TREATED WASTEWATER EFFLUENT AS A POTENTIAL SOURCE OF VIRULENT AND ANTIBIOTIC RESISTANT *YERSINIA* SPECIES IN RECEIVING SURFACE WATER

GCINILE ZAMANTUNGWA KHUMALO

Submitted in fulfilment of the academic requirements for the degree of Master of Science (MSc) in the Discipline of Microbiology, School of Life Sciences, College of Agriculture, Engineering and Science at the University of KwaZulu-Natal (Westville Campus), Durban, South Africa.

As the supervisor of the candidate, I approve this dissertation for submission

Signed: _____ **Name:** _____ **Date** _____

PREFACE

The experimental work described in this dissertation was carried out in the Discipline of Microbiology, School of Life Sciences, College of Agriculture, Engineering and Science at the University of KwaZulu-Natal (Westville Campus), Durban, South Africa from March 2013 – December 2015, under the supervision of Prof B. Pillay and Prof. A.O Olaniran.

These studies represent original work of the author and have not been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE

DECLARATION 1– PLAGIARISM

I, Gcinile Zamantungwa Khumalo declare that:

1. The research reported in this dissertation except where otherwise indicated, is my original research.
2. This dissertation has not been submitted for any degree or examination at any other University.
3. This dissertation does not contain other person's data, pictures, graphs or other information, unless specifically acknowledged as being sourced or adapted from other persons.
4. This dissertation does not contain other person's writing, unless specifically acknowledged as being sourced from other researchers. Where other sources have been quoted, then:

a. Their words have been re-written but the general information attributed to them has been referenced.

b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.

5. This dissertation does not contain text, graphics or tables copied and pasted from the internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the reference sections.

Signed

……

Declaration Plagiarism 22/05/08 FHDR Approved

COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE

DECLARATION 2– PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this dissertation (include publications in preparation, submitted, in press and published and give details of the contributions of each authors to the experimental work and writing of each publication).

Publication 1:

Publication 2:

Signed:

……

TABLE OF CONTENTS

CHAPTER 2: PHYSICO-CHEMICAL ANALYSIS AND ENUMERATION *YERSINIA* IN TREATED WASTEWATER EFFLUENT AND RECEIVING WATER BODIES.

CHAPTER 3: ANTIBIOTIC RESISTANCE AND VIRULENCE DETERMINANTS OF *YERSINIA ENTEROCOLITICA* ISOLATES RECOVERED FROM TREATED WASTEWATER EFFLUENT AND RECEIVING RIVERS

ACKNOWLEDGEMENTS

I am grateful to the Almighty God for His grace and mercies that strengthened me to further my studies and write this thesis. My sincere thanks goes to the following special people who contributed to this success.

- I am exceptionally grateful to my supervisors, Prof A. O. Olaniran and Prof B. Pillay, from whom I have learnt a lot through their guidance and support.
- My utmost gratitude to my best friend Thembelani Sithole, thank you for the support and love I had received from you throughout this journey.
- My sister Sebenzile Mathe - Mohlala, for being my mother through it all, love and support and endless prayers not to forget her beloved husband David Mohlala.
- My son Bayanda Amukele Sithole and my friend Noyise Ntshobeni for the support through this journey.
- I'm grateful for the support frrom Bonga, Dumi, Lu, Lindo, Khethiwe and the family at large and my friends for believing in me.
- I am also grateful for the assistance received from staff in the Microbiology Department.
- I am also grateful for the support from the students in Lab 1 and 4.
- I acknowledge the funding received for my studies from National Research Foundation (NRF).

LIST OF FIGURES

List of Tables

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

South Africa is located in a semi-arid part of the world. The climate varies from desert and semi-desert in the west to sub-humid along the eastern coastal area, with an average rainfall for the country of about 450 mm per annum (mm/a), well below the world average of about 860 mm/a, while evaporation is comparatively high (Turpie and Visser, 2013). As a result, South Africa's water resources are, in global terms, scarce and enormously limited. The country has no truly large or navigable rivers, and the combined flow of all the rivers in the country amounts to approximately 49 000 million cubic metres per annum (m^3/a), less than half of that of the Zambezi River, the closest large river to South Africa. Groundwater plays an essential role in especially rural water supplies, due to the predominantly hard rock nature of the South African geology, only about 20 percent of groundwater occurs in major aquifer systems that could be utilised on a large scale. Natural availability of water across the country is also uneven due to the poor distribution of rainfall. This condition is compounded by the strong seasonality of rainfall, as well as high within-season variability, over the entire country. Consequently surface runoff is also uneven. As a result, stream flow in South African rivers is at relatively low levels most of the time. The irregular high flows that do occur limit the amount of stream flow that can be relied upon to be available for use (Le Roux and van Hyussteen, 2010).

Historically, the degree of development of potable water infrastructure has varied significantly among different geographical areas in South Africa (Schreiner and Van Zyl 2006). Prior to 1994, the majority of the black population was confined to homelands and townships with limited if any infrastructure (DWAF, 1994). This has resulted in the development of large settlements with no potable water infrastructure. After 1994, the South

African government started to address these issues and to date major improvements have been achieved. By 2002, 84.5% of the population had access to piped and tap water inside their homes or within 200 m of their residence from communal or neighbours' taps or boreholes. A coverage increase to 89.3% was achieved by 2010 (Stats SA, 2011). This contributed to a decrease in the average risk of contracting diarrhoeal diseases among children under 5 years of age from 17.8% in 2002 to 11.2% in 2010. Around 65% of all economic activity is concentrated in the metropolitan areas of the Gauteng Province and the municipalities of Cape Town, eThekwini and Nelson Mandela Bay (Le Roux and van Hyussteen 2010).

A considerable percentage of the South African population is likely to occasionally rely on water resources of poor microbial quality to meet their demand for domestic use (Haarhoff, 2008). Domestic water use includes drinking, laundry, cooking and personal hygiene. The water resources in question include streams, springs, and rivers, *i.e.*, surface water resources. The public health risk is measured by the amount of indicator organisms such as faecal coliforms and *Escherichia coli* present in water (DWAF, 1996). Raw water obtained from the surface sources can meet the microbial criteria for domestic use after minimal treatment, *i.e.*, boiling, addition of bleach or sand filtration (Murray *et al*, 2004). Boiling can, however, be time and energy consuming at the household level and bleach is often too expensive for poor households (Monyai, 2004). Thus treatment is frequently not done before domestic use, which in turn increases the chances of a waterborne disease outbreak, e.g., from hepatitis E and cholera (Dalsgaard *et al*, 2001). The major health risks associated with surface water are from microbial pathogens derived from human and animal faeces. Pathogenic organisms found in water with high counts of faecal coliforms include *Escherichia coli*, V*ibrio cholerae, Aeromonas hydrophilla*, *Shigella dysenteria*, *Salmonella typhimurium*,

Pseudomonas spp. and *Klebsiella* spp. and all these organisms can cause waterborne diseases. Therefore monitoring of microbial quality of surface water in South Africa is an important exercise in public health protection (Momba and Notshe, 2003).

1.2 Current water availability and use in South Africa

Water in South Africa is obtained from the following three sources in the order of magnitude: surface water (77%), return flows (14%) and groundwater (9%). There is a 98% assurance level which proposes that any peaks in future demand will exceed supply and this is a source of vulnerability that needs to be addressed (REF). The available water resources in South Africa are distributed as detailed in Table1.1

Table 1.1: Water resource allocations per water user group in South Africa.

Water user/sector	Proportion of allocation
Agriculture	62%
Domestic	27%
Urban	23%
Rural	4%
Industrial	3.5%
Afforestation	3.0%
Mining	2.5%
Power generation	2.0%

Source: South African Department of Water Affairs & Forestry (2004).

1.3 Quality of drinking water and sanitation in South Africa

Access to clean drinking water and basic sanitation, including toilets, wastewater treatment and recycling; influence a country's developmental progress in terms of human health, education and gender equality. The provision of sustainable drinking water and sanitation are insufficient in various parts of Africa and, where available, water supply and sanitation services are differentiated according to urban, rural or informal settlements. The lack of an

adequate water supply at home or in its immediate surrounding area is a particular problem in South Africa. In rural regions of Africa, people spend, on average, three hours a day engaged in the collection of water for a family of six and this restricts their opportunities for education. Lack of access to good quality and inadequate sanitation can cause several diseases which are transmitted from human and animal waste to humans via contaminated hands, soil, water, etc. There are several diseases that can be prevented with good quality water supply and basic sanitation, e.g., diarrhoea and cholera which are estimated to causing 2 million deaths per year (WHO, 2009).

The provision of water and sanitation services addresses the critical needs of people. Safe water and good sanitation are essential to protect human health and maintaining a disease free environment (Okonkwo, 2010).

1.4 Wastewater treatment plants and wastewater effluent

As from 1956, South Africa set a mandate through the South African Water Act (Act 54 of 1956) that the discharge effluent be treated to acceptable standards and returned to the water course of its origin, clean and safe (Morrison *et al*, 2001). As the demand for water increased, due to economic expansion and population growth, the operation of wastewater and sewage treatment plants has been operated under stress in South Africa. This exerted pressure on water and sanitation authorities to find ways to sustain the quality of water resources (Turton, 2008). A number of studies conducted thus far have indicated that wastewater and sewage effluent from treatment plants and deteriorating infrastructure are a major source of pollution, contributing to a number of pollutants found in water resources (Ngwenya, 2006). The deteriorating state of municipal wastewater and sewage treatment infrastructure in South Africa is the largest factor contributing to the water contamination experienced in most parts of the country and a major contributor to health problems in poor communities (Coovadia *et al*, 1992). In many parts of South Africa, there have been outbreaks of typhoid fever

including KwaZulu-Natal, Limpopo and the Transkei (Coovadia *et al*, 1992). Another incident occurred in the Eastern Cape where 94 patients were treated with diarrhoea symptoms while 18 babies died (Ukhahlamba District Municipality Addendum, 2008).

1.4.1 Environmental and public health implications of poor quality wastewater

The world is facing problems regarding the proper/ recommended management of wastewater. This is due to extensive industrialization, ever-increasing population density and high urbanized societies (McCasland *et al*., 2008). The effluents from domestic and industrial activities amount to the key sources of the natural water pollution load. This is a crisis in terms of wastewater management and can lead to a pollution problem, which not only increases treatment cost, but also introduces a range of chemical pollutants and microbial contaminants to water sources (Amir *et al*., 2004).

The prevention of pollution of water sources and protection of public health by maintaining water supplies free from diseases, are the two essential reasons for treating wastewater. This is accomplished by removing organic substances that have a high demand for oxygen from the system through the metabolic reactions of microorganisms, the separation and settling of solids to create an acceptable quality of wastewater effluents, and the collection and recycling of microorganisms back into the system, or removal of excess microorganisms from the system (Abraham *et al*., 1997). In municipal wastewater treatment systems, the common water quality variables of concern are biological oxygen demand (BOD), chemical oxygen demand (COD), dissolved oxygen (DO), suspended solids, nitrate, nitrite and ammonia nitrogen, phosphate, salinity and a range of other nutrients and trace metals (Brooks, 1996).
The presence of high concentrations of these pollutants exceeding the values stipulated by national and international regulatory bodies is considered unacceptable in receiving water bodies. This is because, apart from causing a major drawback in wastewater treatment

systems, they also lead to eutrophication and various health impacts in humans and animals (EPA, 2000). In recent years, the reuse of treated effluent that is discharged to the environment from municipal wastewater treatment plants is receiving an increasing attention as a dependable water resource. In many countries, wastewater treatment for reuse is an important aspect of water resources planning and implementation. This is intended for discharging high quality water supplies for potable use. Furthermore, wastewater reuse is increasingly becoming important for supplementing drinking water needs in some countries around the world. The option of reuse of wastewater is becoming a necessity as a result of increased climate change leading to droughts and water scarcity (Rietveld *et al*., 2009).

1.4.2 Characteristics of wastewater effluents

The physicochemical characteristics of wastewater that are of concern are pH, dissolved oxygen (DO), oxygen demand (chemical and biological), solids (suspended and dissolved), nitrogen (nitrite, nitrate and ammonia), phosphate, and metals (DeCicco, 1979; Larsdotter, 2006). A pH less than 7 in wastewater influent is an indication of septic conditions while values less than 5 and greater than 10 indicate the presence of industrial wastes and non-compatibility with biological operations. The pH concentration range for the existence of biological life is quite narrow (typically 6-9) (Akpor and Muchie, 2011). Extreme pH is detrimental to biological processes in wastewater treatment units (EPA, 1996; Gray, 2002).

Another parameter that has a significant effect on the characteristics of water is dissolved oxygen. It is required for the respiration of aerobic microorganisms as well as all other aerobic life forms. Dissolved oxygen concentration is governed by the solubility, temperature, partial atmospheric pressure and the concentration of impurities such as salinity and suspended solids in the water (Metcalf and Eddy, 2003).

Oxygen demand, which may be in the form of BOD or COD, is the amount of oxygen used by microorganisms as they feed upon the organic solids in wastewater (FAO, 2007). The 5-day BOD test (BOD_5) is the most widely used test to measure organic pollution parameter applied to wastewater. It involves the measurement of dissolved oxygen used by microorganisms in the biochemical oxidation of organic matter. The presence of sufficient oxygen promotes the aerobic biological decomposition of an organic waste (Metcalf and Eddy, 2003). Although BOD test is widely used, it has a number of limitations, which include the requirement of a high concentration of active acclimated microorganisms and the need for treatment when dealing with toxic wastes thus, reduces the effects of nitrifying organisms. The BOD measures only the biodegradable organics and requires a relatively long time to obtain test results (Metcalf and Eddy, 2003). Similarly, the COD test measures the oxygen equivalent of the organic material in wastewater that can be oxidized chemically. The COD will always be higher than the BOD. This is because the COD measures substances that are both chemically and biologically oxidized. The ratio of COD: BOD provides a useful guide to the proportion of organic material present in wastewaters, although some polysaccharides, such as cellulose, can only be degraded anaerobically and so will not be included in the BOD estimation.

Heavy and trace metals are also of importance in water. The metals of importance in wastewater treatment are As, Cd, Ca, Cr, Co, Cu, Fe, Pb, Mg, Mn, Hg, Mo, Ni, K, Se, Na, V and Zn. Living organisms require different amounts of these metals (Ca, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni and Zn) as nutrients (macro or micro) for proper growth. Other metals (Ag, Al, Cd, Au, Pb and Hg) have no biological role and therefore are non-essential (Metcalf and Eddy, 2003). Heavy metals are one of the persistent pollutants in wastewater. Unlike organic pollutants they cannot be degraded but build up throughout the food chain, producing

potential human health risks and ecological disturbances. Their presence in wastewater is due to discharges from residential dwellings, groundwater infiltration and industrial discharges. The build-up of these metals in wastewater depends on many local factors such as the type of industries in the region, way of life and awareness of the impact on the environment through the careless disposal of wastes (Silvia *et al*., 2006).

1.4.3 Effect of untreated or partially treated water in the environment and public health

There is a significant relationship that exists between the quality of the final effluent and the receiving water bodies. Chemical and microbiological changes take place in water bodies as a result of a variety of anthropogenic activities due to the discharge of raw wastewaters into the receiving water bodies such as streams, rivers, lakes and ponds (Momba *et al.,* 2006). These activities increase treatment costs and discharge of a variety of potentially pathogenic microorganisms to waters, thereby causing waterborne diseases with many health impacts and socio-economic effects (Craun, 1991) and a reduction in the quality of water (Simpson and Charles, 2000). Igbinosa and Okoh (2009) from their study revealed that there was an adverse impact on the physico-chemical characteristics of the receiving watershed as a result of the discharge of inadequately treated effluents from the wastewater treatment facility which poses a health risk to several rural communities which rely on the receiving water bodies primarily as their sources of domestic water.

1.4.4 Efficiency of wastewater treatment plants

The waste stabilization efficiency of a wastewater treatment plant (WWTP) is dependent on the type of sewer collection system, type of waste entering the sewer, type of wastewater treatment technology, the quality of domestic water and the standard of living of the community (Hammer, 1996). The most important factor that affects the removal efficiency of

treatment plants includes seasonal changes. In a previous study, It was reported that the final effluents were less polluted in the rainy season due to dilution by the rain, than that recorded during the dry season (Kantachote *et al*., 2009). There are other factors that affect the removal efficiency of wastewater treatment plants which include the type of wastewater treatment system, temperature, dissolved oxygen, pH, the time of sampling during the day and light intensity available.

1.4.5 Inefficiency of wastewater treatment plants

The high quality of the final effluent results in a high quality of the receiving water body. The inefficiency of sewage treatment plants and their effluents negatively affect the receiving water bodies (Momba *et al.,* 2006). The prevalence of infective agents in the final effluents after the treatment process is an indication of the inefficiency of the wastewater treatment plants for the removal of the pathogens, a result of poor disinfection process and poor maintenance of the infrastructure (Pearson and Idema, 1998) especially when the receiving water is used for domestic, recreational and agricultural purposes (Tchobanogeuos, 1979). The efficiency of sewage treatment plants is measured in terms of removal of organic matter determines the general efficiency indicator in terms of average TSS, COD, BOD and ammonia removal efficiencies (Sincero and Sincero 1996). There are several factors that are responsible for the inefficiency of a wastewater treatment plant viz., poor conditions of sewerage system, improper design of the plant and organizational problems (Storhaug, 1990), overloading and discharge of industrial effluents (Bataineh *et al*., 2002), chemical shock, inadequate mixing in the equalization tank and inappropriate C/N/P ratio in anaerobic and aerobic tanks (Sadeghpoor *et al.,* 2009), short retention time and the treatment efficiency may be affected when the system is hydraulically under loaded (Kapur, 1999).

1.5 Waterborne bacterial pathogens

Infectious diseases are commonly transmitted via the faecal-oral route. Infected humans or animals excrete viable pathogens. If faecal contamination occurs on the water supply, infections result from drinking water or using the water for domestic purposes. The cost, the complexity of diagnostic and detection methods make it difficult to know which microorganism is responsible for a certain episode, and also some of the microorganisms are more likely to cause diseases than others leading to death. The World Health Organization has identified several orally transmitted waterborne pathogens that have high health significance. These pathogens include bacteria, viruses, protozoa, and one helminth (Vissscher, 1990) as detailed in Table 1.2.

1.5.1 Bacteria

Bacteria are organisms that usually consist of a single prokaryotic cell. They have many different shapes; i.e rods, cocci, helical, etc. They are typically small between 0.1 – 5.0 µm compared to eukaryotic cells. The WHO (World Health Organisation) identified six bacteria that have high health significance.

1.5.1.1 *Escherichia coli*

Eschericia coli are Gram-negative, rod-shaped bacteria belonging the family Enterobacteriaceae. There are four known classes of virulent *E. coli* that cause infection in humans. These are the enteroinvasive (EIEC) strains, the enteropathogenic (EPEC) strains, the enterotoxigenic (ETEC) strains, and the enterohemorrhagic (EHEC) strain designated *Eschericia coli* O157:H7. E. coli infection results in mild to severe diarrhoea which can lead to dehydration. Infection of O157:H7 can cause hemolytic uremic syndrome, resulting in

severe anemia and kidney failure. Healthy cattle are a significant reservoir. The detection of *E coli* O157:H7 in environmental samples is a major public health concern (Nicolai, 2002).

1.5.1.2 *Salmonella* and *Shigella* species

Salmonella is a rod-shaped, motile Gram-negative bacterium. There is widespread occurrence in animals, especially in poultry and swine. *Salmonella typhi,* the most virulent of the *Salmonella* species, causes Typhoid fever. Symptoms include weakness, confusion, headache, and most notably a very high fever. Other *Salmonella* species generally produce milder symptoms: nausea, vomiting, abdominal cramps, mild fever, and headache. Some strains of *Salmonella* have developed antibiotic resistance.

Shigella is a rod-shaped, non-motile, Gram-negative bacterium. The major symptom is watery or blood streaked diarrhea. *Shigella* spp. can only grow in the intestines of humans but can survive for a long time in water. Only a small number of organisms (200 cells) are needed to cause infection. *Salmonella* and *Shigella* species have been reported to be prevalent at all stages of treatment in conventional wastewater treatment plants including the final effluents indicating the inefficiency of wastewater treatment plants in totally eliminating these pathogens from wastewater (Pant and Mittal 2007).

1.5.1.3 *Vibrio cholerae*

Vibrio cholerae is a Gram-negative motile rod shaped bacterium. Infection causes large secretions of chloride into the intestines. This in turn causes water and electrolytes to leave the body via osmosis. Diarrhea can be very severe and cause death in less than a day. Humans are the main reservoir but *Vibrio cholerae* can multiply in water, which is the natural habitat for *V. cholera* and can become a pathogen by horizontal gene transfer due to environmental stress (Boles *et al*., 2004). These organisms transfer genetic material from one

another through mechanisms such as conjugation, transformation, or transduction and it is incorporated into the recipient chromosome by recombination. These genes may contain single or multiple nucleotide mutation. The change in DNA is due to the fact that Vibrios contain large chromosomal integrons (Cambray *et al*., 2010) and belong to the group of naturally competent bacteria, which allows them to absorb free DNA from their surrounding environment and recombine it into their genome (Seitz and Blokesch, 2013a). This organism can also cause wound infections and bacteremia. Cholera has re-emerged as a potential infectious disease in the recent past with a worldwide increase in its occurrence (Nicolai, 2002).

Table 1.2: Major waterborne pathogens and diseases caused (Yates, 2013).

Microorganisms	Pathogenic organism	Diseases caused
Bacteria	*Salmonella*	Typhoid,
	E. coli (enterotoxigenic)	Bacillary dysentery
	Yersinia	Gastroenteritis
	Campylobacter	Gastroenteritis
	Vibrio	Cholera
	Leptospira	Leptospirosis
Viruses	Polio virus	Paralysis
	Rota virus	Infantile gastroenteritis
	Hepatitis A virus	Infectious hepatitis
	Norwalk virus	Gastroenteritis
	Adenovirus	Conjunctivitis
	Reovirus	Respiratory disease
	Echovirus	Aseptic Meningitis
	Giardia lamblia	Diarrhea, malabsorption
	Entamoeba coli	Diarrhea, ulceration
	Entamoeba histolytica	Amoebic dysentery
Protozoa, helminths and other parasites	*Cryptosporidium parvum*	Diarrhea
	Ascaris lumbricoides	Ascariasis
	Ancylostoma	Anemia
	Nectar	Anemia
	Trichuris	Diarrhea, anemia
	Tenia solium	Teniasis
	Trichuris trichuria	Trichuriasis

1.6 Historical background of *Yersinia* species

In 1944, Van Loghem proposed that a new genus designated *Yersinia*, be separated from the genus *Pasteurella*. This proposition became effective in 1974. The first species identified in this genus by Malassez and Vingalin 1883 was *Yersinia pseudotuberculosis.* The second species, *Yersinia enterocolitica*, was identified in 1939 by Schleifstein and Coleman. This species was found to be heterogeneous and to contain several related species (*''Y. enterocolitica-like'')* that were subsequently designated *Y. intermedia, Y. frederiksenii, Y. kristensenii, Y. aldovae, and Y. rhodei*. More recently, *Y. mollareii* and *Y. bercovieri* were also separated from *Y. enterocolitica.* Finally, the species *Y. ruckeri* was included in the genus. Members of the genus *Yersinia* are non-spore forming, Gram-negative or Gram-variable, facultative anaerobic, rod-shaped or coccoid cells of 0.5-0.8 µm in width and 1-3 µm in length. *Y. enterocolitica* are of pathogenic importance of humans and certain warm blooded animals, whereas the other species are of environmental origin and may generally be classified as opportunists (Fenwick and Murray, 1991; Wauters *et al*., 1988).

Yersinia is well established as a food borne pathogen of human concern and is mostly associated with pork products (Nesbakken *et al*., 1985). *Yersinia spp*. have been reported as contaminants of raw meats and isolated from beef (Fukushima *et al*., 1987), lamb (Ibraham and Mac Rae, 1991), and poultry (De Boer *et al*., 1982). *Yersinia* spp. have also been isolated from cooked meats including turkey, chicken, pork and lamb (Hudson *et al*., 1992). The detection of *Yersinia* spp. in meats and other products is of particular concern in relation to consumer safety as these organisms are capable of growth on both raw and cooked meats at refrigeration temperatures which could lead to the propagation of significant numbers of the organism (Manu-Tawiah *et al*., 1993; Hudson and Mott, 1993).

Bacteria of the genus *Yersinia* cause diseases ranging from enteritis to bubonic plague (Black Death). The initial characterization of this genus was performed in 1894 in Hong Kong, when Alexandre Emile John Yersin together with Shibasaburo Kitasato identified *Yersinia pestis* (formerly known as *Pasteurella pestis*) as the causal agent of the bubonic plague (Bottone, 1997).

