed by Kainer *et al.*, (2007) and Werner *et al.*, (2008).

Enterococci often acquire antibiotic resistance through exchange of resistance-encoding genes carried on conjugative transposons, pheromone-responsive plasmids, and other broad-host-range plasmids (Rice *et al.*, 1995). Antimicrobial resistance in enterococci is of two types: inherent/intrinsic resistance and acquired resistance. Intrinsic resistance is species characteristics and thus present in all members of a species and is chromosomally mediated High percentage of antibiotic resistance was observed in seven different antibiotics out of the 18 antibiotics that were tested in 123 isolates.

The result observed in this study for susceptibility analyses (Figure 4.5) showed that these organisms have been well exposed to the tested antibiotics and they have developed mechanisms to evade or avoid the effects of these antibiotics. This high antimicrobial resistance is of concern because as antibiotic resistance genes can be transferred from pathogens to non-pathogenic (indigenous) microorganism. Results were similar to Marothi *et al.*, (2005) as in their study they have observed high resistance of antibiotics by bacteria for penicillins, cephalosporins, lincomasides, nalidixic acid, tetracyclines and aminoglycoside. Results were also similar to those observed by Kainer *et al.*, (2007) and Werner *et al.*, (2008) where resistance to similar antibiotics were also reported.

Resistance genes detected in soil bacteria (Table 4.9.1 to Table 4.9.3) e.g. *Burkholderia Cepacia* may be attenuated to that soil microorganism may serve as reservoirs for the propagation and possibly the amplification of antibiotic resistance and this possess a hazard to the surrounding environment (Krista *et al.*, 1996). As soil bacteria like *Burkholderia Cepacia* are usually used in bioremediation of soil, the acquiring of antibiotic resistance gene renders

this soil bacteria unsafe for bioaugmentation application (Krista *et al.*, 1996). Similar results were also observed by Mathew *et al.*, (1998) and Kelley *et al.*, (1998). Bacterial pathogens such as *E. coli, Proteus* spp., *Salmonella* spp., *Enterobacter* spp., were observed to have multiple resistance genes to most of the antibiotics testes.

However, the detection of van genes (Table 4.9.2) may be an indication that enrichment for van operons is possible under alternative conditions. *Tet* and *sul* resistance genes have been reported as the most frequently detected ARGs pig farm seepage by Zhu *et al.*, (2013) and this was also observed in this study as *sul* resistance gene (Table 4.9.3) was also detected in most of the water and soil samples from pig farm. In a study conducted by Chee-Sanford *et al.*, (2001) *OtrA* resistance gene was the most abundant and detected in surface soil in pig farm and this was consistent with the observation in this study where *OtrA* resistance gene (Table 4.9.3) was observed in most surface soil sample isolates. The results for *sul* resistance gene detection in this study (Table 4.9.3) were lower than those observed by McKinney *et al.*, (2010) where in their study they found a high abundance of sulphonamide (*sul1 and sul2*) resistance genes in pig farm seepage. Results in this study for resistance gene detection were consistence to those observed by Munir and Xagoraraki (2011) and Zhu *et al.*, (2013). In a study conducted by Faldynova *et al.*, (2013), *sul1* and *aadA* resistance genes were very abundant in pig farm soil and seepage while *sul2* resistance gene was less observed in isolates from pig farm surrounding environment. Results observed in this study were not consistent with those observed by Faldynova *et al.*, (2013), as *Aad* resistance genes (Table 4.9.1) were less abundant and *sul1* and *sul2* resistance genes were more or less equally observed in isolates from soil and water samples in pig farm. The results obtained in this study shows that ARGs in pig farms are not only diverse but are also remarkably abundant, which together offers a higher probability of dispersal, further selection, and/or horizontal transfer in the environment. Li *et al.*, (2013) observed an abundance of *InuF* resistance gene in all soil and water samples

collected in pig farm and this was not the case in this study as *InuF* resistance gene was less detected in both soil and water samples from pig farm. Similar results reported by Li *et al.*, (2013) were also reported by Cheng *et al.*, (2013) where widespread of *Inu F* resistance gene was also observed in soil and water samples proximal to pig farm. The abundance of *Inu F* resistance gene in this study were lower than those observed by Cheng *et al.*, (2013) and Li *et al.*, (2013). The results for *InuA* resistance gene (Table 4.9.2) in this study were similar to those reported by Li *et al.*, (2013) where the gene was the most observed *Inu* gene detected in isolate from pig farm. Detection of *InuD* resistance gene (Table 4.9.2) in this study were similar to those observed by Li *et al.*, (2013) where *InuD* resistance was detected sporadically in adjacent environment proximal to pig farm. The results in this study were also consistent with those observed by Sun *et al.*, 2014 where *aadA, aph(3')-lv, aph (3')-IIIa, Inu F, InuA, bla$_{TEM}$ and bla$_{OXA}$* resistance genes were detected in all soil and seepage samples in pig farm.