1.6.1 Classification of *Yersinia enterocolitica*

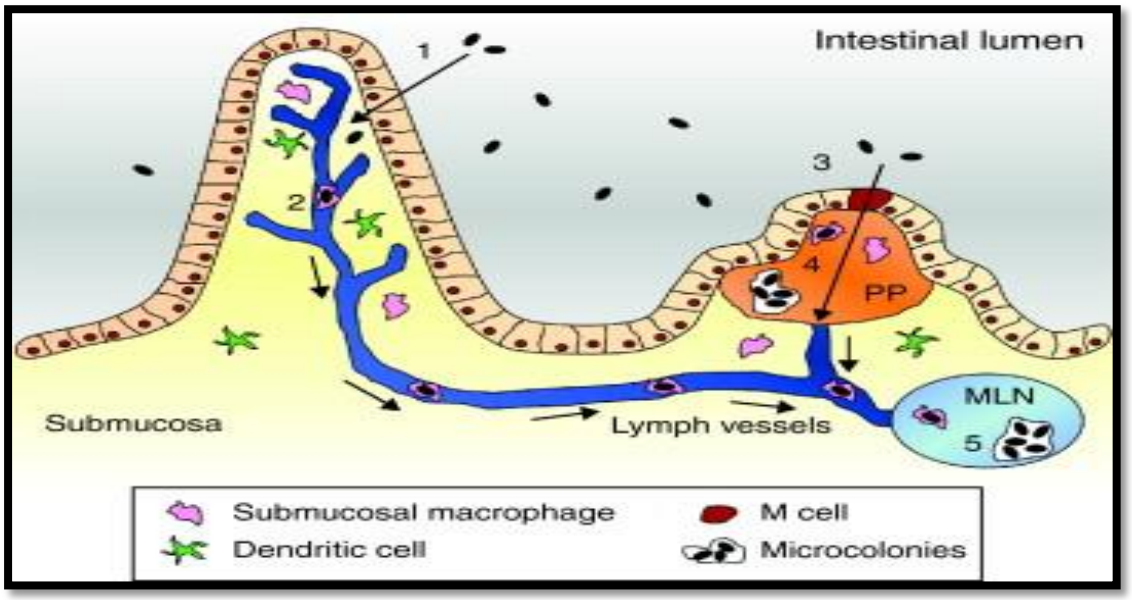
Yersinia enterocolitica is the most predominant *Yersinia* species associated with disease in humans (Bottone, 1999; Robins-Browne, 2001). It has also frequently been isolated from animals, food and the environment (Fredriksson-Ahomaa *et al*., 1999; Thoerner *et al*., 2003; Falca˜o *et al*., 2004). *Yersinia enterocolitica* is particularly well-adapted to survive in a wide range of natural and host environments. As one of the few human pathogens that can proliferate at refrigeration temperatures, it is particularly significant for food microbiology and in blood transfusion (Robins-Browne, 2001).

Y. enterocolitica is divided in 6 biogroups that can be differentiated by biochemical tests: 1A (non-pathogenic), 1B (highly pathogenic), 2, 3, 4 and 5 (weakly pathogenic). Serologically, they can be seperated into approximately 60 serogroups based on the variability of the O side chain (O-antigen) of LPS. Eleven serogroups have been associated with human infections (Bottone, 1999), with the majority being caused by serogroups O:3, O:9, O:5,27 and O:8. More rarely encountered virulent serogroups are O: 4, 32, O:13, O:18, O:21 (Skurnik and Toivanen, 1993). *Y. enterocolitica* is widely distributed in nature in aquatic and animal reservoirs, with pigs serving as a major reservoir for the human pathogenic strains. The majority of non-porcine isolated bacteria are of the non-pathogenic group 1A (Bottone, 1997). This species encompasses three grades of pathogenicity mostly non-pathogenic strains (biotype 1A), weakly pathogenic strains of biotypes 2 to 6, and highly pathogenic strains (biotype 1B). The high pathogenicity is attributed to the Yersiniabactin-siderophore-mediated

iron uptake system. Yersiniabactin is maintained by genes located in the *Yersinia enterocolitica* high-pathogenicity island (Carniel, 2001).

1.6.2 Pathogenesis model of *Yersinia enterocolitica*

The bacteria invades the epithelial cells, binding to intestinal brush-border membranes, from where they penetrate M cells and gain access to and multiply in Peyer's patches (Fig 1.1). Bacteria taken up by M cells are usually phagocytosed and killed by macrophages of Peyer's patches; however, pathogenic *Y. enterocolitica* strains have several surface components which enable them to resist phagocytosis and escape from complement-mediated death. Continuing proliferation of the bacteria results in an inflammatory reaction, which leads to local micro abscess formation and ulceration of the overlying epithelium. Finally, the bacteria may spread to the mesenteric lymph nodes and enter the bloodstream (Robins-Browne 2001).



Source: (Sabina *et al*, 2011)

Fig 1.1: Pathogenesis model of *Yersinia enterocolitica*

1.6.3 Clinical picture of *Yersinia enterocolitica*

Yersinia enterocolitica causes a wide array of gastrointestinal syndromes such as enteritis, enterocolitis, acute mesenteric lymphadenitis and terminal ileitis depending partly on the age and condition of the host and serogroup of bacterial strain. Acute enteritis with fever and inflammatory diarrhoea is the most frequent occurrence in children, while acute terminal ileitis and mesenteric lymphadenitis, often mimicking appendicitis, is more common in young adults (Lee *et al*., 1990; Chandler and Parisi, 1994). Illness may last for 3 – 28 days in infants and 1 to 2 weeks in adults. Septicemia may occur in immunosuppressed hosts or those with an underlying disorder especially when an iron overload was induced (Blei and Puder, 1993). Infection with *Yersinia enterocolitica*, predominantly serogroup O:3, can also lead to secondary immunologically induced sequelae such as arthritis, erythema nodosum, glomerulonephritis or myocarditis (Laitenen *et al*., 1972). In most cases, *Y. enterocolitica* infections and their sequelae are self-limiting.

1.6.4 Virulence and epidemiology of the *Yersinia* species

The virulence of pathogenic biotypes is attributed to the presence of plasmidial and chromosomal genes. The virulence plasmid of *Yersinia* (*pYV*) encodes adhesin A (*YadA*), *Yersinia* outer proteins (*Yops*) from the type III secretion system, and transcriptional regulator gene (*virF*) (Cornelis, 2001). The chromosomal virulence genes include *inv* (invasin), *ail* (attachment and invasion locus), *ystA* (Yersinia stable toxin) and *myfA* (mucoid *Yersinia* factor) (Ravell, *et al*, 2001). Some of these factors are restricted to pathogenic *pYV*- bearing strains of *Yersinia enterocolitica*, such as *ail*, *ystA* and *myfA*, while the *inv* gene is common to pathogenic and non-pathogenic strains (Falca˜o*et al*., 2006). *Yersinia enterocolitica* biotype 1A strains are classically considered as non-pathogenic, since they do not bear pYV plasmid and chromosomal virulence genes, such as *ail*, *myfA*, *yst*A and the *ysa* locus (Robins-Browne, 1989).

1.6.5 Plasmid-encoded virulence factors

All virulent *Y. enterocolitica* strains carry an approximately 70-kb virulence plasmid, termed pYV (plasmid for *Yersinia* virulence), which is required for expression of virulence. Virulence plasmids of pathogenic *Yersinia* species are closely related to each other, sharing functional similarities and a high degree of DNA homology. The presence of pYV enables the bacteria to survive and multiply in lymphoid tissues of their host (Sato *et al*, 2006).

The *YadA* protein promotes binding to epithelial cells. The gene codes for the major outer membrane protein *YadA*, which forms a fibrillar matrix on the surface of *Y. enterocolitica* and can be expressed at 37°C.

YadA plays a protective role in *Y. enterocolitica*, with different functions such as, serum resistance, surface hydrophobicity autoagglutination, adhesion to epithelial cells, expression of fibrils on the surface, haemagglutination, binding to intestinal brush border membranes and resistance to killing by polymorphonuclear leukocytes **(**Heise and Dersch, 2006). One major role of *YadA* is to protect *Y. enterocolitica* against killing by polymorphonuclear leukocytes. Although the mechanism is unknown, *YadA* has been suggested to act by binding to eukaryotic cells, and in doing so, allow delivery of the *Yops*, thus preventing phagocytosis (Grosdent *et al*, 2002).

The *Yop* genes located on the pYV are well-known to code for at least 14 Yops proteins, previously called *Yersinia* outer membrane proteins because they are found in the outer membrane fraction of bacterial extracts (Lee *et al*, 2001). Some of the Yops form pores in the eukaryotic cell membrane, while others Yops are effector proteins delivered through these pores into the cytosol of the target cell. A minimum of different Yop effectors are injected by the Ysc secretion translocation apparatus (Tardy e*t al*, 1999). The genes specific for the type

III machinery (*ysc*) are also located on the pYV. The *yop* and *ysc* genes are temperature and calcium-regulated and can be maximally expressed at 37°C in response to the presence of a low calcium concentration (Venecia and Young, 2005).

1.6.6 Chromosome-encoded virulence factors

Chromosome-encoded factors also play a role in pathogenicity. The chromosome encoded factors are involved in the adhesion and invasion to the host. Adherence to and invasion of epithelial layers require at least two chromosomal genes, *inv* (invasion) and *ail* (attachment invasion locus).

(a). The invasion (*inv*) codes for Inv, an outer membrane protein found on the surface of *Yersinia*, which plays a vital role in promoting propagation and entry into epithelial cells of the stomach lining during the initial stage of infection, that is responsible for binding to β-1-integrins on the apical surface of M cells and initiating uptake of the organism. Entrance through these cells leads to accumulation of bacteria in the Peyer's patches and spread to the mesenteric lymph nodes (Hamzaoui *et al*, 2004). This gene is found in all *Yersinia* species; however non-pathogenic strains lack functional *inv* homologous sequences (Revell and Mille, 2000). Expression of *inv* in *Y. enterocolitica* responds to both temperature and pH, *inv* expression is higher at 26°C compared to 37°C during *in vitro* growth (Logue *et al*, 2000).

(b). The attachment invasion locus (*ail*) codes for the surface protein ail, which is produced at 37°C which is in contrast to the *inv*. The *ail* is known to be restricted to strains of particular biotypes and serotypes associated with disease (Jourdan *et al*, 2000).

(c) The heat-stable enterotoxin role in pathogenesis of *Y. enterocolitica* infection is unclear. Non-pathogenic strains of *Y. enterocolitica* and strains of related species have been found to produce *Yst*. Absence of enterotoxin production *in vitro* at temperatures exceeding 30°C suggests that the toxin is not produced in the intestinal lumen (Amirmozafari and Robertson, 1993).

1.7 Significance of the *Yersinia* species in drinking-water

Although most *Yersinia* spp. detected in water are probably non-pathogenic, in certain circumstances evidence has been presented to support transmission of *Y. enterocolitica* and *Y. pseudotuberculosis* to humans from untreated drinking-water. The most likely source of pathogenic *Yersinia* spp. is human or animal waste. The organisms are sensitive to disinfection processes. Within a Wastewater Sewage Plant, control measures that can be used to minimize the presence of pathogenic *Yersinia* spp. in drinking-water supplies include protection of raw water supplies from human and animal waste, adequate disinfection and protection of water during distribution. Owing to the long survival and/or growth of some strains of *Yersinia* spp. in water, *E. coli* (or, alternatively, thermotolerant coliforms) is not a suitable index for the presence/absence of these organisms in drinking-water.

1.8 Scope of the current study

Due to urbanization and population growth, water shortage is becoming a serious concern, especially in arid and semi-arid regions. Also, rapid increase in urbanization and population result in the generation of more wastewater. It is known that wastewater contains a wide range of pathogens and, sometimes, heavy metals and organic compounds that are hazardous to human health and the environment. Hence, the discharge of inadequately treated wastewater into the environment degrades quality of water and the surrounding environments (Bartram *et al.,* 2005). Since large amounts of wastewater effluents are passed through sewage treatment systems on a daily basis, there is a need to remedy and diminish the overall impacts of these effluents on receiving water bodies. This can be achieved through the application of appropriate treatment processes, which will help to minimize the risks to public health and the environment. To achieve unpolluted wastewater discharge into receiving waterbodies, there is the need for careful planning, adequate and suitable treatment and

regular monitoring. The current study was undertaken to investigate the prevalence of *Yersinia enterocolitica* in treated effluent of two WWTP's and its receiving surface water in Durban, South Africa, and to correlate this finding with physicochemical properties of the wastewater effluent. The correlation between antibiotic resistance patterns, virulence determinants of *Yersinia enterocolitica* and their fingerprinting profiles using RAPD-PCR was also determined.

1.9 Hypotheses

It was hypothesized that treated effluent from wastewater treatment plants are a potential source of *Yersinia* spp in surface water. It was further hypothesized that the *Yersinia* species isolated from the treated wastewater effluent harbour various virulence determinants and are resistant to the commonly used antibiotics.

1.10 Objectives

The objectives of this study were:

1.10.1 To determine the distribution and abundance of *Yersinia* species in treated wastewater effluent and receiving surface water resources.

1.10.2 To determine the phenotypic antibiotic resistance and virulence determinants of *Yersinia eneterocolitica* recovered from treated effluent and receiving surface water.

1.10.3 To determine genetic relatedness of selected *Yersinia enterocolitica* isolates and correlate with their phenotypic resistant attributes.

1.11 Aims

The aims of this study were:

1.11.1 Enumeration of *Yersinia* species by membrane filtration and selective plating on Yersinia selective agar (YSA).

1.11.2 Identification and confirmation of *Yersinia* species isolates via biochemical tests and PCR.

1.11.3 To differentiate the isolates into different important species using PCR targeting specific gene.

1.11.4 To determine the antibiotic susceptibility profiles of the isolates using the Kirby-Bauer disk diffusion assay.

1.11.5 Investigation of the absence or presence of virulence genes in the isolates via PCR.

1.11.6 To differentiate between species of the emerging bacterial pathogens using Random Amplified Polymorphic DNA (RAPD) analysis.

CHAPTER 2

PHYSICO-CHEMICAL ANALYSIS AND ENUMERATION OF *YERSINIA* IN TREATED WASTEWATER EFFLUENT AND RECEIVING WATER BODIES

Abstract

Waterborne diseases are predominantly caused by enteric pathogenic microorganisms including bacteria, viruses and protozoa, which are introduced into surface water through disposal of improperly treated wastewater or agricultural waste and runoff. A large part of the population in South Africa relies on water resources of inadequate microbial quality to meet their domestic and recreational needs. This study investigated the physicochemical properties and *Yersinia* spp. population of treated effluent from two wastewater treatment plants in Durban and the relevant receiving rivers. The temperature of the water samples tested ranged from 15 - 23ºC for the New Germany treatment works (NGTW) and 17 - 24ºC for the Northern Wastewater treatment plant (NWWTP), while pH ranging from 6.8 - 7.8 and 6.7 - 9.0 was recorded for NGTW and NWWTP, respectively. The COD values ranged from 37 - 309 mg/L for NGWT and 52 - 292.3 mg/L for NWWTP. Biological oxygen demand (BOD) values for the NGTW ranged from 4.4 - 282.9 mg/L, while that for NWWTP ranged from 106 - 205.1 mg/L. The correlation matrices showed a significant ($p<0.05$) positive correlation ($r = 0.898$) between pH and BOD. A stronger ($p<0.01$) negative correlation ($r = -0.938$) was observed between the *Yersinia* species load and the total dissolved solids (TDS) in samples collected before chlorination at the NGTW. There was a strong significant ($p<0.01$) positive correlation ($r = 1.000$) between pH and COD for the upstream point of the NWWTP. The high microbial counts obtained before chlorination (BC), was 8-37% after chlorination (AC) for both plants. The *Yersinia* population ranged between 4.3 - 7.8 log cfu/ml and 4 -7.3 log cfu/ml for NGTW and NWWTP, respectively. The highest counts were observed at the upstream points for both treatment plants. A total of 473 isolates were identified on the basis of colony morphology on Yersinia selective agar (YSA). Following biochemical tests, 274 isolates were presumptively identified as belonging to *Yersinia* species. The presumptive isolates were further confirmed by PCR with 171 as the *Yersinia* spp and 40.93% (70/171) isolates confirmed as *Yersinia enterocolitica*. This study showed that the independent treatment plants monitored met some of the standards for water quality such as pH and temperature but fell short for the discharge effluent microbial quality standard. The study also demonstrated that *Yersinia* spp. survived the treatment process and confirmed that Ethekwini municipal wastewater treatment plants still discharge *Yersinia* pathogens into surface water of the Umngeni and Aller rivers. This could pose a health risk to the communities which rely on the receiving water bodies as their primary sources of domestic water. There is a need for the intervention by appropriate regulatory agencies to ensure the production of good quality treated final effluents.

2.1 Introduction

South Africa is a water stressed country because of the low average rainfall of 465 mm per annum received which is below the global average of 860 mm per annum (Pitman, 2011). The availability of good quality water is of paramount importance to minimise or eliminate the risk of contamination of surface water bodies with pathogenic microorganisms (Pitman, 2011). In South Africa, nearly 80% of the population rely on surface water as the main source of water for their domestic and recreational needs (Luyt *et al*., 2012). Most of these people are poor and rely on state intervention for improved water supply. Demand for this important scarce resource is expected to increase due to rapid industrial development, increasing human population and increase in consumption increase, and the resulting impact of human activities on the environment (Adewumi *et al.,* 2010; Ngwa *et al.,* 2013). Natural water bodies such as rivers are subject to dramatic changes in microbial and physico-chemical qualities as a result of a variety of anthropogenic activities on the watershed. These changes are caused by discharges of municipal raw waters or improperly treated effluent at a specific point-source into the receiving surface waters (Igbinosa and Okoh 2009).

Yersinia is a bacterial genus belonging to the family Enterobacteriaceae. *Yersinia* species are Gram-negative, rod-shaped bacteria, a few micrometers long and are facultative anaerobes (Ryan and Ray, 2004). At present, the genus includes 11 established species: *Y. pestis, Y. pseudotuberculosis, Y. enterocolitica, Y. frederiksenii, Y. intermedia, Y. kristensenii, Y. bercovieri, Y. mollaretii, Y. rohdei, Y. aldovae* and *Y. ruckeri*. Among these species only *Y. pestis, Y. pseudotuberculosis* and some strains of *Y. enterocolitica* are of pathogenic importance to humans and certain warm blooded animals, whereas the other species are of environmental origin and may best act as opportunists (Murray *et al*., 1991; Wauters *et al*., 1988).

Yersinia enterocolitica is the most prevalent *Yersinia* species linked to disease in humans (Bottone, 1999; Robins-Browne, 2001). It has also frequently been isolated from animals, food and the environment (Fredriksson-Ahomaa *et al*., 1999; Thoerner *et al*., 2003; Falca˜o *et al*., 2004). *Yersinia enterocolitica* is particularly well-adapted to survive in a wide range of natural and host environments and can proliferate at refrigeration temperatures. It is particularly significant in food microbiology and in blood transfusion (Robins-Browne, 2001). *Yersinia enterocolitica* is an enteric bacterium that has been identified as an emerging waterborne pathogen (Theron *et al*., 2002; Sharma *et al*., 2003), however, reports of waterborne disease caused by *Y. enterocolitica* are few. Studies have documented the occurrence of various *Yersinia* spp. in environmental waters (Zanfaly *et al.*, 2008; Amasiani *et al.*, 2013; Sahota *et al.*, 2014). The majority of *Yersinia* isolates recovered from water are considered to be non-pathogenic, due to the different subtypes and some strains which are named *Y. enterocolitica*-like strains due to a few phenotypic similarities they have with the *Yersinia enterocolitica* isolates. However, previous studies have suggested that *Y. enterocolitica* subtyping analyses may not be a reliable indication of pathogenicity but the detection of the *pYV* virulent plasmid can differentiate the pathogenic from non-pathogenic strains (Grant *et al.*, 1998; Thoerner *et al.*, 2003; Bhagat and Virdi., 2007).

Considering that *Y. enterocolitica* is associated with animal hosts and shed in the faeces of infected animals, it is reasonable to assume that waterborne transmission of *Y. enterocolitica* may be occurring (Bottone, 1997). Illness caused by *Y. enterocolitica* infection is referred to as yersiniosis and can result in a wide variety of disease outcomes. Typical disease symptoms include those associated with gastrointestinal disease, such as fever, abdominal pain and diarrhoea (Bottone, 1997). However, the consequences of infection can be very serious, particularly in sensitive patients like the young, the elderly and the immunocompromised (Sharma *et. al.*, 2003). Previous studies conducted in some provinces in South Africa have

implicated treated wastewater as a point source of contamination of receiving watershed with pathogenic and emerging pathogenic microorganisms (Igbinosa and Okoh, 2009) .However; there is a shortage of information on the prevalence of *Yersinia* spp. in treated wastewater effluents of some treatment plants in Durban, South Africa. This study therefore investigated the prevalence of *Yersinia* spp., especially *Yersinia enterocolitica* in treated wastewater effluent of two independent WWTP's in Durban and evaluate the impact of the effluent quality on receiving water surfaces. This study also evaluated the correlation between certain physico-chemical parameters of the wastewater and the *Yersinia* spp. counts.

2.2 Materials and Methods

2.2.1 Plant description

The two wastewater treatment plants investigated in this study were the Northern wastewater treatment plant (NWWTP) and the New Germany treatment works (NGTW) located in Durban, South Africa. The NWWTP is located at geographical coordinates 29°48′45.62″S and 30° 59′ 45.62″ E and processes 70 ML per day of industrial and domestic wastewater and the treated effluent is discharged into the Umgeni River. The NGTW is located at geographical coordinates 29°48′ 21.68″S and 30°53′ 50.44″E and treats more of the domestic waste than industrial waste. The capacity of the plant is about 7 ML per day but running at a capacity less than 1ML at the time of sampling. Treated effluent from this plant is released into Aller River, Alongside the river is an informal settlement with inadequate sewage and waste removal system and the residing community is believed to rely on the receiving river effluent for some of their day-to-day domestic activities.

2.2.2 Collection of water samples

Wastewater sample collection was done for six consecutive weeks (February – March), for both plants, at four sampling points of interest namely: before chlorination (BC), treated effluent discharge point after chlorination (DP), upstream (US) and downstream (DS) of the river receiving the treated effluent. Samples were collected in 2 X 1L plastic containers pre-sterilized by soaking in 70% (v/v) ethanol. During sampling, the containers were first rinsed with water from the respective sampling point. The containers were not filled to the top but a headspace was left to allow for proper mixing during analysis. The samples were placed in a cooler box filled with ice packs and transported to the Department of Microbiology at the University of KwaZulu-Natal (Westville campus). Samples were stored at 4 °C and analysed within 24 h of collection.

2.2.3 Physico-chemical Parameter Analysis

Temperature of the water samples was measured on site using a portable mercury thermometer (Scientific, USA); turbidity was measured with a turbidity meter 21000P (HACH, USA); pH was determined using a Microprocessor pH meter 211 (HANNA, USA). Chemical oxygen demand (COD) was measured using a Spectroquant Nova 60 **(**Merck, USA) according to the manufacturer's instructions. A 3 ml sample was added to COD test cell (Merck), thoroughly mixed and heated for 2h in a TR420 spectroquant thermo-reactor and cooled to room temperature in the dark. The COD test cells were shaken and cooled further for another 10 minutes and read using the Spectroquant Nova 60 (Merck, USA). Total dissolved solids (TDS) were measured using the CDC 401 probe and HQ40d multimeter (HACH Colorado, USA). Dissolved oxygen (DO) was determined using the LDC 101 probe with an HQ40d multimeter (HACH) for day 0 and day 5 after incubation at 20 ºC and the biological oxygen demand (BOD_5) calculated thereafter using the two values using the following equation:

$$BOD_5\,(mg/L) = D1\text{-}D2 / P$$

Where, (D1= DO of diluted sample immediately after preparation, D2= DO of the sample after 5 days incubation at 20 ºC and P = volumetric fraction of the sample used).

2.2.4 Microbial Analysis

2.2.4.1 Enumeration and isolation of *Yersinia* species

Five hundred millilitres of each water sample was filtered through a 0.45 µm pore membrane (PALL Life Sciences, Mexico). Using sterile forceps, filters containing the residue from the filtrate were rolled such that sample residue was on the inside. Rolled filters residue were placed into test tubes containing 10 mL modified tryptic soy broth (mTSB) and incubated at 12 °C for 2-3 days. After 24 h of incubation, 10 µL of irgasan stock solution (4 mg/mL in

methanol) was added to each enrichment culture, to achieve a final concentration of 4 µg/mL (Bhaduri *et al.*, 1997). To reduce the background flora, a 0.5 ml portion of the enrichment culture was transferred into 4.5 ml of 0.5% potassium hydroxide solution (KOH) and mixed gently for 20 s (Söderqvist *et al.*, 2011). Thereafter, 1 ml of the treated samples was used for 10- fold serial dilutions in sterile distilled water and 100 µl of each dilution was then spread plated onto Yersinia selective agar (YSA) and plates were incubated at 28 ℃ for 16-24 h. After incubation, the plates were observed for colonies displaying typical *Yersinia* spp. morphology of deep red centre with a transparent margin, often referred to as having "bulls-eye" appearance. The colonies were enumerated and expressed in colony forming units per millilitre (cfu/ml).

2.2.4.2 Biochemical Identification and Molecular Confirmation of *Yersinia* spp. by PCR

The presumptive colonies were further identified by the biochemical reactions that would assist in eliminating organisms that may grow and resemble *Yersinia* spp on the YSA agar. The biochemical tests performed include urease test, citrate utilization, lactose fermentation and oxidase test**.** DNA extraction was done from the presumptive colonies tested positive from biochemical tests screening. The isolates were grown on tryptic soy agar (TSA) at 37 °C for 24 h. Following incubation, 5 single colonies were picked and transferred into 100 µL nuclease-free water in 1.5 ml Eppendorf tube and homogenized by vortexing. The tubes were then placed in a boiling water bath at 100 ℃ for 10 min, cooled down centrifuged at 12000 rpm for 3 min at 25 ℃ and immediately placed on ice. The supernatant was transferred into a new tube and used directly as DNA template in PCR assay (Sambrook and Russell, 2001) for *Yersinia* spp. identification. Each 25 µl reaction mixture contained 20 pmol of each primer (Table 2.1), 0.20 mM dNTPs, 4 mM $MgCl_2$, 2.5 U of *Taq* DNA polymerase, 2.5 µl of 10X PCR buffer and 1 µl of the DNA template. Cycling conditions were initial denaturation (80

°C, 5 min), 30 cycles of amplification; 1 min of denaturation at 94 °C, 1 min of annealing at 63 °C and 2 min of extension at 72 °C. For all the reactions 5 µl of each PCR product was analyzed by electrophoresis on 1.5% (w/v) agarose gels. The products were visualized by UV transillumination (Syngene, UK) after staining in 1 mg/ml ethidium bromide for 15 min.

2.2.4.3 Molecular identification of *Yersinia enterocolitica*

DNA was extracted as described in section 2.2.4.2. To specifically amplify the *Y. enterocolitica* 16S rRNA gene, a primer set by Neubauer *et al*. (2000) was used (Table 2.1). Each 25 µl reaction contained the *Y. enterocolitica* 16S rRNA-specific primers at a concentration of 80 nM each, 200 µM dNTPs, 0.5 U of *Taq* polymerase, 2.5 µl 10x PCR buffer and 2 µl of DNA template. Cycling conditions were: denaturation step at 94 °C for 5 min, followed by 36 cycles consisting of heat denaturation at 94 °C for 45 s, annealing at 62 °C for 45 s, and extension at 72 °C for 45 s. A final extension was performed at 72 °C for 7 min. For all the reactions, 5 µl of each PCR product was analyzed by electrophoresis on 1.5% (w/v) agarose gels. The products were visualized by UV transillumination (Syngene, UK) after staining in 1 mg/ml ethidium bromide for 15 min.

2.2.4.4 Multiplex PCR for identification of *Y. pseudotuberculosis* and *Y. pestis*

DNA was extracted as described in section 2.2.4.2. A multiplex PCR was performed for the confirmation of the *Y. pseudotuberculosis* and *Y. pestis* using primer sets shown in Table 2.1. Each 25 µl contained 1 µl of genomic DNA, 1X PCR buffer, 0.2 mM dNTPs, 0.3 mM of each primer and 0.75 U *Taq* polymerase. The cycling conditions were 95 °C for 5 min, 40 three-step cycles, 94 °C for 20 s, 60 °C for 20 s, 72 °C for 15 s. For all the reactions, 5 µl of each PCR product was analyzed by electrophoresis on 1.5 % (w/v) agarose gels. The products

were visualized by UV transillumination (Syngene, UK) after staining in 1 mg/ml ethidium bromide for 15 min.

Table 2.1: Primers used in this study for PCR detection of genes specific to certain *Yersinia* species.

Target organism	Primer Sequence (5´-3`)	Amplicon size (bp)	Reference
Yersinia spp.	GCGGCAGCGGGAAGTAGTTTA TACAGCGTGGACTACCAGGGT	749	Kalheinz *et al*., (1998)
Y. enterocolitica	AATACCGCATAACGTCTTCG CTTCTTCTGCGAGTACGTC	330	Neubauer *et al*., (2000)
Y. pseudotuberculosis	GTCTGGGCTTTGCTGGTC ACGTCGTCTGTCATGATTCG	756	Steknova *et al.,* (2008)
Y. pestis	GTCTGGGCTTTGCTGGTC CTTGTTAGCGATAGTATCAGAGAAG	510	Steknova *et al.,* (2008)

2.2.5 Statistical Analyses of data

Mean and standard deviation calculations were carried out using Microsoft excel office 2010. The Pearson's correlation of the microbial counts and the physicochemical parameter data was done using the SPSS 18.0 software for windows program (SPSS, Inc. USA) and correlations were considered statistically significant at P values < 0.05 and P values <0.01.

2.3. Results

2.3.1 Physicochemical parameters of water samples

The physicochemical profiles of the treated effluents and receiving rivers of NWWTP and NGTW are presented in Table 2.2 and Table 2.3, respectively. The pH values were between 6.7 ± 0.0 – 8.8 ±.01 for NGTW and 6.7 ± 0.6 - 9.0 ± 0.08 for NWWTP. These values varied between both plants and were within the recommended limits for surface water. The lowest pH (6.7) recorded was from the UP point in NGTW and the BC point of the NWWTP while the highest (9.0) was recorded at the UP point in NWWTP. The temperature of the water samples fluctuated from 15 °C to 23 °C and 17 °C to 24 °C for the NGTW and NWWTP, respectively.

The turbidity readings varied from plant to plant and ranged between 3.2 ± 0.08 to 452 ± 0.8 and 2.23 ± 0.12 to 16.2 ± 0.08 NTU for NGTW and NWWTP, respectively. The high turbidity values were recorded for the NGTW in comparison to the NWWTP which had much lower readings (Table 2.2 & 2.3). TDS profiles varied for both plants as the readings were between 183.5 ± 0.1 to 772.3 ± 0.4 and 226.3 ± 1.2 to 2156 ± 0.8 NTU for the NGTW and NWWTP respectively.

The COD values obtained ranged between 37 – 309 mg/mL and 52 – 292.3 mg/mL for both NGTW and NWWTP, respectively (Table 2.2 & 2.3). The BOD values ranged between 4.4 – 314 mg/mL and 10.6 – 205.1 mg/mL for NGTW and NWWTP, respectively.

Table 2.2: Physicochemical profiles of treated effluents of the NGTW and its receiving water bodies over the sampling period.

Sampling Time (Weeks)	Point	COD (mg/L)	Temperature (˚C)	Turbidity (NTU)	TDS (mg/L)	pH	BOD (mg/L)
1	UP	269 ± 4.1	23 ± 0.0	13.2 ± 0.0	429.7 ± 0.5	8.8 ± 0.1	23.2 ± 0.4
	BC	94 ± 22.2	22 ± 0.0	21.2 ± 0.00	540.7 ± 0.5	7.2 ± 0.0	314 ± 2.6
	AC	309 ± 0.8	21 ± 0.0	14.6 ± 0.3	534 ± 1.4	8.2 ± 0.1	105.5 ± 1.2
	DS	292 ± 24.8	22 ± 0.0	13.4 ± 0.1	553.7± 5.4	7.4 ± 0.0	162.1 ± 8.6
2	UP	37.0 ± 4.5	18 ± 0.0	2.05 ± 0.05	214.1 ± 0.1	8.5 ± 0.3	13.7 ± 0.4
	BC	72.0 ± 8.0	23 ± 0.0	263 ± 0.82	659.7 ± 0.5	7.4 ± 0.1	282.9 ± 2.5
	AC	92.7 ± 17.6	22 ± 0.0	1.25 ± 0.01	476.3 ± 0.5	7.6 ± 0.1	104.3± 0.5
	DS	75.7 ± 4.6	19 ± 0.0	36.9 ± 1.52	375.0 ± 0.0	8.3 ± 0.1	161.2 ± 5.8
3	UP	222.7 ± 6.9.	17 ± 0.0	3.2 ± 0.08	197.4 ± 0.1	8.0 ± 0.0	13.4 ± 0.05
	BC	185.3 ± 15.1	22 ± 0.0	280.7 ± 3.4	772.3 ± 0.4	8.1 ± 0.0	159.3 ± 1.34
	AC	100.7 ± 4.8	22 ± 0.0	10.2 ± 0.04	582.6 ± 1.2	7.3 ± 0.0	26.4± 0.4
	DS	187.0 ± 41.8	18 ± 0.0	94.6 ± 1.61	401 ± 1.4	7.7 ± 0.0	59.8 ± 9.4
4	UP	229.0 ± 36.3	15 ± 0.0	5.00 ± 0.02	189.6 ± 0.3	7.4 ± 0.0	11.3 ± 1.2
	BC	187.7 ± 20.2	20 ± 0.0	9.99 ± 0.00	590.3 ± 0.5	6.8 ± 0.0	123.3 ± 12.1
	AC	152.37 ± 13.8	20 ± 0.0	34.5 ± 0.16	576.7 ± 0.5	6.8 ± 0.0	33.6 ± 2.4
	DS	72.3 ± 16.4	17 ± 0.0	53.2 ± 1.61	432 ± 0.5	7.1 ± 0.0	79.6 ± 1.4
5	UP	161 ± 2.4	16 ± 0.0	21.4 ± 0.4	282.7 ± 0.5	6.7 ± 0.0	4.4 ± 0.29
	BC	216 ± 8.1	20 ± 0.0	19.6 ± 0.0	550.7 ± 1.7	6.9 ± 0.1	73.4 ± 12.5
	AC	170 ± 1.6	20 ± 0.0	8.0 ± 0.0	380.0 ± 0.8	7.0 ± 0.0	31.5 ± 1.9
	DS	287 ± 4.0	15± 0.0	22.1 ± 0.3	343.3 ± 0.5	7.0 ± 0.0	43.2 ± 1.6
6	UP	303.3 ± 0.5	21 ± 0.0	8.6 ± 0.1	183.5 ± 0.1	7.5 ± 0.0	7.3 ± 0.1
	BC	297 ± 0.0	20 ± 0.0	268.7 ± 0.5	619.3± 0.5	6.9 ± 0.1	91.4 ± 1.6
	AC	300.6± 0.5	21 ± 0.0	93.3 ± 1.7	480.3 ± 1.2	7.4 ± 0.0	16.6 ± 1.1
	DS	185.0 ± 0.8	22 ± 0.0	452 ± 0.8	465.6 ± 0.5	7.4 ± 0.0	26.8 ± 1.5

Key: UP- upstream, BC- before chlorination, AC- after chlorination, DS-downstream

Values represent means of triplicate data ± standard deviation.

Table 2.3: Physicochemical profiles of treated effluents of the NWWTP and its receiving water bodies over the sampling period.

Sampling Time (Weeks)	Point	COD (mg/L)	Temperature (˚C)	Turbidity (NTU)	TDS (mg/L)	pH	BOD (mg/L)
1	UP	152.3 ± 1.9	22 ± 0.0	9.17 ± 0.02	293.7 ± 0.5	7.3 ± 0.1	22. ± 0.4
	BC	58.3 ± 2.1	22 ± 0.0	2.88 ± 0.00	344.0 ± 0.8	6.7 ± 0.6	40.7 ± 5.0
	AC	292.3 ± 6.3	21 ± 0.0	2.41 ± 0.02	344.7 ± 0.5	7.7 ± 0.4	110 ± 0.9
	DS	138.7 ± 10.3	22 ± 0.0	9.20 ± 0.01	346.0 ± 0.0	7.4 ± 0.3	205.1 ± 3.8
2	UP	254.7 ± 13.5	19 ± 0.0	16.2 ± 0.08	294.3 ± 0.5	9.0 ± 0.08	21.5 ± 0.1
	BC	52.0 ± 16.5	19 ± 0.0	13.9 ± 0.12	369.0 ± 0.8	8.9 ± 0.1	91.1 ± 0.06
	AC	96.0 ± 24.9	20 ± 0.0	11.0 ± 0.04	3743 ± 0.5	8.2 ± 0.08	12.2± 0.1
	DS	56.3 ± 15.06	19 ± 0.0	7.15 ± 0.02	2156 ± 0.8	7.7 ± 0.1	178.9 ± 0.8
3	UP	67.3 ± 17.7	19 ± 0.0	2.7 ± 0.4	293.7 ± 0.9	7.5 ± 0.1	12.7 ± 0.1
	BC	175.7 ± 2.9	19 ± 0.0	2.23 ± 0.12	338.3 ± 5.4	7.6 ± 0.2	111.3 ± 0.8
	AC	123.3 ± 9.3	18 ± 0.0	2.4 ± 0.03	345.3 ± 0.5	7.7 ± 0.03	67.4 ± 0.6
	DS	87.3 ± 12.7	19 ± 0.0	9.9 ± 0.02	226.3 ± 1.2	7.2 ± 0.02	81.1 ± 6.8
4	UP	84.0 ± 8.0	17 ± 0.0	2.56 ± 0.1	292.3 ± 2.4	7.2 ± 0.0	10.8 ± 0.3
	BC	181.3 ± 2.1	18 ± 0.0	4.70 ± 0.09	353.7 ± 0.9	7.1 ± 0.0	158.5 ± 3.5
	AC	85.3 ± 9.9	17 ± 0.0	5.01 ± 0.11	355.7 ± 0.5	7.2 ± 0.0	70.7 ± 0.8
	DS	76.7 ± 22.4	17 ± 0.0	6.18 ± 0.12	1744.7 ± 6.0	7.4 ± 0.0	55.9 ± 1.5
5	UP	159 ± 4.1	21 ± 0.0	5.0 ± 0.1	1133.7± 2.0	7.2 ± 0.0	11.7± 0.5
	BC	267 ± 3.3	21 ± 0.0	13.4 ± 0.5	366 ± 0.9	7.3 ± 0.0	33.4 ± 4.2
	AC	268 ± 5.7	21 ± 0.0	10 ± 0.0	370 ± 0.5	7.2 ± 0.0	23.5 ± 0.9
	DS	119 ± 3.3	21 ± 0.0	5.0 ± 0.1	326 ± 0.5	7.2 ± 0.0	60.2 ± 1.1
6	UP	156 ± 5.7	23 ± 0.0	4.3 ± 0.3	321.3 ± 0.5	7.3 ± 0.02	10.6 ± 0.41
	BC	198 ± 6.5	23 ± 0.0	5.2 ± 0.02	368.3 ± 0.5	7.0 ± 0.05	23.5 ± 71.5
	AC	152.7± 3.5	22 ± 0.0	4.9 ± 0.02	371.7 ± 1.9	7.1 ± 0.02	34.2 ± 2.8
	DS	195.0 ± 5.7	24 ± 0.0	5.1 ± 0.02	265 ± 0.8	7.6 ±0.01	37.4± 4.9

Key: UP- upstream, BC- before chlorination, AC- after chlorination, DS- downstream

Values represent means of triplicate data ± standard deviation.

2.3.2 Microbial analysis of water samples

2.3.2.1 Presumptive counts of *Yersinia* spp. in wastewater samples

The presumptive *Yersinia* spp. population obtained for the different effluent samples at the NGTW is shown in figure 2.1. The upstream (UP) had counts ranging from 4.3 - 7.8 log cfu/ml throughout the sampling periods. The pre- chlorination (BC) point for all 6 sampling intervals had counts ranging from 5.3 - 7.4 log cfu/ml. After chlorination (AC), the range was 4.3 - 5.7 log cfu/ml and finally the downstream (DS) point had the log cfu/ml counts ranging from 5.6 - 6.6 log cfu/ml. A reduction in *Yersinia* spp. counts was observed after the treatment process for this particular plant as displayed in Wk 1 from the (BC) at 7.4 log cfu/ml counts, which was reduced to 5.7 log cfu/ml at the (AC) sampling point. A similar trend was observed for all sampling intervals. The wastewater treatment process from the plant was effective in reducing the microbial load but did not completely remove all the *Yersinia* spp. The effluent (DS) samples had relatively lower *Yersinia* spp counts compared to the (BC) points but were higher than the (AC) points. There could be other sources influencing the higher counts from the (DS) point, these could include the rain wash off from the informal households and improper sanitation.

Figure 2.2 shows the log cfu/ml plot for the presumptive *Yersinia* population from NWWTP. The upstream (UP) had values ranging from 5 - 7.3 log cfu/ml throughout the sampling intervals. The effluent sample before chlorination (BC) had counts ranging from 6.3 - 6.4 log cfu/ml. The post chlorination (AC) point had a range of 4 - 5.6 log cfu/ml and finally the downstream (DS) point had the log cfu/ml counts ranging from 5.6 - 6.2 log cfu/ml. The (BC) points had higher counts and reduction was observed in (AC) points for all the sampling intervals. It was observed that the AC point for Wk 1 and 2 had the lowest *Yersinia* spp counts (4 log cfu/ml) lower than all the

points reduced from the BC (6.3 log cfu/ml) points. The microbial counts for the DS points in Wk 5 and 6 was higher (6.7 log cfu/ml) compared to the former sampling intervals.

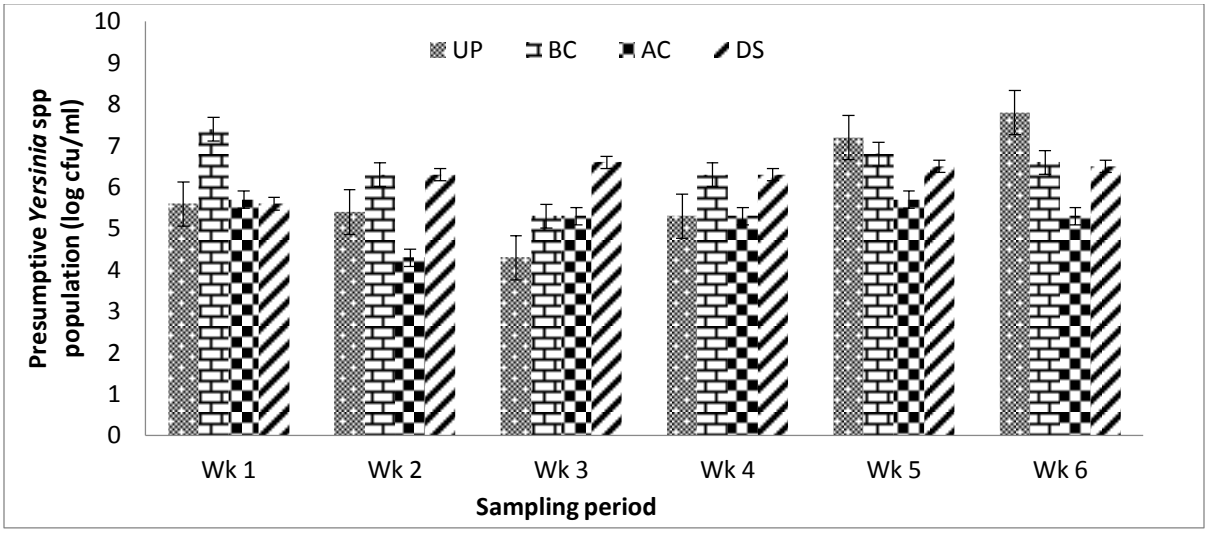


Figure 2.1: Presumptive *Yersinia* spp. population in NGWT and receiving water bodies.

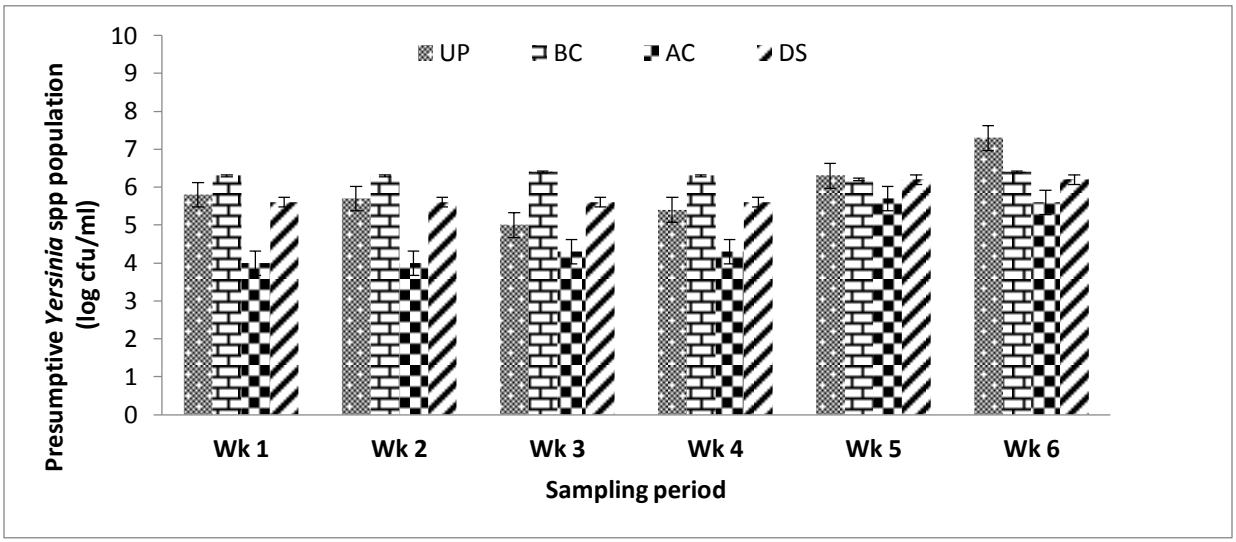


Figure 2.2: Presumptive *Yersinia* spp. population in NWWTP and receiving water bodies.

2.3.2.2 Biochemical and molecular identification of *Yersinia* spp.

A total of 473 isolates presumptively identified based on their bull's eyes appearance on YSA was further identified using biochemical reactions. After biochemical screening only 274 isolates displayed positive reaction by being able to utilize urea, oxidase negative, non- lactose fermenter and the inability to utilize citrate. These isolates were further confirmed by genus specific PCR for both the 16S rDNA of the subgenus *Yersinia* and the kingdom bacteria. Of the 274 biochemically identified isolates, only 62.41 % (174/274) were confirmed as *Yersinia* spp. based on the amplification of the expected fragment size of 749 bp (Figure 2.3).

Of the 171 Y*ersinia* spp. isolates, 40.94% (70/171/) were positively identified as *Yersinia enterocolitica* based on correct amplification of the expected fragment (330 bp) specific to *Yersinia enterocolitica* (Figure 2.4).

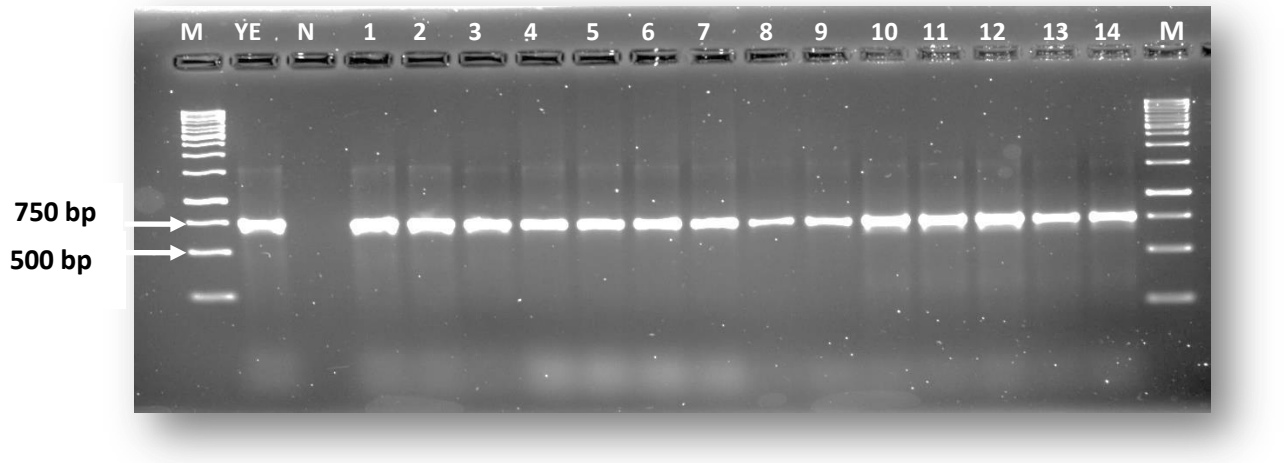


Figure 2.3: Agarose gel showing the expected amplicon size (749 bp) for the 16S gene conserved in all *Yersinia* spp. identification Lane M contains the marker 1kb (Thermo Scientific), Lane YE contains a *Yersinia enterocolitica* positive control, Lane N is a negative control, Lane 1 to 14 are representative *Yersinia spp.* isolates.

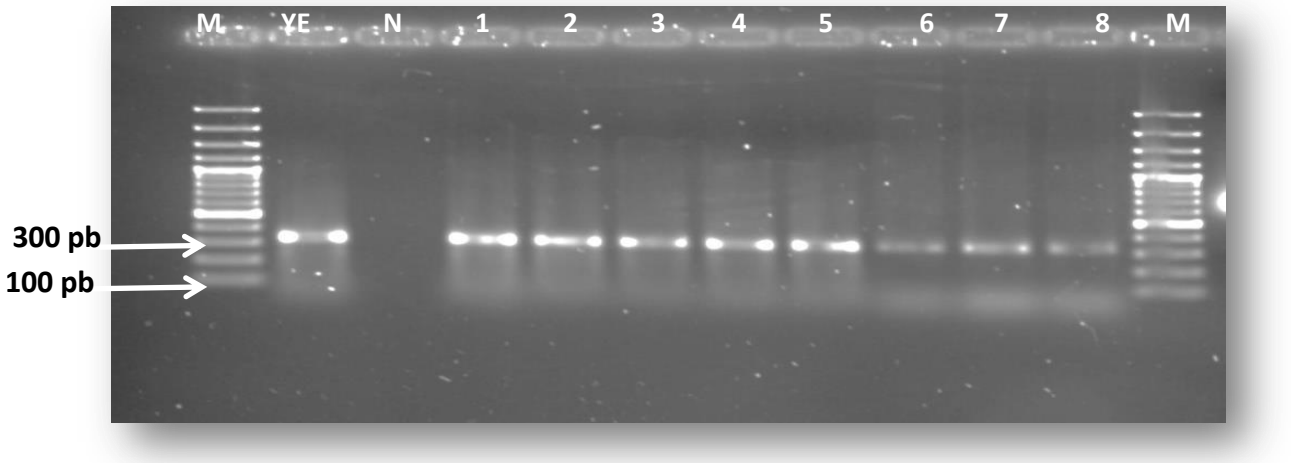


Figure 2.4: Agarose gel showing the expected amplicon size (330 bp) for the *Yersinia enterocolitica* species specific gene. Lane M contains the 100 bp marker (Thermo Scientific), Lane YE contains a *Yersinia enterocolitica* positive control, Lane N is a negative control, Lanes 1 to 8 are representative *Yersinia enterocolitica* isolates.

2.3.4 Correlation between physico-chemical parameters and *Yersinia* counts

Tables 2.4 and 2.5 show the correlation matrices of tested physicochemical parameters with presumptive *Yersinia* spp counts. At the NGTW (Table 2.4), there was a significant ($p < 0.01$) positive correlation between TDS and BOD (r = 0.580) as well as with temperature (r = 0.646). TDS correlated significantly ($p< 0.05$) with turbidity (r = 0.445) and again a significant ($p< 0.05$) positive correlation was observed for BOD and temperature (r = 0.441). At NWWTP (Table 2.5), TDS correlated significantly ($p< 0.05$) with temperature (r = 0.473) and with turbidity (r = 0.503) but correlated strongly ($p < 0.01$) with BOD (r = 0.626).