5.2 Conclusion

Pig farms are known to produce seepage with high concentration of pollutants and recycling of this seepage in a sustainable manner remains a major challenge in agricultural sectors. Thus treatment of seepage using constructed wetlands has been developed to manage and control the contamination of the environment. Bacteriological pollution is serious hazard to the natural environment as it renders it unsafe to use by flora and fauna. Bacteriological pollution at pig farm was observed to be high in natural environment in the vicinity of pig farm at enclosure soil, soil 20 m from pig enclosures, and soil 20 m from constructed wetland treating pig farm seepage most water samples. This study showed that viable cell counts were higher than those recommended by DWAF (1996c) and FME (2011) for water and soil samples from pig farm. As observed this will have a negative impact on the environment as nutrients availability to indigenous microorganism will be compromised. Pathogenic bacteria such as *E. coli1, Salmonella* spp, *Proteus* spp, *Pseudomonas* spp etc. isolated and identified from water and soil

samples in the vicinity of pig farm indicate that bacterial pollution of pathogenic bacteria is occurring in pig farm. This may have a serious impact as this pathogens have a tendency to survive for longer periods in favourable environmental condition. If this pathogens can be exposed to water system by surface runoff, wind, leaching to ground water, and to soil by leaching, they can cause waterborne diseases and impact on the physicochemical parameters of the receiving environment. The results for the antibiotic susceptibility test and detection for resistance genes, showed that most isolates were resistant to more than three antibiotics and had multiple antibiotic resistance genes. An introduction of such isolate in the environment may have a negative impact as this isolates can transfer their resistance genes either by horizontal gene transfer or vertical gene transfer to indigenous microorganism which are very important for soil remediation. In this study *Burkholderia cepacia* was isolated from soil samples in pig farm, this bacteria occurs naturally in soil and is usually used for soil remediation and was observed to have multiple resistance genes that were also observed in pathogens such *as Proteus vulgaris, Salmonella* spp*, Pseudomonas* spp*, and Klebsiella pneumonia* etc. This indicates that contamination of the natural environment in the vicinity of pig farm with pathogenic bacteria with an antibiotic resistance gene is occurring and thus causing a transfer of resistance genes to the indigenous microorganism. The physicochemical parameter results of water samples also showed that temperature (in March) , BOD, COD, TDS, DO, orthophosphate, nitrate and nitrite and the physicochemical parameters of soil showed that pH, salinity, TDS, COD, orthophosphate, nitrate and nitrite were higher than the recommended standards. With regards to soil samples the physicochemical parameters analysed were high in enclosures, soil 20 m away from enclosure and soil 20 m away from constructed wetland treating pig farm seepage and thus shows that the seepage from the pig farm is impacting on the environment. The results will have a negative impact on the environment as it will cause reduction in available oxygen, increase solubility of heavy metals

and increase toxicity of other chemicals, increase the sensitivity of living organisms to other toxic substances, cause osmotic stress to the natural environment and affects osmoregulatory capability of organisms, and may cause eutrophication of water systems and soil. Pig farm seepage causes bacterial pollution in the natural environment in the vicinity orthophosphate, nitrate and nitrite of pig farm by introducing bacterial pathogens that have an antibiotic resistance gene which impacts on indigenous organisms and the physicochemical parameters for soil and water samples in pig farm.

5.3 Recommendation

Pig farm production in South Africa is growing at a fast rate due to the demand in pork meat, and the use of antibiotics as growth promoters and for treatment of diseases for pigs is also increasing. Research of bacterial pollution and introduction of antibiotic resistance genes caused by pig farms has never been reported in South Africa but has been reported in parts of Asia (e.g. China). It is therefore recommended that more research to be done on environmental pollution emanating from pig farms in South Africa. It was also observed in this study that the seepage in the pig farm was not managed properly thus the high bacterial pollution around the natural environment in the vicinity of pig farm in ARC-API. It is also recommended that the pig farm should consider the need of intervention by appropriate regulatory agencies to ensure regular monitoring of the qualities of final effluents of wastewater treatment facilities and soil pollution control at the pig farm in order to safe guard the natural environment in the vicinity of pig farm. With high number of pathogenic bacteria that was isolated from pig farm that had multiple antibiotic resistance genes it was a clear indication of the increase use and mismanagement of antibiotics in pigs at the pig farm. It is therefore recommended that the pig farm in ARC-API stop using antibiotics and consider using probiotics instead. It is also recommended that more research on bacteria, protozoa, viruses and parasites pollution in pig farms should be conducted in South Africa. More research on the bioremediation of antibiotics

residues introduced in pig farm soil should be conducted in future. More research on seepage management in pig farms should be conducted and new and cheaper technologies for wastewater treatment should be introduced to pig farms that will assist constructed wetlands for treating pig farms wastewater

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