Table 2.4: Correlation matrix between the physicochemical parameters and *Yersinia* population counts obtained for the NGTW.

Parameter	COD	Temp	Turbidity	TDS	pH	BOD	*Yersinia*
COD (mg/L)	1						
Temperature (˚C)	-0.010	1					
Turbidity (NTU)	0.003	0.327	1				
TDS (mg/L)	-0.054	0.646**	0.445*	1			
pH	-0.029	0.196	-0.040	-0.190	1		
BOD (mg/L)	-0.321	0.441*	0.201	0.580**	-0.013	1	
Yersinia (log cfu/ml)	0.077	-0.053	0.142	-0.072	-0.342	0.259	1

*. Correlation significant at 0.05 level (2 tailed), **. Correlation significant at 0.01 level (2-tailed).UP- upstream, BC- before chlorination, AC- after chlorination, DS- downstream

Table 2.5: Correlation matrix between the physicochemical parameters and *Yersinia* population obtained for the NWWTP.

Parameter	**COD**	**Temp**	**Turbidity**	**TDS**	**pH**	**BOD**	***Yersinia***
COD (mg/L)	1						
Temperature (˚C)	-0.066	1					
Turbidity (NTU)	-0.006	0.029	1				
TDS (mg/L)	-0.038	0.473*	0.503*	1			
pH	0.048	0.120	0.207	-0.110	1		
BOD (mg/L)	-0.251	0.320	0.249	0.626**	0.033	1	
Yersinia (log cfu/ml)	0.135	0.074	0.150	-0.104	-0.259	-0.096	1

*. Correlation significant at 0.05 level (2 tailed), **. Correlation significant at 0.01 level (2-tailed) UP- upstream, BC- before chlorination, AC- after chlorination, DS- downstream

2.4 Discussion

The pH values observed in this study for all the sampling points fell within the recommended standards irrespective of the sampling intervals and the activities at each sampling point for both wastewater treatment plants and similar to previously reported values for final effluents and their receiving water bodies by Osode and Okoh (2009); Manios *et al*. (2006). The values were also within the acceptable limits of 6 – 9 recommended by the Department of Water Affairs and Forestry in South Africa (DWAF, 1996b) as indicated in Table 2.2 and 2.3. The above results may suggest that the effluent may not have negative impact on the domestic use of the receiving water bodies such as fishery and recreational purposes with reference to pH standards. The neutral to alkaline pH values obtained from the current study were also similar to the pH values reported by Lokhande *et.al*, (2011), Morrison *et al.,* 2001 and Igbinosa and Okoh (2009).

The temperature observed in this study was in a range of 15 ℃ – 23 ℃ for NGTW and 17 ℃ – 24 ℃ for NWWTP. The highest temperature of 23 ℃ was observed for the UP and BC samples and the lowest temperature of 15 ℃ was observed at the UP and DS of the NGTW. Temperature is an important water quality parameter due to its influence on other parameters. Temperature affects the solubility of solids, gas and and differently consequently affecting the availability of oxygen in water (Akan *et al*. 2008). It also affects the toxicity of some chemicals in water systems as well as the sensitivity of living organisms to toxic substances (Dojlido and Best, 1993; Mayer and Ellersieck, 1988). The temperature values of the WWTP's in the current study were within the acceptable limit of no risk ($\leq 25^{o}$C) for domestic water uses in South Africa (WRC, 1995). This observation implies that the discharged effluent was of standard quality with respect to temperature and may not significantly offset the homeostatic balance of the receiving

ecosystems; neither will it adversely affect the use of the receiving watershed for domestic purposes. The results obtained in this study are in agreement with to the results obtained by Jaji *et al*. (2007). The current observations are in agreement with the findings of with Odjajare and Olaniran, (2015) who reported temperature ranges between 13-27 °C from the same WWTP's used in this study.

Turbidity values obtained for both plants ranged from 1.25- 452 NTU and 2.4 – 16.2 NTU for NGTW and NWWTP, respectively and were higher than the acceptable limits for domestic use. Increased turbidity obtained at downstream point of NGTW may be attributed to poor quality pipes through which the final treated effluent travels prior to discharge into the receiving water bodies. None of the receiving water bodies met the South African guideline of 0 to 1 NTU for turbidities in water for domestic use (DWAF, 1998). However, South Africa does not have guidelines for the turbidity values accepted for treated effluent (Government Gazette, 1984). The World Health Organization standard for domestic water supply is 5 NTU (WHO, 2004). These results show that the water was not of good quality with respect to turbidity. Excessively high turbidity of the effluent samples at the discharge point of the NGTW is of serious concern as the values were relatively higher than the typical values from similar studies in different plants (Odjadjare and Okoh, 2009). Highly turbid conditions may increase the possibility for waterborne diseases, since particulate matter may harbor microorganisms and may stimulate the growth of bacteria (Hoko, 2005), thereby posing some health risk to the effluent users. Also, the excessive turbidity in water can cause problems with water purification processes, such as flocculation, filtration and disinfection which may increase treatment cost (DWAF, 1998). Chlorination of water may result in an increase of trihalomethane (THM) precursor.

Trihalomethanes are by-products of chlorination and include several chemicals such as chloroform, bromodichloromethane, dibromochloromethane and bromoform. Since these substances are suspected to cause cancer in humans, their concentrations in drinking water must carefully be controlled before release to surface water bodies (Hacioglu and Dulger, 2009).

Chemical oxygen demand (COD) and biological oxygen demand (BOD) levels serve as an indication of both the organic and inorganic pollution within the water source, thus serving as a useful indicator of potentially toxic conditions as well as the presence of biologically organic and inorganic resistant substances (Sawyer *et al.*, 2003). COD is a measure of the amount of oxygen required by a strong oxidant (e.g. H_2SO_4) to breakdown both organic and inorganic matters in a water system (Akan *et al*., 2008). Elevated levels of COD in water systems lead to drastic oxygen depletion which adversely affects the aquatic biota (Fatoki *et al*., 2003). The COD values of the effluent samples in this study ranged between 37-309 mg/L for NGTW and 52.0 – 292.3 mg/L for NWWTP. The South African water quality guidelines specify COD concentrations of 30 mg/L for wastewater discharge but also specify a general standard of 75mg/L (DWA, 2010). Almost similar values were observed from similar study by Igbinosa and Okoh (2009) whereby their COD values were between 34.82 – 238 mg/L. The current observation agrees with the previous works of Fatoki *et al.* (2003) who reported a mean COD value of 122.9 mg/L.

Biochemical oxygen demand (BOD), the amount of oxygen needed by bacteria to oxidize the organic matter present in the water is a basic means of measuring the degree of water pollution (Hach, 1997). EU guidelines stipulate the BOD target limits of 3.0 to 6.0 mg/L (Chapman, 1996) and South Africa guidelines recommends 5 mg/L for BOD (Government Gazette, 1984). Except for the UP point at NGTW which had a BOD of 4.4 mg/L at week 5 of the sampling period,

which fell within the EU stipulated range but were within the SA guidelines. Even so the BOD levels recorded in the current study were much higher than those indicated in the EU and SA guidelines. These high levels of BOD from the investigated WWTP's and receiving water bodies disqualify these water sources for use as aquatic ecosystems. An increase in BOD may be due to heavy discharge of industrial wastewater effluent, animal and crop wastes and domestic sewage. BOD values have been commonly adopted as a measure of effects of pollution. The greater the BOD, the more rapidly oxygen is depleted in the water, which may lead to anoxic conditions, and consequent disruption of balance of the aquatic ecosystem (Momba *et al.,* 2006).

Chlorination of the final effluent prior to discharge into receiving surface water as practised by the treatment plants investigated in this study was effective in reducing the number of presumptive *Yersinia* species after tertiary treatment but failed to totally eliminate them as these organisms were recovered after chlorination as well as in downstream points of the receiving points (Figures 2.2 and 2.3). It was noted that the upstream point (UP) of the NGTW along the Aller river had the highest counts ranging between 4.6 – 7.8 log cfu/ml compared to the UP point of the NWWTP along Umgeni river which had the *Yersinia* population ranging between 5 – 7.3 log cfu/ml. Upstream of the river at the NGWTP has informal settlements with poor sanitation and inadequate sewage disposal system which contaminate the river with human and animal waste. Storm runoff from the informal settlements and discharge of inadequately treated wastewater influence the high counts obtained for this point. The higher turbidity values measured for the NGTW water samples relative to the NWWTP could also explain the high microbial population observed. Hoko (2005) previously indicated that highly turbid conditions may increase the possibility for waterborne diseases, since particulate matter may harbor microorganisms and may stimulate the growth of bacteria.

The downstream (DS) point had *Yersinia* species population ranging from 5.6 – 6.6 log cfu/ml and 4 - 6.2 log cfu/ml at the NGTW and NWWTP, respectively. The higher counts of *Yersinia* spp obtained for NWWTP could be attributed to the surrounding environment nearby the sampling site, as trace or evidence of human and animal faecal matter was found in the area. The high microbial count in the DP indicates that apart from the cross contamination from the surrounding environment the discharge from the plant itself may also be a contributing factor.

Higher amount of total dissolved solids leads to increased turbidity (Kumar and Bahadur, 2009). This was observed from the BC (Wk 2) point at NGTW where TDS value was 659.7 mg/L and the turbidity was also high at 263 NTU. Higher turbidity hinders the chlorination/disinfection process (Asano, 2007). Bacteria utilize these suspended solids as sites of attachment there by increasing the microbial load (Hurst, 1996 and Kurup *et al*, 2010). As observed throughout the sampling interval that the BC points in both plants had higher microbial counts that could be correlated with the high turbidity values observed.

Previous reports have also suggested that wastewater treatment plants in South Africa are either dysfunctional or non-functional (Bateman, 2010). Sewage discharge is one of the problems presently facing South Africa, and several efforts are being vigorously pursued to control it. Water contaminated by effluents from various sources is associated with heavy disease burden (Okoh *et al.*, 2007) and this could influence the current shorter life expectancy in the developing countries when compared with developed nations (WHO, 2002). The issue of treatment efficiency is of major importance if the reclaimed water is intended for recreational or potable reuse or is to be discharged into natural water bodies because disposal of inadequately treated

wastewater into surface water recipient is one of the major sources of pathogens in the environment (Touron *et al.,* 2005; Ottoson *et al.,* 2006; Odjadjare *et al.,* 2012).

Chlorine disinfection of sewage effluent at the two wastewater treatment plants (WWTPs) under investigation showed that there was a reduction in viable counts of presumptive *Yersinia* spp. at the discharge point during the sampling period but failed to totally eliminate them. Presumptive *Yersinia* spp. were also recovered downstream of the rivers receiving the treated effluent indicating the negative impact of the wastewater treatment plants on the microbial quality of the river. The DS points had 5.6 – 6.6 log cfu/ml and 5.6 – 6.2 log cfu/ml for both NGTW and NWWTP, respectively (Figures 2.2and 2.3). It is known that water with high turbidity is often difficult to disinfect properly (Ekholm and Krogenus, 1998). This indicates that the high turbidity values of (1.25 ± 0.01 – 452 ± 0.8 NTU) and (5.0 ± 0.1 – 9.9 ± 0.02 NTU) obtained in the final effluent may have contributed to the high microbial load detected. There could be other contributing factors that may have increased the microbial load such as the upstream of the river of NGWTP has an informal settlement with poor sanitation and inadequate sewage disposal system, which contaminates the river with human and animal wastes. Whilst at NWWTP the Umgeni River downstream is contaminated by faecal waste and run-off from the informal settlement contributing to high counts of presumptive *Yersinia* spp. observed. Although the treatment plants studied succeeded in removing *Yersinia* species from the influent water but discharge effluent had traces of *Yersinia* species still detected, this could be a potential threat of incidences of infectious diseases.

2.5 Conclusion

This study was carried out to evaluate the efficiency of the treatment process of two independent WWTPs in eliminating *Yersinia* spp. from the influent received and the input of the treated effluent on the receiving rivers. The results revealed that the treatment plants exhibited effluent qualities that meet acceptable standard in some parameters, like pH and temperature. It was also observed that the effluents fell short of standard requirements that are critical to the provision of clean and safe water such turbidity, COD, BOD and total dissolved solids (TDS). Findings from this study therefore show that the effluent could pose significant health and environmental risks to communities who use the receiving water for domestic purposes and may also affect the health status of the aquatic milieu in the receiving water. The study therefore reiterated the need for continuous monitoring and improvements of the treatment regime employed in wastewater treatment plants.

CHAPTER 3

ANTIBIOTIC RESISTANCE AND VIRULENCE DETERMINANTS OF *YERSINIA ENTEROCOLITICA* ISOLATES RECOVERED FROM TREATED WASTEWATER EFFLUENT AND RECEIVING RIVERS

Abstract

Yersinia enterocolitica is a potentially pathogenic bacterium transmitted through the faecal-oral route. Typical symptoms include those associated with gastrointestinal disease, although infection can also lead to more serious and invasive illnesses, particularly in sensitive populations. Previous studies have detected *Y. enterocolitica* in surface water in various parts of the world, and studies have reported the intake of untreated water to be one of the potential risk factors for *Y. enterocolitica* infection. This study investigated the antibiotic resistant patterns and the virulence determinants of the previously identified *Y. enterocolitica* in treated wastewater effluents and the receiving rivers. In addition, the antibiogram and virulence factors of these isolates were determined in order to establish the possible effects posed by these isolates to the users of receiving surface waters. Finally, the genetic relatedness of the isolates was established by Random Amplified Polymorphic DNA (RAPD-PCR). The antibiotic susceptibility assays revealed that the isolates were resistant to ampicillin (100%), amoxicillin (98%), cefuroxime (96%), cefalothin (90%), streptomycin (93%), chloramphenicol (100%), tetracycline (100%) and trimethoprim (100%). The calculated multiple antibiotic resistance (MAR) indices of the *Y. enterocolitica* isolates ranged from 0.5-0.66, suggesting high multi-antibiotic resistance among the isolates. A high prevalence (59%) of class 2 integrin was found among the isolates, with 26 and 6% of the isolates in possession of class1 and class 3, respectively. The integrase genes detection showed that the isolates possessed 3 classes of integrons, detected in 59%, 26% and 6% of the isolates, respectively. The virulence determinant assays using crystal violet staining showed that only 21% (15/70) of the isolates could retain the purple colour suggesting that they may be the virulent strain of *Y. enterocolitica*. The negative MBL activity suggests that the tested isolates do not demonstrate any hydrolytic activity for the degradation of cephalosporins. Virulence gene detection via PCR showed that the most abundant gene is the *ystA* (56%) followed by *ail* (34%), both chromosomally located. The plasmid located genes were detected in 3% of the isolates for both *Vir/Lcr* and *yadA*. The genotypic characterization of the tested isolates revealed two main clusters (A and B), with cluster A comprising the majority of the isolates (68%) and include the *Y. enterocolitica* positive control, whilst cluster B grouped 31% of the isolates. had 31% similarity to the control.

3.1 Introduction

Yersinia enterocolitica, are gram-negative, oxidase-negative and facultative anaerobes. They are highly heterogeneous and can be divided into several biotypes, with only a few biotypes known to be associated with human disease. *Y. enterocolitica* is primarily a gastrointestinal tract pathogen and has become a major cause of diarrhoea in most of the industrialised world (Bolton *et al.,* 2013). *Y. enterocolitica* are also known to be zoonotic pathogens (Simonova *et al*., 2008). Since drug resistance in zoonotic pathogens has affected the therapeutical interventions in humans; antimicrobial resistance in food-borne pathogens has recently become a public health issue (Simonova *et al*., 2008). One tenth or more of the world's population is believed to consume food produced by irrigation with recycled wastewater; partially treated and/or in some instances using untreated hospital waste effluents. However, it is common knowledge that hospital waste effluents, even if treated, may contain pathogenic drug-resistant bacteria, which constitute the most dangerous risk factor for spreading of pathogenic and drug resistant organisms to the environment (Rahman *et al.,* 2005).

Antimicrobial resistance is a major global health concern requiring urgent interventions especially in developing countries, where health and related sectors are challenged with a number of constraints, including access to better or new drugs when resistance is suspected or has developed or even when the priority is to provide basic health care (Moore, 2010; Suma et al, 2014). Spreading of antibiotic resistance genes by horizontal transfer has led to the prevalence of antibiotic resistance among bacterial isolates (Ploy *et al.*, 2000). The spread of these resistance genes is significantly greater when they are part of a mobile gene cassette. Known mechanisms of horizontal gene transfer include: (a) mobilization of individual cassettes by the integron-

encoded integrase (Collis, 1992), (b) movement when the integron containing the cassette relocates (targeted transposition) (Brown *et al*., 1996; Craig. 1996; Minakhina *et al*., 1999), (c) distribution of larger transposons such as Tn21 carrying integrons (Liebert, 1999), and (d) movement of conjugative plasmids containing integrons among bacterial species. It is therefore not surprising that many of the antibiotic resistance genes found in clinical isolates of gram-negative microorganisms are part of a gene cassette inserted into an integron (Recchia, 1995).

The overall objective of this chapter was to determine the prevalence of antibiotic resistance and virulence determinants among *Y. enterocolitica* recovered from treated effluents and receiving rivers as well as determining the level of genetic relatedness of the isolates using rapid fragment length polymorphic analysis. Understanding the antibiotic-resistance profiles of *Y. enterocolitica* in different locations of the wastewater treatment system will establish an understanding of both the baseline and potential movement of antibiotic resistance of these organisms and their possible spread into different ecosystems. The results of this study will establish a preliminary environmental assessment of wastewater treatment plants in eliminating or increasing the spread of antibiotic resistance of *Y. enterocolitica* recovered from the treated effluent and the surrounding receiving water bodies.

3. 2 Materials and Methods

3.2.1 Maintenance of identified *Y. enterocolitica* isolates

The PCR confirmed *Y. enterocolitica* isolates recovered from the treated wastewater effluent and the receiving surface water bodies were maintained on 40% glycerol and kept at -70°C. When required, the isolates were streaked onto TSA plates and incubated at 37°C. Individual colonies were used for the subsequent assays.

3.2.2 Antibiotic susceptibility testing

Antibiotic susceptibility profiles of the isolates was determined using the Kirby-Bauer disk diffusion assay as previously described (Bauer *et al.*, 1966). The isolates were screened against a panel of antibiotics listed in Table 3.1 using *Escherichia coli* ATCC 25922 as a positive control. The isolates were grown on nutrient agar for 24 h at 37^{o}C, then few distinct colonies were suspended in 5 ml of 0.8% NaCl solution and turbidity was standardized to 0.5 McFarland standards. The suspension was spread onto Muller-Hinton agar plate using a sterile swab. The plates were allowed to air dry by opening the plate at 45° angle in a sterile zone before antibiotic disks were placed at equidistance onto the bacterial lawn and incubated for 24 h at 37°C. After incubation the zones of inhibition were measured, recorded to the nearest millimeters and interpreted as resistant (R), intermediate (I) and susceptible (S), according to the Clinical Laboratory Standards Institution (CLSI, 2008) guidelines.

Table 3.1: List of antibiotics used in this study.

Class	Antibiotic (abbreviation)	Concentration (µg)
β-lactams	Cefalothin (C)	30
	Cefuroxime (CXM)	30
	Ampicillin (AMP)	10
	Amoxycillin (AMC)	30
Aminoglycosides	Gentamicin (CN)	10
	Kanamycin (K)	30
	Streptomycin (S)	10
Phenicols	Chloramphenicol (KF)	30
Tetracyclines	Tetracycline (TE)	30
Quinolones	Ciprofloxacin (CIP)	5
Sulfonamides	Trimethoprim (W)	5
3rd Generation Cephalosporins	Ceftriaxone (CRO)	30

The MAR index values were calculated as described by Blasco *et al*. (2008) as follows: MAR = a/b, where a = number of antibiotics the isolate was resistant against; and b = total number of antibiotics tested. The resistant phenotypes were created based on the resistant patterns generated for the individual isolates against the tested antibiotics.

3.2.3 DNA extraction

DNA extraction was done following a protocol described by Akinbowale *et al.* (2007) with a few modifications. Single isolated colonies (5) were suspended in 80 µl of sterile deionized water, boiled in a water bath at 100 °C for 10 min and cooled on ice. Thereafter, the suspension was centrifuged at 14 000 rpm in a micro-centrifuge for 5 min. The supernatant was transferred into a sterile Eppendorf tube and used as a template in PCR assays.

3.2.4 Integrase genes and gene cassette detection

The integrase genes of interest (*intI1, intI II* and *intI III*) were detected using the primers listed in Table 3.2. The 50 µl PCR mixture contained 5 µl of 10X PCR buffer, 4 µl of 25 mM $MgCl_2$, 10 µl of 1 mM dNTP's, 0.2 µl of 250 U Supertherm *Taq* polymerase, 1 µl of 10 mM of each primer and 2µl of DNA template. Integrase gene detection (*intI1, intI II, intI III*) was done in a 25 µL PCR mixture at the following conditions: 94 ℃ for 5 min, 30 cycles of 1 min of denaturation at 94 ℃, 1 min of annealing at 59 ℃, 1 min of extension at 72 ℃ and a final elongation for 8 min at 72 ℃. Amplification products were analyzed by electrophoresis in a 1.5% agarose gel using 1X TAE buffer at 60V for 90 min. The amplicons were visualized using a GENE*Sys* version V1.2.0.0 gel documentation system using UV transillumination (Syngene, UK) after staining in 0.5 mg/ml ethidium bromide for 15 min.

Isolates detected to harbor the integrase genes were further screened for the presence of the gene cassette as described by Laveqsue *et al*. (1995) using the primers listed in Table 3.2 in a 25 µl PCR mixture containing 5 µl of 10 X PCR buffer, 3.8 µl of 25 mM $MgCl_2$, 10 µl of 1 mM dNTP's, 0.2 µl of 250 U Supertherm *Taq* polymerase, 1 µl of 10 mM of each primer (5'-CS and 3'-CS) and 2µl of DNA template. The PCR was carried out in a 25 µl PCR mixture under the following conditions: 94 ℃ for 2 min, 20 s of denaturation at 94 ℃, 30 s of annealing at 57 ℃ and 90 min of extension at 68 ℃ for a total of 30 cycles. The products were separated in a 2% (w/v) agarose gel at 70V for 90 min in 1% TAE buffer, and visualized using a GENE*Sys* version V1.2.0.0gel documentation system using UVtransillumination (Syngene,UK) after staining in 0.5 mg/ml ethidium bromide for 15 min.

Table 3.2: The list of primers used for the integrase genes detection and gene cassette arrays.

Primer	Primer sequence (5ˈ-3ˈ)	Product size(bp)	Reference
Int1F	CAG TGG ACA TAA GCC TGT TC	160	Koeleman *et al*. (2001)
Int1R	CCC GAG GCA TAG ACT GTA		
Int2 F	CAC GGA TAT GCG ACA AAA AGG T	788	Mazel *et al*. (2000)
Int2 R	GTA GCA AAC GAG TGA CGA AAT G		
Int3 F	GCC TCC GGC AGC GAC TTT CAG	979	Mazel *et al*. (2000)
Int3 R	ACG GAT CTG CCA AAC CTG ACT		
5'-CS	GGCATCCAAGCAGCAAG	Variable	Levesque *et al*. (1995)
3'-CS	AAGCAGACTTGACCTGA		

3.2.5 Restriction Fragment Length Polymorphism for the gene cassette array characterization

Isolates with PCR products that correspond to the gene cassette regions were digested using *EcoR1*as per manufacturer's guidelines. Briefly, each 30 µl reaction contained 3 µl (20 U) of *EcoR1*, 10 µl of PCR-amplified product 2 µl of enzyme buffer and 15 µl of double-distilled water. The reaction was incubated at 37°C for 3h for digestion. The products were separated in a 2% (w/v) agarose gel at 70V for 90 min in 1% TAE buffer, and visualized using a GENE*Sys* version V1.2.0.0 gel documentation system using UVtransillumination (Syngene, UK) after staining in 0.5 mg/ml ethidium bromide for 15 min.

3.2.6 Expression of pyv-associated phenotypic virulence determinants and metallo-β-lactamase (MBL) production by *yersinia enterocolitica*

Yersinia enterocolitica isolates were subcultured onto TSA plates and incubated at 37 ℃ for 24 h. After incubation, the isolates were tested for the presence of the PVY virulence determinant by flooding the plates with crystal violet (100 mg/ml) solution following the procedure by Bhaduri and Sommers, 2011. The isolates that retained the crystal violet and produced dark violet colonies were assumed to be pYV^{+} bearing isolates while pYV^{-} colonies remained white.

The MBL production of the *Y. enterocolitica* isolates was performed following the procedure by Yong *et al*. (2002). A disk diffusion assay was carried out using the Impenem antibiotic disk and the Impenem + EDTA (pH 8.0) disk. The isolates were grown on Muller- Hinton broth overnight at 37°C. Following incubation, the inoculum was standardized to 0.5 McFarland and streaked onto the Muller- Hinton agar plates and air-dried. The two disks were placed onto the agar plates and incubated at 37°C for 24 h. The zones of inhibition (in millimeters) were measured. The MBL producing isolates were determined by an increase of ≥ 7 mm of the zone of inhibition from the disk that had Impenem + EDTA compared to the zone of the Impenem disk alone (Yong *et al*. 2002).

3.2.7 PCR detection of virulence genes

Plasmid extraction was performed using plasmid mini-prep protocol as described by Kotchoni (2003), while Genomic DNA was extracted using a boiling method as described in section 3.2.3. Primers specific for the *ystA*, and *ystB* chromosomal encoded virulence genes, and *yadA* and *virF*/*lcrF* plasmid located virulence genes of *Y. enterocolitica* were synthesised by Inqaba biotech as per the sequences described by Theoner *et al.* (2002) and used for the detection of the virulence genes. PCR reactions were performed in 25 µl volumes containing 2µl of DNA template, 0.2 mM dNTPs, 5µl of 10X PCR buffer, 3 mM $MgCl_2$, 1mM concentrations of each primer, and 1.25 U of Supertherm *Taq* polymerase. The thermal cycling conditions were: Pre-denaturation at 95°C for 10 min; 25 cycles of denaturation at 95°C for 15 s, annealing for 30 s at appropriate temperatures (Table 3.3) depending on the primer pair used, elongation at 72°C for 30 s; and a final extension at 72°C for 10 min. The third chromosomal gene *ail* gene was detected using the primers described by Wannet *et al*. (2001). Briefly each 25 µl PCR mixture contained *ail*-specific primers at a concentration of 160 nM, 200 µM dNTPs; 0.5 U of Supertherm *Taq* Polymerase; 1µl of 10xPCR buffer; 3 mM $MgCl_2$ and 2 µl of DNA sample. Cycling conditions were as follows: Pre-denaturation at 94°C for 5 min followed by 36 cycles consisting of heat denaturation at 94°C for 45 s, primer annealing at 62°C for 45 s, extension at 72°C for 45 s and a final extension was performed at 72°C for 7 min. The amplicons were separated in a 2% (w/v) agarose gel at 70V for 90 min in 1% TAE buffer, and visualized using a GENE*Sys* version V1.2.0.0 gel documentation system using UVtransillumination (Syngene, UK) after staining in 0.5 mg/ml ethidium bromide for 15 min.

Table 3.3: Primers used for detection of the virulence genes.

Primer	Primer sequence (5'-3')	Annealing temp (°C)	Amplicon size (bp)	Reference
***ystA* Forward**	ATCGACACCAATAACCGCTGAG	61	79	Thoerner *et al.*(2002)
***ystA* Reverse**	CCAATCACTACTGACTTCGGCT			
***ystB* Forward**	GTACATTAGGCCAAGAGACG	61	146	Thoerner *et al.*(2002)
***ystB*Reverse**	GCAACATACCTCACAACACC			
***Vir/Lcr*Forward**	GGCAGAACAGCAGTCAGACATA	63	561	Thoerner *et al.*(2002)
***Vir/ Lcr*Reverse**	GGTGAGCATAGAGAATACGTCG			
***yadA* Forward**	CTTCAGATACTGGTGTCGCTGT	60	681, 759, 849	Thoerner *et al.*(2002)
***yadA*Reverse**	ATGCCTGACTAGAGCGATATCC			
***A1* Forward**	TTAATGTGTACGCTGGGAGTG	62	425	Wannet *et al.*(2001)
***A2* Reverse**	GGAGTATTCATATGAAGCGTC			

3.2.8 Genotypic fingerprinting of Integron positive *Yersinia enterocolitica* isolates by RAPD-PCR

Integron positive *Yersinia enterocolitica* isolates were further characterized for genotypic similarities and variations. Genomic DNA for fingerprinting was extracted from the isolates using the ZR Fungal/ Bacterial DNA MiniPrep kit as per manufacturer's instruction. The RAPD-PCR was performed using a 10 bp primer-II (5'GAGACGCACA3'). Each 25 µl reaction mix contained 30 ng genomic DNA, 1 U *Taq* DNA polymerase, 1 X PCR buffer, 2.5 mM $MgCl_2$, 400 µM dNTPs and 20 pmol / µl primer. The RAPD - PCR conditions were according to Leal *et al.* (2004) as follows: initial denaturation for 1 min at 94°C, annealing for 1 min at 55°C, extension for 1 min at 72°C, and a final extension at 72°C for 7 min. The PCR products were separated in a 2 % (w/v) agarose gel at 70V for 90 min in 1% TAE buffer and visualized using a GENE*Sys* version V1.2.0.0 gel documentation system using UVtransillumination (Syngene,UK) after

staining in 0.5 mg/ml ethidium bromide for 15 min. Genotypic similarities were analysed using the GelCompareII version 6.0 software package (Applied Maths) using Jacquard coefficient and Unweighted Pair Group Method with Arithmetic mean (UPGMA) cluster analysis to produce a dendrogram.

3.3 Results

3.3.1 Antibiotic susceptibility profiles of *Y. enterocolitica* isolates

The antibiotic susceptibility testing results as displayed in Table 3.4 showed that all *Y. enterocolitica* isolates were susceptible to gentamicin, ciprofloxacin and ceftriaxone. In addition, susceptibility to kanamycin, an aminoglycoside, was observed in 84% of the isolates. However, 93% of the isolates were resistant to streptomycin, a member of the aminoglycoside class of antibiotics. All *Y. enterocolitica* isolates showed high resistance pattern towards the β-lactam class of the antibiotics, with percentage resistance of 100%, 98%, 96% and 90% obtained for ampicillin, amoxicillin, cefuroxime, cefalothin, respectively. All the *Yersinia enterocolitica* isolates tested were resistant to chloramphenicol, tetracycline and trimethoprim. The multiple antibiotic resistance index calculated showed that each tested isolate is resistant to at least 50% of the test antibiotics with MAR index values varying between 0.5-0.66 amongst the isolates. It was also observed was that 85% of the tested isolates were grouped on the same antibiotic resistance phenotype with isolates resistant to 7 of the 12 tested antibiotics (Table 3.5).

Table 3.4: Antibiotic resistance profile of *Yersinia enterocolitica* isolates recovered from treated wastewater effluents and receiving surface waters (n = 70).

Class	Antibiotic	Resistant (%)	Susceptible (%)	Intermediate (%)
β-lactams	Cefalothin (30 µg)	63 (90)	0 (0)	7 (10)
	Cefuroxime (30 µg)	67 (96)	0 (0)	3(4)
	Ampicillin (10 µg)	70 (100)	0 (0)	0 (0)
	Amoxycillin (30 µg)	69 (98)	0 (0)	1 (2)
Aminoglycosides	Gentamicin (10 µg)	0 (0)	70 (100)	0 (0)
	Kanamycin (30 µg)	4 (6)	59 (84)	7 (10)
	Streptomycin (10 µg)	65 (93)	0 (0)	5 (7)
Phenicols	Chloramphenicol (30 µg)	70 (100)	0 (0)	0 (0)
Tetracyclines	Tetracycline (30 µg)	70 (100)	0 (0)	0 (0)
Quinolones	Ciprofloxacin (5 µg)	0 (0)	70 (100)	0 (0)
Sulfonamides	Trimethorprim (5 µg)	70 (100)	0 (0)	0 (0)
3rd Generation Cephalosporins	Ceftriaxone (30 µg)	0 (0)	70 (100)	0 (0)

Table 3.5: Distribution of the resistance phenotype and multiple antibiotic resistance index amongst *Yersinia enterocolitica* isolates.

Phenotype	No. of isolates	Resistant Profiles								MAR index
A	59	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**
B	2	AMC	AMP	CXM	KF	S	TE	W		**0.58**
C	1	AMC	AMP	CXM	C	K	TE	W		**0.58**
D	1	AMC	AMP	CXM	C	KF	TE	W		**0.58**
E	2	AMC	AMP	C	KF	S	TE	W		**0.58**
F	1	AMC	AMP	CXM	C	S	TE	W		**0.58**
G	1	AMP	CXM	C	KF	TE	W			**0.5**
H	1	AMC	AMP	C	KF	TE	W			**0.5**
I	1	AMC	AMP	KF	S	TE	W			**0.5**
J	1	AMC	AMP	CXM	KF	TE	W			**0.5**

Key: AMC- amoxycillin, AMP- ampicillin, CXM-ceftriaxone, C- cefalothin, KF- chloramphenicol, S- streptomycin, TE- tetracycline, W- trimethoprim, K- kanamycin

3.3.2 Integrase genes and gene cassette arrays

The detection of the integrase genes showed that of the 70 *Y. enterocolitica* isolates tested, 76 % (53/70) were found to contain integrase genes (*Int I*, *Int II* or *Int III*). Of the three integrase genes tested, *Int II* was most dominant as it was detected in 59% (41/70) of the isolates. This was followed by *Int I* detected in 26% (18/70) of the isolates while *Int III* was only found in 6% (4/70) of the isolates, with 10% (7/70) of the isolates lacking the integrase genes. The RFLP digestion of integron positive isolates using *EcoR1* showed that there were four different banding patterns with the product sizes of 250, 300, 400, 500 and 800 bp.The gene cassette array found associated with class 1, 2 and 3 integrons, were *aacA7, aadB, aadA* and *aadA1*. The *aadA1*cassette array contained an *orfD* gene, which is a hypothetical protein for aminoglycoside-3`-adenyltransferase. The most prevalent gene cassette arrays were *aacA7, aadB, aadA4* and the least dominant was gene cassette array *aadA1* (Table 3.6*)*.

Table 3.6: Characterization of integrons and antibiotic resistance patterns of integron positive *Yersinia enterocolitica* isolates recovered from wastewater treated effluent.

Isolate designation	Integron	Resistant Pattern								Integron variable part (bp)	RFLP Pattern (bp)	Gene Cassette array
YE 12	*Int 1*	AMC ,	AMP,	CXM,	C,	KF,	S,	TE,	W	**1500**	400,500	-
YE 14	*Int 2*	AMC,	AMP,	CXM,	C,	KF,	S,	TE,	W	**1500**	400,500	-
YE 15	*Int 2*	AMC ,	AMP,	CXM,	C,	KF,	S,	TE,	W	**1500**	400,500	-
YE 16	*Int 2*	AMC ,	AMP,	CXM,	C,	KF,	S,	TE,	W	**1500**	400,500	-
YE 17	*Int 2*	AMC ,	AMP,	CXM,	C,	KF,	S,	TE,	W	**1500**	400,500	-
YE 18	*Int 3*	AMC ,	AMP,	CXM,	C,	KF,	S,	TE,	W	**1500**	250,300,500,800	*aadA1*
YE 20	*Int 2*	AMC ,	AMP,	CXM,	C,	KF,	S,	TE,	W	**1500**	400,500	-
YE 21	*Int 2*	AMC ,	AMP,	CXM,	C,	KF,	S,	TE,	W	**1500**	400,500	-
YE 22	*Int 1*	AMC ,	AMP,	CXM,	C,	KF,	S,	TE,	W	**1500**	250,300,500,800	*aadA1*
YE 23	*Int 1*	AMC,	AMP,	CXM,	C,	KF,	S,	TE,	W	**1500**	400,500	-
YE 32	*Int 2*	AMC ,	AMP,	CXM,	C,	KF,	S,	TE,	W	**1500**	250,300,500	*aacA7, aadB, aadA4*
YE 35	*Int 2*	AMC ,	AMP,	CXM,	C,	KF,	S,	TE,	W	**1500**	250,300,500	*aacA7, aadB, aadA4*
YE 37	*Int 2*	AMC ,	AMP,	CXM,	C,	KF,	S,	TE,	W	**1500**	250,300,500,800	*aadA1*
YE 46	*Int 2*	AMC,	AMP,	CXM,	C,	KF,	S,	TE,	W	**1500**	250,300,500	*aacA7, aadB, aadA4*
YE 50	*Int 1*	AMC,	AMP,	CXM,	C,	KF,	S,	TE,	W	**1500**	250,300,500	*aacA7, aadB, aadA4*
YE 51	*Int 2*	AMC,	AMP,	CXM,	C,	KF,	S,	TE,	W	**1500**	400,500	-
YE 53	*Int 2*	AMC,	AMP,	CXM,	C,	KF,	S,	TE,	W	**1500**	400,500,1000	-
YE 55	*Int 3*	AMC ,	AMP,	CXM,	C,	KF,	S,	TE,	W	**1500**	400,500	-
YE 56	*Int 2*	AMC ,	AMP,	CXM,	C,	KF,	S,	TE,	W	**1500**	250,300,500	*aacA7, aadB, aadA4*

Table 3.6: Continued……

Isolate designation	Integron	Resistant Pattern								Integron variable part (bp)	RFLP Pattern (bp)	Gene cassette array
YE 59	*Int 2*	AMC ,	AMP,	CXM,	C,	KF,	S,	TE,	W	**1500**	400,500	-
YE 64	*Int 3*	AMC,	AMP,	CXM,	C,	KF,	TE,	W,		**1500**	250,300,500	*aacA7, aadB, aadA4*
YE 66	*Int 1*	AMC,	AMP,	CXM,	C	S,	TE,	W		**1500**	250,300,500	*aacA7, aadB, aadA4*
YE 2	*Int 1*	AMC ,	AMP,	C,	KF,	S,	TE,	W		**1500**	250,300,500	*aacA7, aadB, aadA4*
YE 67	*Int 3*	AMP,	CXM,	C,	KF,	TE,	W			**1500**	250,300,500,800	*aadA1*
YE 70	*Int 1*	AMC,	AMP,	CXM,	KF,	TE,	W			**1500**	250,300,500	*aacA7, aadB, aadA4*
YE 3	*Int 2*	AMC ,	AMP,	CXM,	C,	KF,	S,	TE,	W	**900**	400,500	-
YE 6	*Int 2*	AMC,	AMP,	CXM,	C,	KF,	S,	TE,	W	**900**	400,500	-
YE 47	*Int 1*	AMC,	AMP,	CXM,	C,	KF,	S,	TE,	W	**900**	400,500	-
YE 45	*Int 1*	AMC,	AMP,	CXM,	C,	KF,	S,	TE,	W	**900**	400,500	-
YE 1	*Int 2*	AMC,	AMP,	CXM,	C,	KF,	S,	TE,	W	**900**	400,500	-
YE 31	*Int 2*	AMC ,	AMP,	CXM,	C,	KF,	S,	TE,	W	**900**	400,500	-
YE 33	*Int 2*	AMC,	AMP,	CXM,	C,	KF,	S,	TE,	W	**900**	400,500	-
YE 48	*Int 3*	AMC,	CXM,	CRO,	C,	KF,	S,	TE,	W	**900**	400,500	-
YE 36	*Int 2*	AMC ,	AMP,	CXM,	C,	KF,	S,	TE,	W	**900**	400,500	-
YE 54	*Int 2*	AMC,	AMP,	CXM,	C,	KF,	S,	TE,	W	**900**	400,500	*aadA1*
YE 39	*Int 2*	AMC ,	AMP,	CXM,	C,	KF,	S,	TE,	W	**900**	250,300,500,800	-
YE 8	*Int 2*	AMC,	AMP,	CXM,	C,	KF,	S,	TE,	W	**900**	400,500	-
YE 60	*Int 2*	AMC,	AMP,	CXM,	KF,	S,	TE,	W		**900**	400,500	-
YE 69	*Int 1*	AMC,	AMP,	KF,	S,	TE,	W			**900**	400,500	

Key: AMC- amoxycillin, AMP- ampicillin, CXM-ceftriaxone, C- cefalothin, KF- chloramphenicol, S- Streptomycin, TE- tetracycline, W- trimethoprim

MAR- Multiple Antibiotic Resistance

Int - Intergron

3.3.3 Expression of pyv associated phenotypic virulence determinants and metallo-*β*-lactamase production by *Yersinia enterocolitica*.

The pYV virulence determinant assay revealed that only 15 (21%) of the isolates were pYV positive while the majority of the isolates (85%) tested negative. For the metallo-*β*-lactamase activity assays, all the isolates (100%) were negative for the MBL activity, since the zones of inhibition for the Imp+EDTA disk compared to the Impenem alone did not show a great difference as expected to be any value ≥7mm.

Table 3.7: Metallo-*β*-lactamase activity and virulence determinant profiles of *Yersinia enterocolitica* isolates.

Sampling point	Isolate code	Crystal violet +/-	Imp + EDTA activity[x] +/-	
BC- N	YE 1	+	-	(4)
BC-NG	YE 2	–	-	(3)
UP-NG	YE 3	–	-	(3)
BC-NG	YE 4	–	-	(3)
DS-N	YE 5	–	-	(3)
UP-NG	YE 6	–	-	(4)
AC-NG	YE 7	+	-	(4)
DS-N	YE 8	–	-	(4)
AC-NG	YE 9	–	-	(4)
UP-N	YE 10	–	-	(3)
DS-NG	YE 11	–	-	(0)
DS-N	YE 12	–	-	(4)
BC-NG	YE 13	–	-	(2)
UP-NG	YE 14	–	-	(3)
DS-N	YE 15	+	-	(2)
UP-N	YE 16	–	-	(3)
AC-N	YE 17	–	-	(2)
DS-N	YE 18	-	-	(0)
BC-N	YE 19	–	-	(3)
UP-NG	YE 20	+	-	(5)
AC-NG	YE 21	–	-	(4)
DS-NG	YE 22	–	-	(4)
BC-NG	YE 23	–	-	(4)
BC-NG	YE 24	–	-	(4)
UP-NG	YE 25	+	-	(3)
DS-NG	YE 26	–	-	(5)
UP-NG	YE 27	–	-	(1)
DS-NG	YE 28	–	-	(3)
UP-N	YE 29	–	-	(5)
DS-NG	YE 30	+	-	(3)

Table 3.7: Continued……

Sampling point	Isolate code	Crystal Violet +/-	Imp + EDTA activity[x] +/-	
UP-N	YE 31	–	-	(4)
AC-N	YE 32	–	-	(3)
DS-N	YE 33	–	-	(3)
DS-N	YE 34	+	-	(4)
BC-N	YE 35	–	-	(4)
AC-N	YE 36	–	-	(3)
DS-N	YE 37	+	-	(4)
AC-N	YE 38	–	-	(3)
AC-N	YE 39	–	-	(3)
AC-NG	YE 40	–	-	(3)
AC-N	YE 41	–	-	(4)
BC-NG	YE 42	–	-	(2)
BC-NG	YE 43	–	-	(5)
DS-N	YE 44	+	-	(4)
AC-NG	YE 45	–	-	(3)
BC-NG	YE 46	+	-	(3)
AC-N	YE 47	–	-	(3)
AC-NG	YE 48	–	-	(3)
UP-NG	YE 49	+	-	(3)
BC-N	YE 50	–	-	(4)
AC-N	YE 51	–	-	(4)
AC-N	YE 52	–	-	(5)
UP-N	YE 53	+	-	(5)
DS-NG	YE 54	–	-	(4)
AC-NG	YE 55	–	-	(4)
AC-N	YE 56	–	-	(4)
AC-N	YE 57	-	-	(5)
AC-N	YE 58	–	-	(3)
AC-N	YE 60	–	-	(3)
AC-NG	YE 61	–	-	(4)
BC-NG	YE 62	–	-	(5)
DS- NG	YE 63	–	-	(1)
UP-N	YE 64	+	-	(3)
UP-NG	YE 65	–	-	(3)
DS-NG	YE 66	–	-	(3)
DS- NG	YE 67	+	-	(1)
DS-NG	YE 68	–	-	(5)
AC-NG	YE 69	–	-	(4)
BC-NG	YE 70	–	-	(4)

x= Difference in inhibition zone of Impenem and EDTA, and EDTA ≥ 7 mm is considered positive

3.3.4 Virulence genes detection

The PCR amplification of the virulence genes yielded the expected amplicon size for the different virulence genes as represented in figure 3.1.

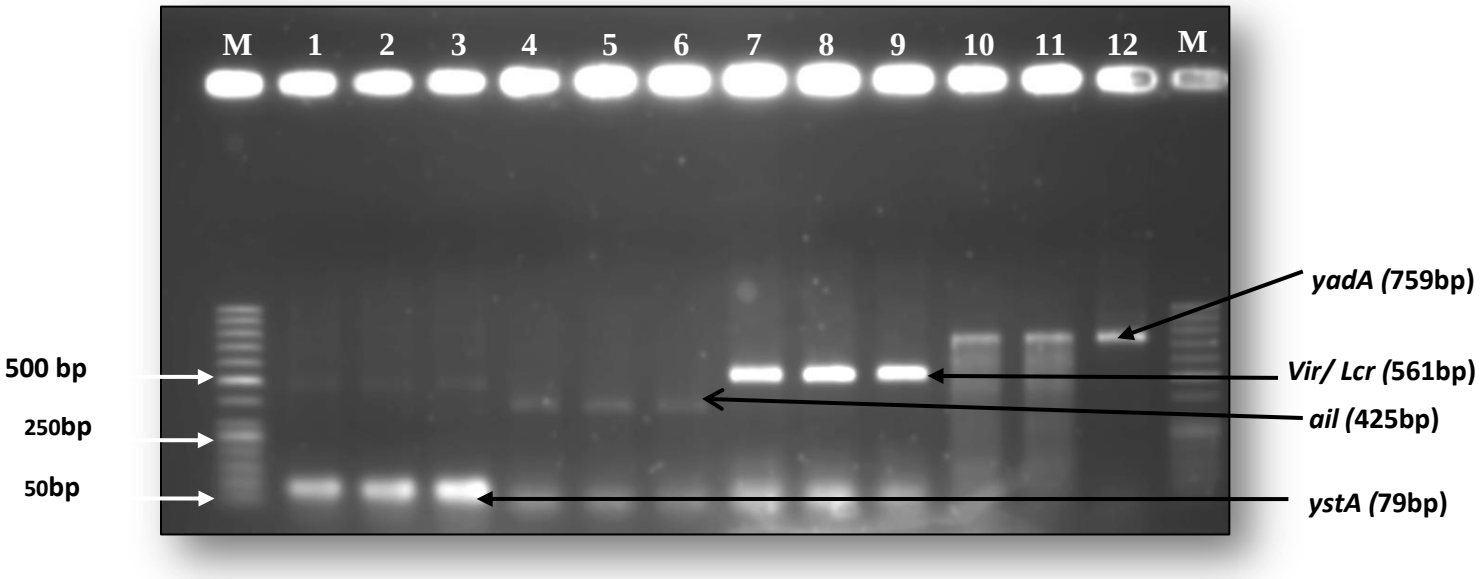


Fig 3.1: Agarose gel electrophoresis of the virulence genes detected in the *Yersinia enterocolitica* isolates recovered from treated wastewater and receiving surface water. Lane M = 50 bp plus ladder, lanes 1-3 represent *ystA* positive isolates at 79 bp, lanes 4-6 represent *ail* positive at 425 bp, lanes 7-9 represent *Vir/Lcr* positive isolates at 561 bp and lanes 10-12 represent *yadA* positive isolates at 759 bp.

The detection of virulence genes amongst the *Yersinia enterocolitica* tested isolates showed that some isolates harbored the virulence genes of interest as represented in Table 3.8.

The most prevalent gene was *ystA* 39/70 (56%), followed by *ail* 27/70 (24 %) and both the plasmid located genes *Vir/Lcr* and *YadA* were detected in only 2/70 (2%) of the tested isolates.

Table 3.8: Prevalence of the virulence genes detected from the *Y. enterocolitica* isolates.

Virulence Gene	No. of positive isolates	Positive isolates (%)
ystA	39	56
YstB	0	0
ail	24	34
Vir/Lcr	2	3
YadA	2	3

3.3.5 Genotypic fingerprint of *Yersinia enterocolitica* isolates by RAPD-PCR

The 53 integron positive *Y. enterocolitica* isolates were further studied for their genotypic similarities using Primer II as detailed in section 3.2.8. Primer II (Akhila *et al.,* 2013) used was capable of amplifying multiple polymorphic DNA fragments from all the tested isolates. RAPD-PCR gel electrophoresis showed different banding patterns with variation in intensity. As presented in figures 3.2 and 3.3, the three major bands (350 bp, 450 bp and 1400 bp) were common to all isolates. The different bands intensities observed were grouped as primary (brighter band), secondary (slightly bright) and tertiary (low intensity) based on their apperance on the gel. The tested isolates had visible bands ranging from 4 to 14 bands.

The agarose gel image obtained from the RAPD-PCR was analyzed using the GelcompareII software. The resulting dendrogram demonstrated 2 major clusters, A and B (Figure 3.4). Obtained from RAPD-PCR analysis sixty eight percentage (37/54) of the isolates are grouped together in cluster A, with most of them showing *aadA1* conserved region and belonged to phenotype A (Table 3.6). Cluster B has 31% (17/54) of the isolates. Although these isolates have

similar profile as isolates in cluster A, most of them did not harbour any of these specified conserved regions.

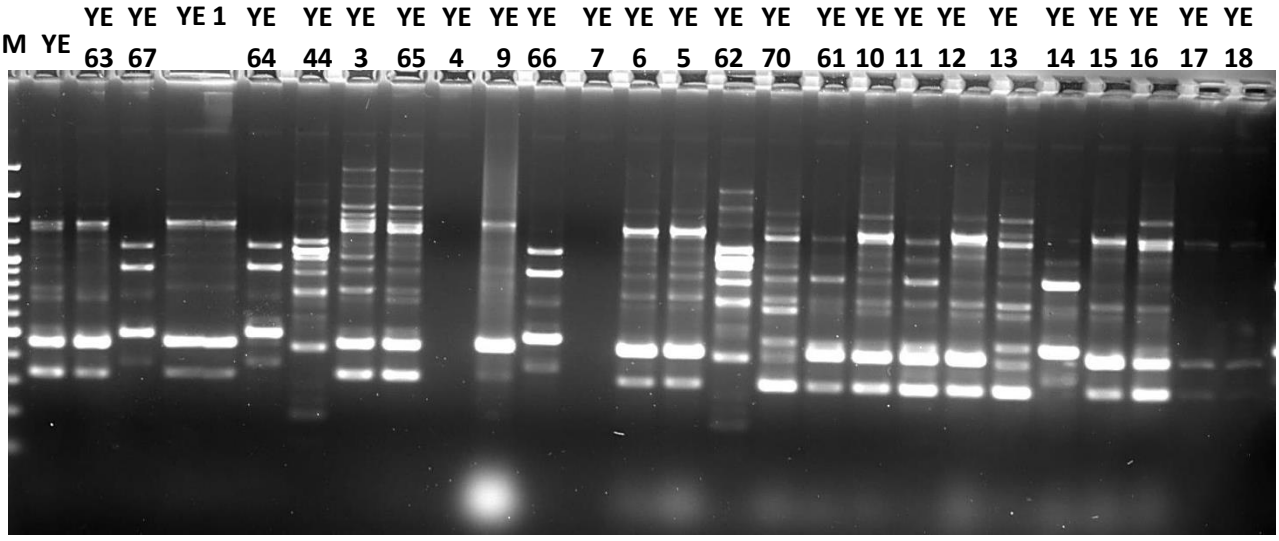


Figure 3.2: Integron positive *Yersinia enterocolitica* isolates RAPD polymorphisms amplified by primer II. M (100bp Plus DNA ladder), YE is the positive control.

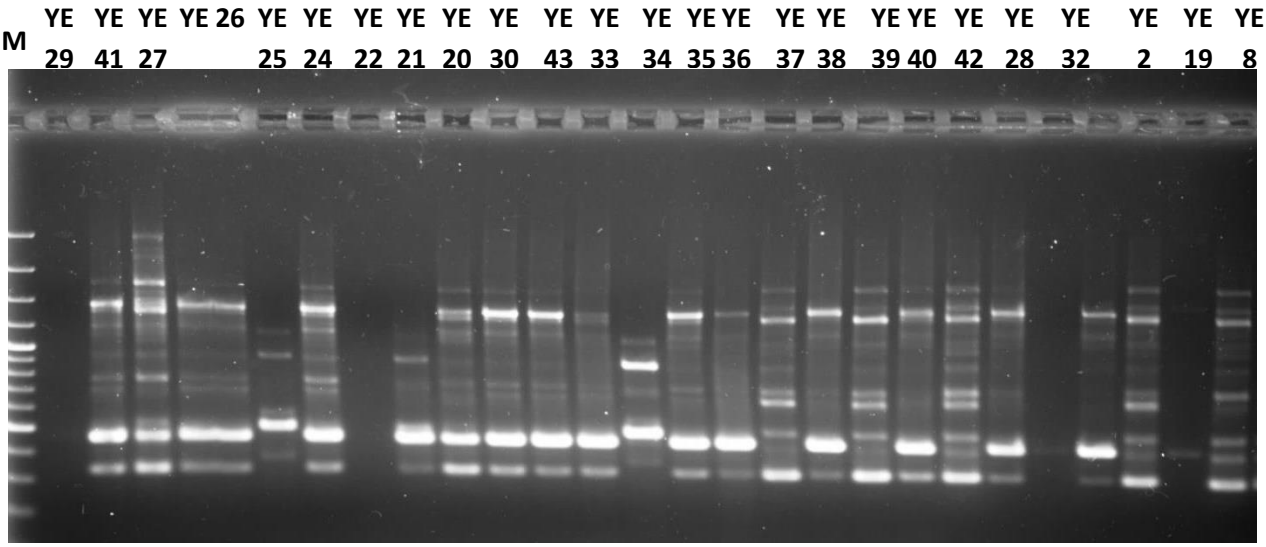


Figure 3.3: Integron positive *Yersinia enterocolitica* isolates RAPD polymorphisms amplified by primer II. M (100bp Plus DNA ladder), YE is the positive control.

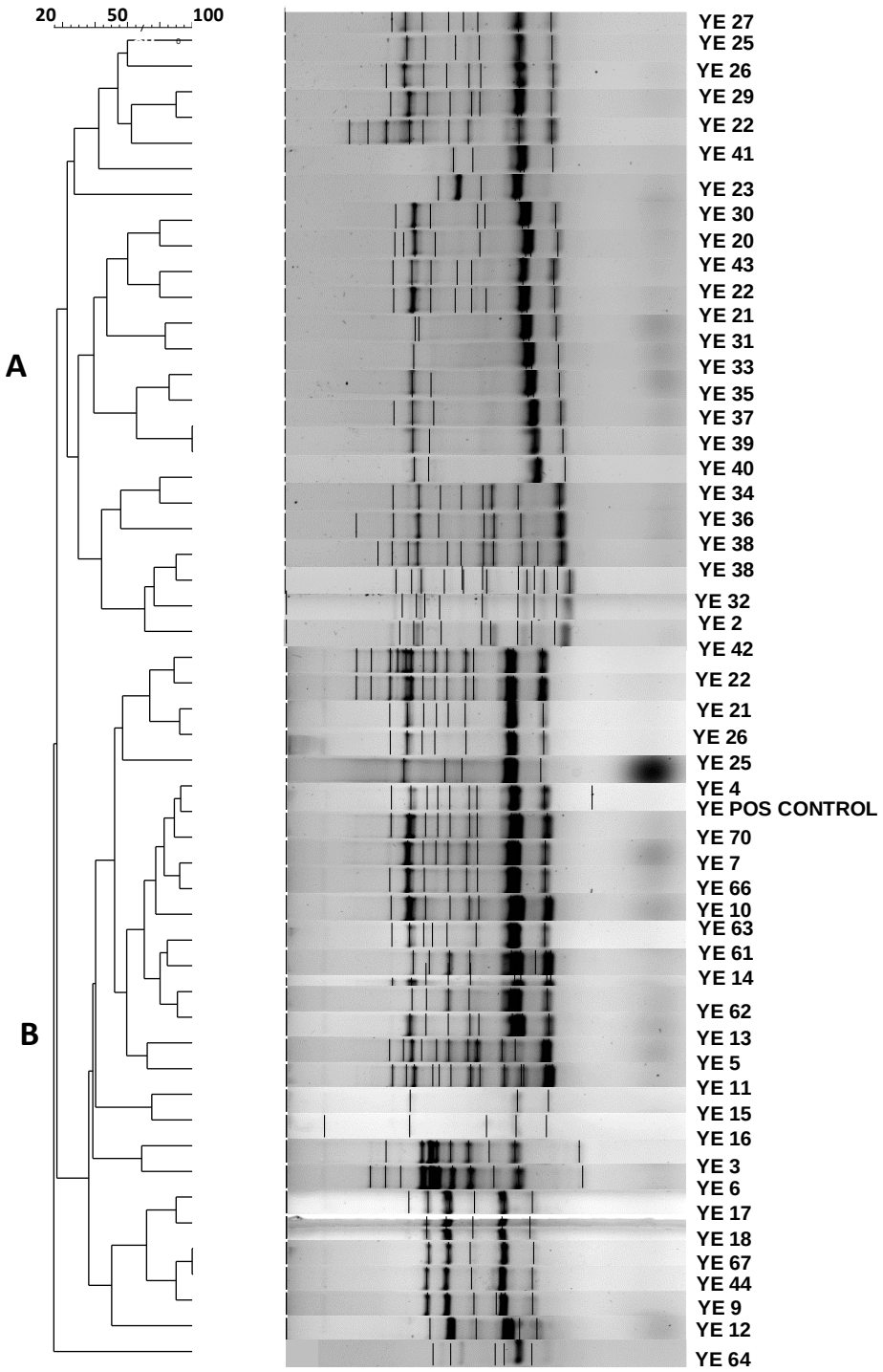


Fig 3.4: Dendrogram analysis showing the genotypic similarities of the *Yersinia enterocolitica* isolates recovered from treated wastewater effluent and receiving surface waters.

3.4 Discussion

An increase in the emergence of multi-antibiotics resistant bacteria in recent years is worrying and the presence of antibiotics resistance genes on bacterial plasmids has facilitated in the transmission and spread of drug resistance among pathogenic bacteria (Zulkifli *et al*, 2009). The increasing problems with antimicrobial drug resistance and the ability to fight against diseases and thus limiting therapeutic options to present-day clinicians (Zulkifli *et al*, 2009). Environments containing antibiotic residues exert selection pressure and contribute to the appearance of resistant bacteria (DebMandal *et al*, 2011). The majority of studies on antimicrobial susceptibility profiles of *Y. enterocolitica* focus on clinical and food isolates with little information in the literature on antibiotic susceptibility profiles of *Y. enterocolitica* isolated from environmental sources such as the discharged effluent from the municipal wastewater treatment plants.

The antibiotic resistance profiling showed that there was a high prevalence of resistance to cefalothin, cefuroxime, ampicillin, amoxicillin, chloramphenicol, tetracycline and trimethoprim among the tested *Y. enterocolitica* isolates. High levels of resistance to trimethoprim were observed in other previous studies (Miranda and Zemelman, 2002; Chen *et al*., 2010). These results reflected the widespread use of these antibiotics in aquaculture worldwide. The high levels of resistance to *β*- lactams among the *Y. enterocolitica* isolates tested may be attributed to the fact that these isolates are able to produce *β*-lactamases. A study by SharifiYazdi *et al*. (2011) on antibiotic susceptibilities of *Y. enterocolitica* isolated from meat and chicken reported high levels of resistance of the isolates to cefalothin and ampicillin. This corroborates results of the current study as a high level of resistance to penicillins (amoxicillin) and aminoglycosides

(streptomycin) was also observed among the isolates. Resistance to the β –lactam antibiotics by *Y. enterocolitica* was also observed by Tzelepi *et al*. (1999) in their study on the antimicrobial susceptibilities of *Y. enterocolitica* isolated from aquatic environments. A resistance of *Y. enterocolitica* against tetracycline was reported by Simonova *et al*. (2008) which is in accordance with the present study. Pandove *et al*. (2012) observed that *Y. enterocolitica* isolates were resistant to ampicillin and ciprofloxacin, which was observed in the present study. Bolton *et al*. (2013) observed that isolates of *Y. enterocolitica* were resistant to sulphonamides and tetracycline. Resistance of *Y. enterocolitica* isolates to tetracycline was observed by many researchers (Mayrhofer *et al*. 2004; Kot and Rainko, 2009; Bolton *et al*., 2013), which are in agreement with the present study. Possible explanation for the high resistance to several antibiotics could be attributed to acquisition of a multi-drug resistance plasmid. Plasmid-mediated transmission of antibiotic resistance has been described as the most common mechanism of horizontal gene transfer (HGT) (Norman *et al*., 2009) with sub-inhibitory concentrations of antibiotics facilitating the process of antibiotic resistance development (Davies *et al*., 2006). Antimicrobials have shown to enhance gene transfer and recombination (Couce and Blazquez, 2009), through the activation of the SOS system (Guerin *et al.*, 2010) and also they have shown to induce phage production from lysogens. Such factors may play an important role in the proliferation of gene exchange in aquatic environments.

None of the isolates tested in this study showed resistance to ceftriaxone, ciprofloxacin and gentamicin, which is in agreement with findings from previous studies (Rastawicki *et al*., 2000). The findings of the current study showed that all tested isolates were susceptible to gentamicin which is also consistent with the results of Funk *et al*. (2000). *Y. enterocolitica* is naturally

resistant to ampicillin (Aarestrup *et al*., 1998), which could explain 100% resistance to ampicillin observed in this study. While previous studies found 100% resistance of *Y. enterocolitica* to cefalothin (Funk *et al*., 2000), 90% of the isolates tested in this study were resistant. The MAR indices calculated from the resistance profiles from the *Y. enterocolitica* isolates ranged from 0.5-0.66, suggesting that the tested isolates were multidrug resistant and could pose serious threat to people who may be exposed to the water.

Detection of integrons within bacterial pathogens has been advocated as a tool in infection control and for studying antibiotic resistance within bacterial microflora (Jones *et al*. 2003). Integrons have been acknowledged as contributors to antibiotic resistance of Gram negative isolates and have been reported in environmental and animal isolates, for example, in diseased poultry (Bass *et al.,* 1999), fish (Schmidt *et al.*, 2001), pigs and cattle (Sandvang and Aarestrup 2000) and retail ground meats (White *et al.,* 2001). This raises concerns as there is a potential for transfer of integron-carrying bacteria from these sources to humans. In the current study, class 2 intergron was found to be the most dominant, as it was detected in 59% (41/70) of the *Y. enterocolitica* isolates tested compared to class 1 and class 3 integron detected in 26% (18/70) and 6% (4/70) of the isolates, respectively. A study by Hansson *et al*. (2002) also found that the most common integron within the Enterobacteriaceae family was class 2 and that more than one gene cassette could be found within a conserved region. Gene cassettes encoding *dfrA1*, *sat*, and *aadA1* was found in *E. coli* (Hansson *et al*., 2002). This is similar to the results of the current study, which indicated the presence of more than one conserved region in these integron positive *Y. enterocolitica* isolates. Genes conferring resistance to aminoglycosides and *β*-lactams are frequently found in integrons from Enterobacteriaceae, and the most common aminoglycoside

resistance gene cassettes belong to *aad* and *aac* families (Severino and Magalha˜es, 2002). Previous reports have shown the presence of aminoglycoside resistance genes associated with integrons found in Gram-negative bacteria (Fluit and Schmitz 2004). The gene cassette conserved regions detected in this study were *aadA1* (known to confer resistance to streptomycin) and recognised as one of the most frequently integrated gene cassettes harboured in class 1 integrons (Partridge *et al*. 2009), *aadB* (gene encoding an aminoglycoside adenylyltransferase and resistance to kanamycin) (AAC), that confers resistance to amikacin, gentamicin, and tobramycin (Hopkins *et al*., 1991) and *aadA4* (for streptomycin resistance). Several reports have shown the presence of *aacA7* gene encoding a type I aminoglycoside acetyltransferase of aminoglycoside resistance genes associated with integrons found in gram-negative bacteria (Severino and Magalhaes, 2002; Fluit and Schmitz, 2004). Resistance to streptomycin could be directly related to the presence of resistance genes within the integron and also the association of antibiotics such as ampicillin and tetracycline with the presence of integrons could be due to the genetic linkage between integrons and conjugative plasmids and transposons (Khosravi *et al*., 2012). In this study, it was observed that the majority of isolates were resistant to streptomycin and also that most of the isolates tested had the *aacA7*, *aadB* and *aadA*4 gene cassette array. With the observations from the tested isolates, it can be inferred that resistance presented by the gene cassette correlated with phenotypic antibiotic resistance profiles obtained in this study. Previously, the association of multidrug resistance with integrons has been specially shown in Enterobacteriaceae (van Loon *et al*, 2004), despite the lack, in some studies, of experimental evidences showing the presence or content of the variable region (Leverstein *et al*., 2003).

The emergence of the (metallo-β- lactamases) MBLs in Gram negative bacilli is becoming a problem as it poses a huge challenge to the therapeutic agents used for treatment. These enzymes are known to possess hydrolytic activity leading to the degradation of the cephalosporins (Vettoretti *et al*., 2009). In this study, the impenem +EDTA synergy assay showed that all *Y. enterocolitica* isolates tested had no MBL activity as all isolates had a zone of clearing less than the recommended value of $\geq$7 mm. These findings could not be compared with any studies as this assay has never been carried out for *Yersinia* spp. These observed findings for the Impenem disk assay correlates or shows a relation to antibiotic resistance profiles observed in this study, as most of the tested isolates had high resistance to the β- lactam class of antibiotics having an ability to produce the lactamases. This explains the poor zones of clearing observed with the Impenem disk known to belong to the β- lactam family.

The virulence abilities of these isolates were evaluated by studying the pYV- encoded phenotypic characteristics using the crystal violet binding assay. It was observed that only 21% (15/70) of the tested isolates had an ability to bind to the crystal violet and change the colony morphology from cream to purple colour. The pYV is known to be unstable in all pathogenic *Yersinia* spp. because after continual cultivation or during processing, the pYV plasmid may be lost resulting in avirulent clones (Bhaduri and Sommers, 2008). The loss of pYV leads to the eventual over growth of cells lacking pYV and resulting in the disappearance of the pYV-associated virulence characteristics (Bhaduri, 2001). It has been found that testing for pathogenicity in cultured isolates can be challenging and the validity of the virulence test has been questioned. A study by Prpic *et al*. (1985) tested the virulence by means of a calcium dependent assay and found that not all calcium dependent strains were virulent. A study by Noble *et al*. (1987) found that the results from the different virulence determinant tests do not

agree on the virulence characteristic of a tested organism. Therefore, the authors found that no individual virulence tests or group of tests could be consistently associated with symptomatic patients. A review by Bottone (1997) summarizes different studies with evidence that results from virulence testing may not be a reliable indicator of whether or not an isolate is of clinical significance to humans.

It has been recognized that *Y. enterocolitica* isolates that lack the classic virulence characteristics may still be of clinical concern to humans and pose different, uncharacterized pathogenic mechanisms within the human host (Sulakvelidze, 2000). Hypothetically, pathogenic strains should contain all virulence genes in chromosomes (*inv*, *ail*, *ystA*) and plasmids (*yadA, virF*), all of which may interact with each other to cause illness in humans. However, in this study, the detection of the virulence genes from the *Y. enterocolitica* isolates showed that isolates have 1 or 2 of the four detected virulence genes. The current study showed that 59% (39/70) of the tested isolates harbored the *ystA* gene which is the most prevalent gene detected amongst the tested isolates. This gene is related to the production of the heat stable enterotoxin (yst). The prevalence of *ystA* gene observed among *Y. enterocolitica* isolates in this study is lower than that reported by Thoerner *et al*. (2003) and Platt-Samoraj *et al*. (2006) where the *ystA* gene was identified in 100% and 99% of the tested isolates, respectively. Grant *et al*. (1998) reported pathogenic biotype 1A strains that lack *yst* gene. The *ail* gene required for attachment and aligning to the host was detected in 34% (24/70) of the tested isolates. Previous studies have reported on the presence of *ail* gene in *Y. enterocolitica* biotype 1A strains, which are commonly considered as non-pathogenic (Kraushaar *et al*. 2011). Tennant *et al*. (2003) suggested that there are two

subgroups of biotype 1A of *Y. enterocolitica*, a group comprising pathogenic strains of clinical origin and another group comprising non-pathogenic strains of environmental origin.

In the current study only 3% (2/70) of the isolates tested positive for each of the *yadA* and *Vir/Lcr* plasmid located virulence genes. The observed low occurrences of these genes have been attributed to the fact that virulence plasmid can easily be lost when the strains are subcultivated at temperatures higher than 30°C, if they are repeatedly subcultivated, or if they are stored over time. Reference strains which are cultivated only at 30°C may also lose the plasmid (Theoner *et al*. 2002). Also, it is possible that virulent strains without a plasmid may facilitate invasion in humans only by traditional and other unknown virulence genes in the chromosome, which is a hypothesis to be validated in the future. Nevertheless, due to different distributions of virulence markers, especially the conflicting occurrence of *virF* in the plasmid (Thoerner *et al*., 2003), it is certain that pathogenesis in virulent *Y. enterocolitica* is not single but diverse.

Analysis of the RAPD-PCR results obtained in this study suggests that considerable genetic diversity exists among the tested *Y. enterocolitica* isolates. Amplification results by RAPD-PCR in *Y. enterocolitica* strains analysed in this present study showed different intensities of amplified bands, suggesting that some DNA fragments had been produced at a higher rate during amplification or these fragments could be the result of multiple copies of identical DNA sequences along the genome, amplification of different sequences of DNA and/or producing fragments of the same size (Sayada *et al*., 1994).Variation in segment size of DNA amplified through RAPD-PCR, between isolates of the same species, could be the result of deletions or insertions, modifying the size of the DNA segments (Welsh and McClelland, 1990). There were

two major clusters (A and B) observed from the dendrogram analysis. Cluster A was a major cluster with 68% of the isolates grouped on this cluster. These isolates were all MBL negative, none of the isolates from this group were positive for class 3 integron, they were negative for the plasmid located gene *Vir/Lcr*. High levels of resistance of the tested isolates were observed in amoxycillin, ampicillin, ceftriaxone, cefalothin, chloramphenicol, tetracycline and trimethoprim and belonging to phenotype A as grouped based on their resistance profiles pattern also this cluster had isolates belonging to phenotypes F and J. Cluster B had 31% of the tested isolates whereby the isolates in this cluster were similar to the ones grouped in cluster A that were under phenotype A, F and J, but this cluster had isolates belonging to phenotype G and D absent in cluster A. (Figure 3.5). Again the isolates in this cluster were MBL negative but positive for both the plasmid encoded virulence genes (*yadA* and *Vir/Lcr*) and some of the isolates grouped in this cluster were positive for class 3 integrons.

3.5 Conclusion

The present study revealed that treatment effluent discharge from the municipal wastewater treatment plants investigated habour *Yersinia enterocolitica*, organisms known to be emerging pathogens. These pathogens have proved to be multiple drug resistant. All *Yersinia enterocolitica* isolates tested in this study showed high resistance (90-100%) to β-lactam class of antibiotics. The isolates also demonstrated resistance to more classes of antibiotics, including chloramphenicol, tetracycline, aminoglycosides (streptomycin) and trimethoprim. The high MAR index values obtained suggests multi-antimicrobial resistance among the isolates. The gene cassette conserved regions detected were *aacA7, aadB*and *aadA4,* encoding a type I aminoglycoside acetyltransferase, aminoglycoside adenylyltransferase and confer resistance to

streptomycin. These 3 conserved regions were found to be conserved on isolates that were class 1 and 2 integron positive showing the abundance and spread of these conserved regions within the *Yersinia enterocolitica* tested isolates. Also *aadA1* known to confer resistance to streptomycin was found on isolates that were class 1and 3 integron positive. The resistance observed can be related to tested isolates to be an integron mediated trait as observed from the results that the conserved regions confer resistance to some of the tested antibiotics.

This study also provided data on the relation between RAPD-PCR profiles of the *Yersinia enterocolitica* isolates treated from wastewater effluents and receiving surface water. Two major clusters were grouped based on the genotypic similarities and the banding patterns. It was also noted that clusters differ based on the class of the integrons harboured by the isolates. Thus it can be concluded from this study that the *Yersinia enterocolitica* tested isolates harbor the gene cassette conserved regions enabling them to be resistant to antibiotics and also that the isolates are able to hydrolyse the β- lactamase o-ring. It was also observed that some of tested isolates habour the virulence genes and it is known that presence of virulence genes indicates that these isolates are capable to cause infections in immunocompromised and susceptible hosts. In general, due to high frequency of occurrence of ampicillin, tetracycline, chloramphenicol and trimethoprim resistant *Yersinia enterocolitica* isolates in this study, these antibiotics could not be drugs of choice for treatment of Yersinosis. Gentamicin, ciprofloxacin and ceftriaxone are recommended as drugs of choice for treatment of Yersinosis as observed from this study.

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

4.1 General Discussion

Currently South Africa is faced with water shortage and the demand for water is growing as the economy expands and the increase population numbers. In order for the country to continue to develop economically, while meeting the needs for water usage, steps must be taken to protect the quality of water resources. It is common knowledge that water sources are exposed to numerous drastic changes in microbial and chemical qualities resulting from a number of activities on the watershed or reservoirs. These changes are mainly influenced by discharges of municipal raw waters or treated effluent at a specific point-source into the receiving waters such as streams, rivers, lakes, ponds etc. (Momba *et al*, 2006). There is a growing awareness of the impact of sewage contamination on aquatic *milieu*; wastewater treatment is now receiving greater attention from the World Bank and government regulatory bodies (Looker, 1998).

South Africa had outbreaks of *Shigella dysenteriae* and *Vibrio cholerae* that resulted in 13 and 288 fatalities, (Pegram *et al*., 1998; DPLG 2001). This raises a need for assessment of water and wastewater to safeguard public health and the environment (Okoh *et al.*, 2005; 2007). Thus monitoring wastewater treatment plants and their receiving water reservoirs of both the physicochemical properties and microbial load is of paramount importance (Okoh *et al.*, 2007). Physicochemical properties such as temperature, pH, DO, salinity, and nutrient loads have been reported to influence biochemical reactions within water systems, hence changes in the concentration of these parameters symbolize changes in the condition of the water system (Hacioglu and Dulger, 2009).

There have been previous studies on the poor physicochemical and microbial quality of treated effluents from wastewater treatment plants and receiving surface bodies in the Eastern Cape province, South Africa (Igbinosa and Okoh, 2009), However to our knowledge, studies on the prevalence of *Yersinia* spp and characterisation of integrons and gene cassette conserved regions of *Yersinia enterocolitica* recovered from wastewater treatment plants in South Africa have not been exhausted to give a clear indication of the prevalence and occurrence in water resources.

Hence the current study investigated the prevalence of *Yersinia* spp., especially *Yersinia enterocolitica* in treated wastewater effluent of two independent WWTP's in Durban and evaluate its impact on receiving water surfaces. This study, also evaluated the correlation between certain physicochemical parameters of the wastewater and the *Yersinia* spp. counts in the wastewater samples.

The results obtained in this study showed that some of the physico-chemical parameters tested such as pH and temperature were within the acceptable limits based on the current guidelines, while turbidity, COD and BOD fell short of standard requirements with values ranging from 1.25 -452 NTU, 37- 309 mg/L and 4.4 – 314 mg/ L, respectively. The microbial analysis showed that *Yersinia* spp. were recovered at after chlorination (AC) points and the discharge point (DS). High *Yersinia* counts were observed for upstream (UP) point for both NGTW and NWWTP, with values as high as 6.8 $x10^7$ and 2.1 $x10^7$ cfu/ml respectively, while downstream (DS) values for NGTW and NWWTP were 4 $x10^6$ and 1.7 $x10^6$ cfu/ml respectively. The detection of *Y. enterocolitica* after tertiary treatment suggests that the process may not have been effective enough to completely remove these emerging bacterial species.

The antibiotic resistance profiles observed for the tested *Yersinia enterocolitica* isolates presents in charpter three showed that these organisms were resistant to tetracycline, trimethoprim and ampicillin. Furthermore, it was observed that the tested isolates were resistant to most of the *β*-lactam antibiotics family such as amoxicillin (98%), cefuroxime (96%) and cefalothin (90%). Relatively high resistance observed for the β- lactamase family could be attributed to the fact that the organism produces β-lactamases that can destroy the o-ring (Hornstein *et al.*, 1985). *Yersinia* strains are usually susceptible *invitro* to co-trimoxazole, aminoglycosides, tetracyclines, chloramphenicol, third-generation cephalosporins, and quinolones (Prats *et al.*, 2000). The high resistance observed for tetracycline and chloramphenicol could be of serious public health concern as these are antibiotics of choice for yersinosis. The MAR indices calculated from the resistance patterns of tested *Y. enterocolitica* isolates ranged from 0.5-0.66, which is higher than the 0.2 limit, signifying that the tested isolates originated from high risks source(s) of contamination where antibiotics are used (Kuete *et al*., 2010).

The detection of the integrase genes showed the presence and distribution of 3 classes of integrase genes amongst the *Yersinia enterocolitica* isolates. The isolates had high occurrences of *IntI II* (56%), followed by *Int1 I* (26%) and *IntI III* (6%). The gene cassette conserved regions of the integron positive isolates after restriction with *EcoR1* showed that the isolates harboured four different regions namely, *aadA1, aacA7, aadB* and *aadA*. Interestingly, each conserved region conferred resistance to a few antibiotics used for susceptibility testing, such as streptomycin and trimethoprim. Genes causing resistance to aminoglycosides and *β*-lactams are often seen in integrons from the members of Enterobacteriaceae family, as most common

aminoglycoside resistance gene cassettes belong to *aad* and *aac* families (Severino and Magalha˜es, 2002).

The MBL activity assay using the antibitotic impenem + EDTA synergy assay showed that all *Yersinia enterocolitica* isolates lacked this activity since the zones of clearing observed were less than the acceptable value of ≥7 mm. The observations from this assay agreed with the resistance conferred by these tested isolates to the *β*- lactam antibiotics used for the susceptibility testing. The virulence associated phenotypic characteristics using crystal violet (CV) binding assay showed that only 15/70 (21%) of the tested isolates had an ability to bind to the crystal violet and change the colony morphology from cream to purple. This could be an indication that only 21% of the isolates bear the pYV protein. The pYV is known to be unstable in all pathogenic *Yersinia* spp. because after continual cultivation or during processing, the pYV plasmid may be lost resulting in nonvirulent clones (Bhaduri and Sommers, 2008). This could explain the low percentage of isolates that could retain the crystal violet.

Virulence genes detection in the *Yersinia enterocolitica* isolates showed that the occurrence of genes varies for each isolate. There was a higher occurrence of the chromosomal genes compared to the plasmid located genes, which could be attributed to the fact that the *Yersinia* spp. lose plasmids during culturing. Results observed in the current study revealed that the occurence of the virulence gene was follows *ystA* (59%), *ail* (34%), *Vir/Lcr* (3%) and *YadA* (3%).

RAPD profiles of *Y. enterocolitica* isolates analysed using BioNumerics (Applied Maths) confirmed the visual differentiation of isolates and grouped them in a cluster based on their

similatities. The genotypic characterization of the *Y. enterocolitica* isolates revealed that there were two major clusters A and B that grouped the isolates based on their phenotypic characteristics such as the antibiotic resistance profile, the occurrence of the intergrons and their conserved regions (Figures 3.3 and 3.4). Both clusters were related to the positive control of *Y. enterocolitica*, with cluster A being the major cluster with 68% of the isolates. These isolates were all MBL negative, none of the isolates from this group were positive for class 3 integron, lacking the plasmid located gene *Vir/Lcr* and included are the isolates belonging to phenotypes F and J (Table 3.5). Cluster B had 31% of the tested isolates grouped, these isolates had patterns of phenotype A, F and J, but this cluster had isolates belonging to phonotype G and D, including isolates that were positive for the plasmid located *Vir/Lcr* gene.

Due to the psychrotrophic nature and the importance of *Y. enterocolitica* as a potential food- and water-borne enteric pathogen, there is a need to perform microbiological controls so that such pathogens do not find ways to enter the water systems accessible to humans. *Yersinia enterocolitica* could be an important drinking water pathogen because of its widespread occurrence and persistence in natural and treated waters (at least in some geographical areas), the existence of animal reservoirs, the evidence for possible waterborne outbreaks, and the lack of definitive data on its reduction via treatment processes (Fredriksson-Ahomaa1and Korkeala 2003).

4.2. Potential future development of study

One of the priorities in the treatment of wastewater is the removal of pathogenic microorganisms in order to comply with the required discharge standards for the treated effluent. In general, the proper implementation and management strategy results in the protection of the quality of water sources; the reduction of the cost of drinking water treatment; and the control, prevention or elimination of waterborne diseases. The current study has proved that the sewage treatment plants investigated discharge potentially pathogenic emerging bacterial species, *Yersinia enterocolitica* into the surface waters which are easily accessible to public and the community at large and this poses a high health risk to the environment.

Certainly this study has set a platform for further investigation in this research area. In order to fully understand the specific strains of *Yersinia enterocolitica* that are discharged in treated effluent, more tests should be done to differentiate the identified organisms into serotype, biotype and biovar. The correlation between phenotypic and genotypic virulence determinant could be better determined using assays such as the autoagglutination test, CR-MOX testing and low calcium response assay. In addition, the presence of antibiotic resistance genes, especially the β- lactamase and the streptomycin resistance genes, should be confirmed in the isolates since most of the gene cassette conserved regions detected were found to confer resistance to streptomycin and most if not all isolates were resistant to the β- lactam class of antibiotics. While the RAPD- PCR could cluster the isolates based on their relatedness, more genetic fingerprinting could be performed to allow for intensive understanding of the genetic similarities among the isolates. This could be achieved using methods such as Enterobacterial repetitive intergenic consensus (ERIC-PCR), Amplified fragment length polymorphism (AFLP) and Pulse field gel electrophoresis (PFGE). A greater effort should also be made in the future to increase the

number of WWTPs to be investigated, frequency of the sampling times and the effect of chlorination on the survival of *Yersinia* species in order to provide more information on the level of distribution of *Yersinia* spp. in treated effluent discharges. Overall, this study has revealed the operational conditions of the WWTPs under investigation and reiterated the need for constant monitoring of the treatment process in order to ensure strict compliance of the treated effluent quality to the stipulated guidelines.

5. References

Aarestrup, F.M., Bager, F., Jensen, N.E., Madsen, M.; Meyling, A., and Wegener, H.C. (1998). Resistance to antimicrobial agents used for animal therapy in pathogenic, zoonotic and indicator bacteria isolated from different food animals in Denmark: a baseline study for the Danish Integrated Antimicrobial Resistance Monitoring Programme (DANMAP). Acta Pathological, Microbiological Immunological. **106**: 745–770.

Adewumi, J.R., Ilemobade, A.A. and Van Zyl, J.E. (2010). Treated wastewater reuse in South Africa: Overview, potential and challenges. Resources, Conservation and Recycling. **55** (2): 221-231.

Akan, J.C., Abdulrahman, F.I., Dimari, G.A., Ogugbuaja, V.O. (2008). Physicochemical determination of pollutants in wastewater and vegetable samples along the Jakara wastewater channel in Kano Metropolis, Kano State, Nigeria. European Journal of Scientific Research, **23** (1): 122-133.

Akhila, S., Shanmuga, S., Priya, T.,Murugn, S. and Thayumanavan, T. (2013). Molecular diversity analysis of *Yersinia enterocolitica* isolated from marine marketed fish. International Journal of Current Microbiology and Applied Science. **2** (9): 204-214.

Akinbowale, O.L., Peng, H., Grant, P. and Barton, M.D. (2007). Antibiotic and heavy metal resistance in motile aeromonads and pseudomonads from rainbow trout (Oncorhynchus mykiss) farms in Australia. International Journal of Antimicrobial Agents. **30** (2):177-182.

Akpor, O.B. and Muchie, M. (2011). Environmental and public health implications of wastewater quality. African Journal of Biotechnology. **10** (13): 2379-2387.

Amasiani, R.N., Agbo, I.C., Ezeifeka, G.O. and Chan, K.F. (2013). Prevalence of *Yersinia enterocolitica* in human population and transmission vehicles in Anambra State, Nigeria. International Journal of Environmental Science and Technology. **2**: 877-885.

Amir, H.M., Ali, R.M. and Farham, K. (2004). Nitrogen removal from wastewater in a continuous flow sequencing batch reactor. Pakistan Journal of Biological Science. **7**(11): 1880-1883.

Amirmozafari, N. and Robertson, D. (1993). Nutritional Requirements for Synthesis of Heat-stable Enterotoxin by *Yersinia enterocolitica*. Applied and Environmental Microbiology. **59**: 3314-3320.

Asano, T. (2007). Water reuse: Issues, technologies, and applications.McGraw-Hill. ISBN 978-0-07-145927-3.

Bartram, J., Lewis, K., Lenton, R. and Wright, A. (2005). Focusing on improved water and sanitation for health. Lancet. **365** (9461): 810-812.

Bass, L., Liebert, C.A., Lee, M.D., Summers, A.O., White, D.G., and Thayer, S.G. (1999). Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in avian *Escherichia coli*. Antimicrobial Agents Chemotherapy. **43** (12): 2925-2929.

Bauer, A.W., Kirby, W.M.M. and Sheris, J.C. (1966).Antibiotics susceptibility testing by standardized single disk method. American Journal of Clinical Pathology. **45** (4): 493-496.

Berzero, R., Volterra, L., and Chiesa, C. (1991). Isolation of *Yersinia* from sewage. Contribution of Microbiology and Immunology. **12**:40-43.

Bhaduri S., Cottrell, B. and Pickard R.A. (1997). Direct detection and isolation of plasmid-bearing virulent serotypes of *Yersinia enterocolitica* from various foods. Applied Environmental Microbiology .**63** (12): 4952- 4955.

Bhaduri, S. (2001). "Pathogenic *Yersinia enterocolitica*" in *Guide to Foodborne Pathogens*, R. G. Labbe and S. Garcia. John Wiley and Sons, New York, NY, USA. p. 245-255.

Bhaduri, S. and Sommers, C.H. (2008). Detection of *Yersinia pestis* by comparison of virulence plasmid (pYV/pCD) - associated phenotypes in *Yersinia* species. Journal of Food Safety. **28**: 453-466.

Bhagat, N. and Virdi, J.S. (2007). Distribution of virulence associated genes in *Yersinia enterocolitica* biovar 1Acorrelates with clonal groups and not the source of isolation. FEMS Microbiology. **266**: 177-183.

Blais, B.W. and Phillippe, L.M. (1995). Comparative analysis of *yadA* and *ail* polymerase chain reaction methods for virulent *Yersinia enterocolitica*. Food Control, **6:** 211-214.

Blasco, M.D., Esteve, C., and Alcaide, E. (2008). Multiresistant waterborne pathogens isolated from water reservoirs and cooling systems. Journal of Applied Microbiology. **105**: 469–475.

Blei, F. and Puder, D. R. (1993). *Yersinia enterocolitica* bacteremia in a chronically transfused patient with sickle cell anemia. Case report and review of the literature. American Journal of Pediatric Hematology and Oncology. **15**:430-434.

Boles, B.R., Thoendel, M., Singh, P.K. (2004). Self-generated diversity produces "insurance effects" in biofilm communities. Proceedings of National Acadamy of Science. USA **10**:16630–16635.

Bolton, D. J., Ivory, C. and McDowell, D. (2013). A small study of *Yersinia enterocolitica* in pigs from birth to carcass and characterisation of porcine and human strains. Food control. **33**: 521- 524.

Bottone, E. J. (1997) *Yersinia enterocolitica*: the charisma continues. Clinical Microbiology Reviews. **10**: 257–276.

Brooks, P.C. (1996). Investigation of temperature effects on denitrifying bacterial populations in biological nutrient removal (BNR) system. MS. Thesis: Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA.

Brown, H. J., Stokes, H. W. and Hall. R. M. 1996. The integrons are defective transposon derivatives. Journal of Bacteriology. **178**:4429-4437.

Cambray G., Guerout A. M. and Mazel, D. (2010). Integrons. Annual Review of Genetics. 44, 141–166. 10.1146/annurev-genet-102209-163504.

Carniel, E. (2001). The *Yersinia* high-pathogenicity island: an iron-uptake island. Microbes and Infection. **3**: 561−569.

Chandler, N.D., Parisi, M.T. (1994). *Yersinia enterocolitica* masquerading as appendicitis. Archive of Pediatrics and Adolescence Medicine Journal. **148**: 527-528.

Chapman , D. (1996). Water Quality Assessments . 2nd Edn. Published by E. & FN Spon, London.

Chen, B., W. Zheng, Y. Yu, W. Huang, S. Zheng, Y. Zhang, X. Guan, Y. Zhuang, N. Chen, and E. Topp. (2010). Class 1 integrons, selected virulence genes, and antibiotic resistance in *Escherichia coli* isolates from the Minjiang River, Fujian province, China. Applied and Environmental Microbiology **77:**148-155.

Clinical Laboratory Standards Institute. (2008). Performance Standards for Antimicrobial Disk and Dilution. Susceptibility Tests for Bacteria Isolated from Animals. 3rd ed., CLSI Document M31-A3. **28** (2008): 1-9.

Collis, C. M., and. Hall, R.M. (1992). Gene cassettes from the insert region of integrons are excised as covalently closed circles. Molecular Microbiology. **6**: 2875- 2885.

Coovadia, Y.M., Gathiram, V., Bhamjee, A., Garratt, R.M., Mlisana, K., Pillay, N., Madlalose, T. and Short, M. (1992). An outbreak of multiresistant *Salmonella typhi* in South Africa. Quarterly Journal of Medicine. **82**: 91-100.

Cornelis G. (2001). The Yersinia Deadly Kiss. Journal of Bacteriology, **180**: 5495- 5504.

Couce, A. and Blazquez, J. (2009). Side effects of antibiotics on genetic variability. FEMS Microbiology Reviews. **33**: 531-538.

Craun, G. F. (1991). Cause of waterborne outbreaks in the United States. Water Science and Technology. **24** (2): 17-20.

Dalsgaard, A., Forslund, A., Sandvang, D., Arntzen, L., Keddy, K. (2001). *Vibrio Cholerae* O1 outbreak isolates in Mozambique and South Africa in 1998 are multiple-drug resistant, contain the SXT element and the aadA2 gene located on class 1 integrons. Journal of Antimicrobial Chemotherapy. **48**: 827–838.

Davies, J. (2012). Sanitation: sewage recycles antibiotic resistance.Nature. **487**: 302

Davies, M. R., McMillan, D. J., Beiko, R. G., Barroso, V.,Geffers, R. and Sriprakash, K. S. (2006). Virulence profiling of *Streptococcus dysgalactiae* subspecies equisimilis isolated from infected humans reveals 2 distinct genetic lineages that do not segregate with their phenotypes or propensity to cause diseases. Clinical and Infectious Diseases. **44**: 1442–1454. doi: 10.1086/516780.

DebMandal, M., Mandal, S. and Pal, N.K. (2011). Antibiotic Resistance Prevalence and Pattern in Environmental Bacterial Isolates. The Open Antimicrobial Agents Journal: **3**:45-52.

DeBoer, E., Hartog, B.J., Oosterm, J. (1982). Occurrence of *Yersinia enterocolitica* in Poultry products. Journal of Food Protection. **45**: 322-325.

Decicco, B.T., (1979). Removal of eutrophic nutrients from wastewater and their bioconversion to bacterial single cell protein for animal feed supplements phase II. Water Resources Research Centre Report 15.

Department of Water Affairs and Forestry of South Africa. (1996) South African Water Quality Guidelines, Volume 1. (Domestic Use);. Available online: http://www.dwa.gov.za/iwqs/wq_guide/Pol_saWQguideFRESH_vol1_Domesticuse.PDF (accessed on 19 February 2010).

Department of Water Affairs and Forestry. (1994). Water Supply and Sanitation Policy White Paper. Available online: http://www.dwaf.gov.za/Documents/Policies/WSSP.pdf (accessed on 21 February 2010).

Dojlido, J.R. and Best, G.A. (1993). Chemistry of water and water pollution. Ellis Horwood Ltd, Great Britain. ISNB- 0-13-878919-3

DWAF. (1996b). South African Water Quality Guidelines (volume 1), Domestic Uses (2nd. Edn). Department of Water Affairs and Forestry, Pretoria.

DWAF. (1998). Quality of Domestic Water Supplies. Assessment Guide (2nd.Edn). Department of Water Affairs and Forestry, Department of Health and Water Research Commission.

DWAF. (2004). Strategic Framework for National Water Resource Quality Monitoring Programmes. Department of Water Affairs and Forestry, Department of Health and Water Research Commission.

Ekholm, P. and Krogenus, K. (1998). Bioavailability of Phosphorus in Purified Municipal Wastewaters. Water Research. **32** (2): 343.

Environment Canada. (2001).The state of municipal wastewater effluent in Canada. Minister of Public Works and Government Services Canada, Ottawa, Ontario K1A 0H3. http://www.ec.gc.ca/soer-ree. Accessed 27 January 2009.

EPA (1996). U.S. Environmental Protection Agency, American Society of Civil Engineers, and American Water Works Association. Technology Transfer Handbook: Management of Water Treatment Plan Residuals. EPA/625/R-95/008. Washington DC.

EPA (2000). Nutrient criteria technical guidance manual-rivers and streams.EPA-822-B-00-002. Washington DC.

Falcão, J. P., Brocchi, M., Proença-Módena, J. L., Acrani, G. O., Correa, E. F. and Falcão, D.P. (2004)**.** Virulence characteristics and epidemiology of *Yersinia enterocolitica* and Yersiniae other than *Y. pseudotuberculosis* and *Y. pestis* isolated from water and sewage. Journal of Applied Microbiology. **96**: 1230–1236.

Fatoki, S. O., Gogwana, P. and Ogunfowokan, A. O. (2003). Pollution assessment in the Keiskamma River and in the impoundment downstream. Water SA. **29** (3):183-187.

Fenwick, S.G. and Murray, A. (1991) Detection of pathogenic *Yersinia enterocolitica* by polymerase chain reaction. Lancet. **337**: 496–497.

Fluit, A.C. and Schmitz, F.J. (2004) Resistance integrons and super-integrons. Clinical Microbiology and Infection. **10**: 272–288.

Food and Agricultural Organistion (FAO). (2007). Wastewater characteristics and effluents qualityparameters. Food and Agricultural Organization of the United Nations. Available from http://www. fao.org/docrep/to55ie/to55ieo3.html. Accessed 06/04/2008.

Fredriksson-Ahomaa M. and Korkeala, H. (2003) Low occurrence of pathogenic *Yersinia enterocolitica* in clinical, food, and environmental samples: A methodological problem. *Clinical Microbiology Review* **16:** 220-229.

Fredriksson-Ahomaa, M., T. Korte, and H. Korkeala. 2001. Transmission of *Yersinia enterocolitica* 4/O:3 to pets via contaminated pork. Letters in Applied Microbiology. **32:**375-378.

Fukushima, H., Hoshina, K., Itogawa, H. and Gomyoda, M. (1987). Introduction into Japan of pathogenic Yersinia through imported pork, beef and fowl, International Journal of Food Microbiology. **35** (3): 205-212.

Funk, J.A., Trout, H.F., Davis, S.A. and Fossler, C.P. (2000). In vitro susceptibility of *Yersinia enterocolitica* isolated from the oral cavity of swine. Journal of Food Protection. **63**:395–399.

Grant, T., Bennett-Wood, V. and Robins-Browne, R.M. (1998). Identification of virulence associated characteristics in clinical isolates of *Yersinia enterocolitica* lacking classical virulence markers. Infection and Immunity.**66:** 1113-1120.

Gray, F.N. (2002). Water Technology: An Introduction for Environmental Scientists and Engineers. Butterworth-Heinemann. Oxford p. 35-80.

Grosdent N., Maridonneau-Parini I., Sory M., and Cornelis G. (2002). Role of Yops and Adhesins in Resistance of *Yersinia enterocolitica* to Phagocytosis. Infection and Immunity, **70**:4165-4176.

Guardabassi, L., Wong, D.M.L.F.and Dalsgaard, A. (2002). The effects of tertiary wastewater treatment on the prevalence of antimicrobial resistant bacteria. Water Research. **36**:1955–1964.

Guerin, E., Cambray, G., Da Re, S., Mazel, D. and Ploy, M.C. (2010). The SOS response controls antibiotic resistance by regulating the integrase of integrons. Medical Science (Paris) **1**: 28-30.

Guidelines for Drinking Water Quality: Recommendations, World Health Organization. (2004).Geneva.

Hach, C.C., Klein, R.L. and Gibbs, C.R. (1997). Introduction to Biochemical Oxygen Demand Technical Information Series. Booklet No. 7.

Hacioglu, N. and Dulger, B. (2009). Monthly variation of some physicochemical and microbial. parameters in Biga stream (Biga, Canakkale, Turkey), African Journal of Biotechnology. **8**:1927-1937.

Hammer, M. J. (1996). Water and Wastewater Technology, 3rd Edn. Prentice–Hall Inc., New York.

Hamzaoui N., Kerneis S., Caliot E., and Pringault E. (2004). Expression and Distribution of b1 Integrins in in vitro -induced M cells: Implications for Yersinia Adhesion to Peyer's Patch Epithelium. Cellular Microbiology. **6**: 817–828.

Hansson, K., Sundström, L., Pelletier, A. and Roy, P.H. (2002). *IntI*2 integron integrase in Tn7. Journal of Bacteriology. **184**: 1712-1721.

Heise T. and Dersch P. (2006). Identification of a Domain in Yersinia Virulence Factor *YadA* that is Crucial for Extracellular Matrix-specific Cell Adhesion and Uptake. Proceedings of the National Academy of Science. **103**: 3375–3380.

Hoko, Z. (2005). An Assessment of the Water Quality of Drinking Water in Rural Districts in Zimbabwe the Case of Gokwe South, Nkayi, Lupane, and Mwenezi Districts, Physics and Chemistry of Earth. **30**: 859 – 866.

Hopkins, J. D., Flores, A., delPilarPla, M., Lester, S., and O'Brien, T.F. (1991). Nosocomial spread of an amikacin resistance gene on both a mobilized, non-conjugative plasmid and a conjugative plasmid. Antimicrobial Agents Chemotherapy. **35**:1605–1611.

Hornstein M.J., Jupeau A.M., Scavizzi M.R., Philippon A.M. and Grimont. J. (1985). Invitro susceptibilities of 126 clinical isolates of *Yersinia enterocolitica* to 2 1 β-lactam antibiotics. Antimicrobial Agents Chemotherapy. **27** (5): 806-811.

Hudson, J.A and Mott S.J. (1993) Growth of *Listeria monocytogenes, Aeromonas hydrophila* and *Yersinia enterocolitica* on cooked beef under refrigeration and mild temperature abuse. Food Microbiology. **10**: 429-437.

Hurst, C. J., Clark, R. M. and Regli, S. E. (1996). Estimating the risk of acquiring infectious disease from ingestion of water. Chapter 4. pp.. In C. J. Hurst (ed.), Modeling Disease Transmission and Its Prevention by Disinfection. Cambridge University Press, Cambridge. p.99-139.

Ibrahim , I and Mac Rae, A. (1991) . Incidence of *Aeromonas* and *Listeria* spp. in red meat and milk samples in Brisbane, Australia. International Journal of Food Microbiology. **12**: 263-269.

Igbinosa, E.O. and Okoh, A.I. (2008). Emerging *Vibrio* species: an unending threat to public health in developing countries. Research in Microbiology **159** (7-8): 495-506.

Igbinosa, E.O. and Okoh, A.I. (2009). Impact of discharge wastewater effluents on the physicochemical qualities of a receiving watershed in a typical rural community. International Journal of Environmental Science and Technology. **6** (2):175-182.

Jaji, M. O., Bamgbose, O., Odukoya, O. O. and Arowlo, T. A. (2007). Water quality assessment of Ogun River, south west Nigeria. Environ. Monit. Assess., **133** (1-3), 447-482

Jones, L.A., McIver, C.J., Rawlinson, W.D. and White, P.A. (2003). Polymerase chain reaction screening for integrons can be used to complement resistance surveillance programs. Communicable Disease Intelligence. S103–S110.

Jourdan, A., Johnson, S., and Wesley, I. (2000). Development of a fluorogenic 5' Nuclease PCR Assay for Detection of the *ail* gene of Pathogenic *Yersinia enterocolitica*. Applied and Environmental Microbiology. **66**: 3750-3755.

Kantachote, D., Dangtago, K. and Siriwong, C. (2009). Treatment efficiency in wastewater treatment plant of Hat Yai Municipality by quantitative removal of microbial indicators Songklanakarin. Journal Science and Technology. **31**(5): 567-576.

Kapur, A., Kansal, A., Prasad, R. K., and Gupta, S. (1999). Performance evaluation of sewage treatment plant and sludge bio-methanation. Indian Journal of Environmental Protection. **19**: 96-100.

Karlheinz T., Harmsen, D., Rakin, A., Schmelz, J., Heesemann, **J.** (1998). Development of rRNA-Targeted PCR and In Situ Hybridization with Fluorescently Labelled Oligonucleotides for Detection of *Yersinia* Species. Journal of Clinical Microbiology. p.2557-2564.

Khosravi, Y., Tay, S.T. and Vadivelu, J. (2012). Analysis of integrons and associated gene cassettes of metallo-β-lactamase-positive *Pseudomonas aeruginosa* in Malaysia. Journal of Medical Microbiology. p.988-994.

Koeleman, J.G.M., Stoof, J. Van Der Bijl, M.W. Vandenbroucke- Grauls, C.M.J.E. and Savelkoul, P.H.M .(2001). Identification of epidemic strains of *Acinetobacter baumannii* by integrase gene PCR. Journal of Clinical Microbiology: **39**: 8- 13.

Kotchoni, S.O., Gachomo, E.W., Betiku, E.and Shonukan, O. (2003). A homemade kit for plasmid DNA mini-preparation. African Journal of Biotechnology. **2** (4): 88-90.

Kraushaar, B., Dieckmann, R., Wittwer, M., Knabner, D., Konietzny, A., Ma¨de, D. and Strauch, E. (2011). Characterization of a *Yersinia enterocolitica* biotype 1A strain harbouring an *ail* gene. Journal of Applied Microbiology. **111**:997-1005.

Kuete, V,. Ngameni, B., Tangmouo, J.G., Bolla, J.M., Alibert-Franco, F., S., Ngadjui, B.T., and Pages, J.M. (2010). Efflux Pumps Are Involved in the Defense of Gram-Negative Bacteria against the Natural Products Isobavachalcone and Diospyrone. Antimicrobial Agents and Chemotherapy. **54** (5): 1749-1752.

Kumar, A. and Bahadur, Y. (2009). Physico-chemical Studies on the Pollution Potential of River Kosi at Rampur (India). World Journal of Agricultural Science. **5** (1):1-4.

Kurup, R., Persaud, R., Caesar, J. and Raja, V. (2010). Microbiological and physiochemical analysis of drinking water in Georgetown, Guyana. Nature and Science. **8** (8): 261-265.

Laitenen, O., Tuuhea, J. and Ahvonen, P. (1972). Polyarthritis associated with *Yersinia enterocolitica* infection. Clinical features and laboratory findings in nine cases with severe joint symptoms. Annals of the Rheumatic Diseases. **31**:34-39.

Larsdotter, K. (2006). Microalgae for phosphorus removal from wastewater in a Nordic climate. A Doctoral Thesis from the School of Biotechnology, Royal Institute of Technology, Stockholm, Sweden, ISBN: 91-7178-288-5.

Le Roux, A. and van Hyussteen, E. (2010). Socio-Economic Landscape: The South African Socio-Economic and Settlement Landscape. In South Africa Risk and Vulnerability Atlas; the South African National Department of Science and Technology Eds., CPD Print: Pretoria, South Africa, pp. 15-21.

Le´ve´sque, C., Piche´, L. and Larose, C. (1995). PCR mapping of integrons reveals several novel combinations of resistance genes. Antimicrobial Agents Chemotherapy. **39**: 185-91.

Leal, N.C., Sobreira, M., Leal-Balbino, T.C., de Almeida, A.M.P.,daSilva, M.J.B., Mello, D.M., Sekia, L.M. and Hofer, E. (2004). Evaluation of a RAPD-based typing scheme in a molecular epidemiology study of *Vibrio cholerae* O1, Brazil. Journal of Applied Microbiology. **96**: 447–454.

Lee, V., Mazmanian S., and Schneewind O., (2001).A Program of *Yersinia enterocolitica* Type III Secretion Reactions Activated by Specific Signals. Journal of Bacteriology. **183**: 4970-4978.

Lee, L.A., Gerber, A.R., Lonsway, D.R., Smith, J.D., Carter, G.P., Puhr, N.D., Parrish, C.M., Sikes, R.K., Finton, R.J.and Tauxe, R.V. (1990). *Yersinia enterocolitica* O:3 infections in infants and children, associated with the household preparation of chitterlings. New England Journal of Medicine. **322**: 984–987.

Leverstein-van Hall, M.A., Blok, H.E.M., Donders, A.R.T., Paauw, A., Fluit, A.C. and Verhoef, J. (2003). Multidrug resistance among Enterobacteriaceae is strongly associated with the presence of integrons and is independent of species or isolate origin. Clinical Infectious Diseases. **187**: 251–259.

Levy, S.B. (1989). Evolution and spread of tetracycline resistance determinants. Journal of Antimicrobial Chemotherapy. **24**: 1-7.

Logue, C., Sheridan, J., McDowell, D., Blair, I., and Hegarty, T. (2000).The Effect of Temperature and Selective Agents on the Growth of *Yersinia enterocolitica* serotype O:3 in Pure Culture. Journal of Applied Microbiology. **88**: 1001-1008.

Lokhande, R.S., Singare, P.U., Deepali, **S.P.** (2011). Study on Physico-Chemical Parameters of Waste Water Effluents from Taloja Industrial Area of Mumbai, India. International Journal of Ecosystem; **1**(1): 1-9.

Looker, N. (1998). Municipal wastewater management in Latin America and the Caribbean, R.J. Burnside International Limited, Published for Roundtable on Municipal Water for the Canadian Environment Industry Association.

Luyt, C.D., Tandlich, R., Muller, W.J. and Wilhelmi, S.B. (2012). Microbial Monitoring of Surface Water in South Africa: An Overview. International Journal of Environmental Research and Public Health. **9** (8): 2669-2693.

Manu-Tawiah, W., Myers, D.J., Olson, D.G. and Molins, R.A. (1993). Survival and growth of *Listeria monocytogenes* and *Yersinia enterocolitica* in pork chops packaged under modified gas atmospheres. Journal of Food Science. **58**: 475-479.

Mayer, F.L. Jr. and Ellersieck, M.R. (1988). Experiences with single-species tests for acute toxic effects in freshwater animal's . Ambio. **17**: 367-375.

Mayrhofer, S., Paulsen, P., Smulders, F. J. M. and Hilbert, F. (2004). Antimicrobial resistance profile of five major foodborne pathogens isolated from beef, pork and poultry. International Journal of Food Microbiology. **97**: 23-29.

Mazel, D., Dychinco, B.,Webb, V.A., Davies, J. (2000). Antibiotic resistance in the ECOR collection: integrons and identification of a novel *aad* gene. Antimicrobial Agents Chemotherapy. **44**:1568– 1574.

McCasland , M., Trautmann, N., Porter, K. and Wagenet, R. (2008). Nitrate: Health effects in drinking water. Available from http://pmep.cee.comell.edu/facts.slides–self/facts/nit-heefgrw85.html. Accessed 05/04/2008.

Metcalf, X., and Eddy, X, (2003). Wastewater Engineering: Treatment and Reuse. In: Wastewater Engineering, Treatment, Disposal and Reuse. Techobanoglous G, Burton FL,

Stensel HD (eds), Tata McGraw-Hill Publishing Company Limited, 4th edition. New Delhi, India.

Miller, V. L., Farmer III, J. J., Hill, W. E. and Falkow, S. (1989). The ail locus is found uniquely in *Yersinia enterocolitica* serotypes commonly associated with disease. Infection and Immunity. **57**: 121-131.

Minakhina, S., Kholodii, G., Mindlin, S., Yurieva, O.and Nikiforov, V. (1999). Tn5053 family transposons are res site hunters sensing plasmidal res sites occupied by cognate resolvases. Molecular Microbiology. **33**:1059–1068.

Miranda, C. D., and R. Zemelman. 2002. Antimicrobial multi resistance in bacteria isolated from freshwater Chilean salmon farms. Science of the Total Environment. **293:** 207-218.

Momba, M. N. B. and Notshe, T. L. (2003). The effect of long storage and household containers on the microbiological quality of drinking water in rural communities of South Africa. Journal of Water Supply: Research and Technology-AQUA. **52** (1): 67.

Momba, M.N.B., Osode, A.N. and Sibewu, M. (2006). The impact of inadequate wastewater treatment on the receiving water bodies—Case study: Buffalo City and Nkokonbe Municipalities of the Eastern Cape Province. Water South Africa. **32**: 687.

Monyai, P., (2004). Health-Related Water Quality and Surveillance Model for the Peddie District in the Eastern Cape; WRC Report No.: 727/1/04; Water Research Commission: Pretoria, South Africa.

Moore, J.E., Moore, P.J.A. and Millar, B.C. (2010). "The presence of antibiotic resistant bacteria along the River Lagan," Agricultural Water Management. **98**: 217–221.

Morris, J.G., Prado, J.V., Ferreccio, C., Robins-Browne, R.M., Bordun, A.M.,. Cayazzo, M., Kay, B.A. and Levine, M.M. (1991). "*Yersinia enterocolitica* isolated from two cohorts of

young children in Santiago, Chile: incidence of and lack of correlation between illness and proposed virulence factors", Journal of Clinical Microbiology. **29**: 2784-2788.

Morrison, G., Fatoki, O. S., Persson, L., Ekberg, A., (2001). Assessment of the impact of point source pollution from the Keiskammahoek Sewage Treatment Plant on the Keiskamma River–pH, electrical conductivity, oxygen demanding substance (COD) and nutrients. Water South Africa. **27** (4): 475-480.

Murray, K., du Preez, M., Kuhn, A.L. and van Niekerk, H. (2004). A Pilot Study to Demonstrate Implementation of the National Microbial Monitoring Programme; WRC Report No. 1118/1/04; Water Research Commission: Pretoria, South Africa.

Nesbakken, T. and Skewe, E. (1985). Control of *Y. enterocolitica* in pigs at herd level and in the slaughterhouse, Nederl. Tijdsch. Medical Microbiology. II, 6, 15.

Neubauer, H., Aleksic, S., Hensel, A., Finke, E.J. and Meyer, H. (2000). *Yersinia enterocolitica* 16S rRNA gene types belong to the same genomospecies but form three homology groups. International Journal of Medical Microbiology. **290**: 61– 64.

Ngwa, G.A., Schop, R., Weir, S., León-Velarde, C.G. and Odumeru, J.A. (2013). Detection and enumeration of E. coli O157:H7 in water samples by culture and molecular methods. Journal of Microbiological Methods **92** (2): 164-172.

Ngwenya, F. (2006). Water Quality Trends in the Eerste River: Masters Mini-thesis.University of the Western Cape., South Africa.

Nicolai, T. (2002). Pollution, environmental factors and childhood respiratory allergic disease. Toxicology. **181-182**: 317-321.

Noble, M.A., Barteluk, R.L., Freeman, H.J., Subramaniam, R. and Hudson, J.B. (1987). Clinical significance of virulence related assay of *Yersinia* species. Journal Clinical Microbiology. **25**: 802–807.

Norman, A., Hansen, H.L. and Sørensen, S.J. (2009). Conjugative plasmids: vessels of the communal gene pool. Philosophial Transaction of the Royal Society. **364**: 2275–2289.

Odjadjare, E.C and Olaniran, A.O. (2015). Prevalence of Antimicrobial Resistant and Virulent *Salmonella* spp. in Treated Effluent and Receiving Aquatic Milieu of Wastewater Treatment Plants in Durban, South Africa. International Journal of Environmental Research and Public Health**. 12**: 9692-9713.

Okoh, A.I., Odjadjare, E.E., Igbinosa, E.O. and Osode, A.N. (2007). Wastewater treatment plants as a source of pathogens in receiving watersheds. African Journal of Biotechnology: **6** (25): 2932-2944.

Osode, A.N. and Okoh, **A.I.** (2009). Impact of Discharged Wastewater Final Effluent on the Physicochemical Qualities of a Receiving Watershed in a Suburban Community of the Eastern Cape Province. Clean. **37** (12): 938 – 944

Ottoson, J., Hansen, A., Björlenius, B., Norder, H. and Stenström, T.A. (2006) Removal of viruses, parasitic protozoa and microbial indicators in conventional and membrane processes in a wastewater pilot plant. Water Research. **40** (7): 1449-1457.

Pandove, G., Sahota, P., Verma, S. K., Mahajan, V., Singh, D. and Singh, J. P. (2012). Epidemiology, virulence and public health significance of *Yersinia enterocolitica* in drinking water. African Journal of Microbiology Research **6**: 5905-5913.

Pant, A. and Mittal, A.K. (2007). Monitoring of pathogenicity of effluents from the UASB based sewage treatment plant. Environmental monitoring and assessment. **133** (1-3), 43-51.

Partridge, S. R., G. Tsafnat, E. Coiera, and J. R. Iredell. (2009). Gene cassettes and cassette arrays in mobile resistance integrons. FEMS Microbiology Reviews. **33:**757-784.

Pearson, I., and Idema, G. (1998). CSIR Division of Water, Environment and Forestry Technology, Pretoria. WRC Report No 649/1/98. Water Research Commission, Pretoria, South Africa, pp. 1-17.

Pegrum, G.C., Rollins, N. and Esprey, Q. (1998). Estimating the cost of diarrhoea and epidemic dysentery in KwaZulu-Natal and South Africa. Water South Africa **24(**1): 11-20

Pitman, V.W (2011). Overview of water resource assessment in South Africa: Current state and future challenges. Water South Africa. **37(**5): 659-664.

Platt-Samoraj A., Ugorski M., Szweda W., Szczerbaturek A., Wojciech K. and Procajlo Z. (2006). Analysis of the presence of *ail*, *ystA* and *ystB* genes in *Yersinia enterocolitica* strains isolated from aborting sows and aborted fetuses. Journal of Veterinary Medicine. **53**: 341-346.

Ploy, M. C., Lambert, T., Couty, J.P. and F. Denis. (2000). Integrons: an antibiotic resistance gene capture and expression system. Clinical Chemistry and Laboratory Medicine. **38**: 483-487.

Prats, G., Mirelis, G., Llovet, T., Munoz, C., Miro, E. and Navarro, F. (2000). Antibiotic resistance terns in enteropathogenic bacteria isolated in 1985–1987 and 1995–1998 in Barcelona. Antimicrobial Agents Chemotherapy. **44**:1140-1145.

Prpic, J.K., Robins-Browne, R.M. and Davey, R.B. (1985). In-vitro assessment of virulence in *Yersinia enterocolitica* and related species. Journal of Clinical Microbiology. **22**: 105–110.

Quality of Domestic Water Supplies: Assessment Guide, Department of Health and Water Research Commission, Department of Water Affairs and Forestry (DWAF). Pretoria 1998.

Rahman M, Huys G, Rahman M, Albert J, Kuhn I and Mollby R. (2005) Wastewater treatment options for paper mills using waste paper /Imported pulp as raw materials. Applied Environmental Microbiology. **58**: 29-37.

Rastawicki,W., Gierczyński, R., Jagielski, M., Kaluzewski, S. and Jeljaszewicz, S., (2000). Susceptibility of Polish clinical strains of *Yersinia enterocolitica* serotype O3 to antibiotics. International Journal of Antimicrobial Agents. **13**: 297-300.

Recchia, G. D. and R. M. Hall. (1995). Gene cassettes a new class of mobile element. Microbiology. **141**:3015-3027.

Requirements for the Purification of Wastewater or Effluent, Government Gazette No. 9225, Regulation No 991, 18 May 1984.

Revell, P. and Mille V. (2000). A chromosomally Encoded Regulator is Required for Expression of the *Yersinia enterocolitica inv* gene and for Virulence. Molecular Microbiology, **35**: 677-685.

Rietveld, L.C., Meijer L, Smeets P.W.M.H, and van der Hoek J.P. (2009). Assessment of *Cryptosporidium* in wastewater reuse for drinking water purposes: a case study for the city of Amsterdam, The Netherlands. Water South Africa. **35**(2): 211-215.

Robins-Browne, R.M. (2001). *Yersinia enterocolitica*. In Food Microbiology: Fundamentals and Frontiers, 2nd edn. pp. 215-245. Washington, DC: American Society for Microbiology Press.

Robins-Browne, R.M. , M. D. Miliotis, S. Cianciosi, V. L. Miller, S. Falkow, J. G. Morris Jr. (1989).“Evaluation of DNA colony hybridization and other techniques for detection of virulence in *Yersinia* species”, Journal of Clinical Microbiology. **27**:644-650.

Ryan, K. J. and Ray, C.G. (2004). Sherris Medical Microbiology (4th Ed.). McGraw Hill. pp. 368-70. ISBN 0-8385-8529-9.

Sabina, Y., Rahman, A. and Chandra, R. (2011). *Yersinia enterocolitica*: Mode of transmission, molecular insights of virulence, and pathogensis of infection, Journal of Pathogens, 429069, doi: 10. 4061/2011/4290069. 10.1128/mBio.01409-14.

Sadeghpoor, M., Hosseini, B., and Najafpour, G.D. (2009). Assessment of wastewater treatment plant's performance in Amol industrial park. American-Eurasian Journal of Agriculture and Environmental Science, **5**: 707-711.

Sahota, P., Garg, V. and Pandove, G. (2014). Prevalence, virulence characterization and antibiotic resistance of hippurate hydrolyzing *Campylobacter jejuni* in drinking water: comparison with standard MPN method. W. S. T. **62** (3):676-683.

Sambrook, J., Russell, D.W. (2001). Molecular Cloning: A Laboratory Manual; 3rd edition. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, USA, 21-55.

Sandvang, D. and Aarestrup, F.M. (2000). Characterisation of aminoglycoside resistance genes and class 1 integrons in procine and bovine gentamicin-resistant *Escherichia coli*. Microbial Drug Resistance. **6**:19-27.

Sato Y., Kaneko K., Sasahara T., and Inoue M. (2006). Novel Pathogenetic Mechanism in a Clinical Isolate of *Yersinia enterocolitica* KU14. Journal of Microbiology. **44**: 98-105.

Sawyer, C. N., McCarty, P.L., and Parkin, G.F. (2003). Chemistry for Environmental Engineering and Science, 5th edition, Tata McGraw-Hill, pp. 625-630.

Sayada, C., Picard, B., Elion, J. and Krishnamoorthy, R. (1994). Genomic fingerprinting of *Yersinia enterocolitica* species by degenerate oligonucleotide-primed polymerase chain reaction. **15**: 562-565.

Schleifstein, J.I. and Coleman, M.B. (1939). An unidentified microorganism resembling *B. lignieri* and Past. *pseudotuberculosis*, and pathogenic for man. New York State Journal of Medicine. **34**: 1749–1753.

Schmidt, A. S., Bruun, M.S., Larsen, J.L. and Dalsgaard, I.(2001). Characterization of class 1 integrons associated with R-plasmidsin clinical *Aeromonas salmonicida* isolates from various geographical areas. Journal of Antimicrobial Chemotherapy. **47**:735–743.

Schreiner, B. and van Zyl, F., (2006). Water Services: Yesterday, Today and Tomorrow. A Strategic Perspective. Presented at The WISA Biennial Conference and Exhibition. Durban, South Africa.

Seitz P., Blokesch M. (2014). DNA transport across the outer and inner membranes of naturally transformable *Vibrio cholerae* is spatially but not temporally coupled. mBio **5**:e01409-14.

Severino, P. and Magalha˜es, V.D. (2002). The role of integrons in the dissemination of antibiotic resistance among clinical isolates of *Pseudomonas aeruginosa* from an intensive care unit in Brazil. Research in Microbiology. **153**: 221–226.

Sharifi Yazdi M. K., Soltan-Dallal M. M., Zali M. R., Avadisians S. and Bakhtiari R. (2011). Incidence and antibiotic susceptibilities of *Yersinia enterocolitica* and other *Yersinia* species recovered from meat and chicken in Tehran, Iran. African Journal of Microbiology Research. **5**: (18), pp. 2649-2653.

Silvia, D., Ana, M., Juan, C.G., (2006). Evaluation of heavy metal acute toxicity and bioaccumulation in soil ciliated protozoa. Environment International.**32** (6): 711-717.

Simonova, J., Borilova, G. and Steinhauserova, I. (2008). Occurrence of pathogenic strains of *Yersinia enterocolitica* in pigs and their antimicrobial resistance. Bull. Vet Tech Institute. **52**: 39-43.

Simpson, E., and Charles, K. (2000). The health threat posed to surrounding community by effluent discharged from rural hospital sewage treatment plants. Proceedings of Water Institute of South Africa Conference, Sun City South Africa.

Sincero, A. P., and Sincero, G. A. (1996). Environmental Engineering: A Design Approach, 4th ed. Prentice-Hall of India Pvt. Ltd., New Delhi, India.

Skurnik, M. and Toivanen, P. (1993). *LcrF* is the temperature-regulated activator of the *yadA* gene of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. Journal of Bacteriology. 174, 2047–2051.

Söderqvist, K., Boqvist, S., Wauters, G., Vågsholm, I., and Lambertz, S. (2011). *Yersinia enterocolitica* in sheep - a high frequency of biotype 1A**.** Acta Veterinaria Scandinavica. **54**:39..

Stenkova, A.M., Isaeva, M.P. and Rasskazov, V.A. (2008). Development of a Multiplex PCR Procedure for Detection of Yersinia Genus with Identification of Pathogenic Species (*Y. pestis, Y. pseudotuberculosis*, and *Y. enterocolitica*). Pacific Institute of Bioorganic Chemistry, Far-East Division, Russian Academy of Medical Sciences. **23** (3): 119-125

Storhaug, R. (1990). Performance stability of small biological chemical treatment plants. Water Science and Technology. **22**: 275-282.

Sulakvelidze, A. (2000).Yersinia other than *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis*: the ignored species. Microbes and Infection **2**: 497-513.

Suma, G.M., Bezuidenhout, C., Mbewe, M. and Ateba, C.N. (2014). Isolation of Environmental Bacteria from Surface and Drinking Water in Mafikeng, South Africa, and Characterization Using Their Antibiotic Resistance Profiles. Journal of Pathogens. Article ID 371208, 11 pages.

Tardy, F., Homble, F., Neyt, C., Wattiez, R., Cornelis, G., Ruysschaert, J., and Cabiaux, V., 1999- *Yersinia enterocolitica* Type III Secretion translocation System: Channel Formation by Secreted Yops. EMBO Journal. **18**: 6793-6799.

Tchobanoglous, G. (1979). Wastewater Engineering: Treatment Disposal Reuse, 2nd edn. McGraw Hill, Boston, pp. 56-141 & 829-864.

Tennant, S.M., Grant, T.H. and Robins-Browne, R.M. (2003). Pathogenicity of *Yersinia enterocolitica* biotype 1A.FEMS Immunology and Medical Microbiology. **38**: 127-137.

Thoerner, P., Bin Kingombe, C.I., Bogli-Stuber, K., Bissig-Choisat, B., Wassenaar, T.M., Frey, J., and Jemmi, T. (2003). PCR detection of virulence genes in *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* and investigation of virulence gene distribution. Applied Environmental Microbiology. **69**: 1810-1816.

Thoerner, P., BinKingombe, C.I., Bo¨gli-Stuber, K., Bissig-Choisat, B., Wassenaar, T. M., Frey J. and Jemmi T. (2002). PCR Detection of Virulence Genes in *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* and Investigation of Virulence Gene Distribution .Applied and Environmental Microbiology. **69** (3): 1810–1816.

Thomas, C. M. (2000a). Paradigms of plasmid organization. Molecular Microbiology **37**:485-491.

Touron, A., Berthe, T., Pawlak, B. and Petit, F. (2005). Detection of Salmonella in environmental water and sediment by a nested-multiplex polymerase chain reaction assay. Research in Microbiology **156** (4):541-553.

Turpie, J. and Visser, M. (2013). The impact of climate change on South Africa's rural areas. Environmental-Economics Policy Research Unit, University of Cape Town.

Turton, A. (2008). Three Strategic Water Quality Challenges that Decision-Makers Need to Know. Science Real and Relevant, 2008. CSIR Report No. CSIR/NRE/WR/EXP/2008/0160/A.

Tzelepi, E., Giakkoupi, P., Sofianou, D., Loukova, V., Kemeroglou, A. and Tsakris, A. (1999). Detection of extended-spectrum beta-lactamases in clinical isolates of *Enterobacter cloacae* and *Enterobacter aerogenes*. Journal of Clinical Microbiology. **38**: 542-546.

Ukhahlamba District Municipality Addendum. (2008). Infant deaths: Barkly East. 17 April 2008. Eastern Cape: Preliminary Epidemological Investigation Report.

Van Loghem, J.J. (1944). The classification of the plague bacillus. Antonie van Leeuwenhoek. **10**: 15-16.

Van Loon, H.J., Box, A.T.A., Verhoef, J. and Fluit, A.C. (2004) Evaluation of genetic determinants involved in β-lactam- and multi resistance in a surgical ICU. International Journal of Antimicrobial Agents. **24**:130-134.

Venecia, K., and Young, G. (2005). Environmental Regulation and Virulence Attributes of the Ysa Type III Secretion System of *Yersinia enterocolitica* Biovar 1B. Infection and Immunity. **73**: 5961-5977.

Vettoretti, L., Floret, N., Hocquet, D., Dehecq, B. and Plésiat, P. (2009). Emergence of extensive drug-resistant *Pseudomonas aeruginosa* in a French university hospital. European Journal of Clinical Microbiology. **28**:1217-1222.

Visscher, J.T. (1990). Slow sand filtration: Design, operation and maintenance. Journal of American Water Works Association. **83**: 67-71.

Wagner, M. and Loy, A. (2002). Bacterial community composition and function in sewage treatment systems. Current opinion in biotechnology. **13**: 218-227.

Wannet, W.J., Reessink, M., Brunings, H.A .and Maas, H.M. (1999). Detection of pathogenic *Yersinia enterocolitica* by a rapid and sensitive duplex PCR assay. Journal of Clinical Microbiology. **39** (12): 4483-6.

Wauters, G., Janssens, M., Steigerwalt, A. and Brenner, D. (1988). *Yersinia mollaretii* sp. and *Yersinia bercovieri* sp. formerly called *Yersinia enterocolitica* biogroups 3A and 3B. International Journal of Systematic Bacteriology. **38**:424-429.

Welsh, J. and McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers.Nucleic Acids Research. **18**: 7213-7218.

White, P.A., and Rawlinson, W.D. (2001). Current status of the *aadA* and *dfr* gene cassette families. FEMS Microbiology Letter. **182**:265-269.

WHO. (2002).Guidelines for Drinking Water Quality- 2nd ed. World Health Organization, Geneva.

WHO. (2009). Diarrhoea: Why children are still dying and what can be done. ISBN 978-92-806-4462-3 (UNICEF).

Yates, M.V. (2013). Pathogens in reclaimed water. Available at: http://www.geoflow.com/wastewater/pathogens.html

Yong, D., Lee, K., Yum, J.H., Shin, H.D., Rossolini, G.M. and Chong, Y. (2002). Impinem-EDTA disk method for differentiation of metallo-β-lactamase producing clinical isolates of *Pseudomonas* spp. and *Acinetobacter* spp. Journal Clinical Microbiology. **40**: 3798-3801.

Zulkifli, Y., Alitheen, N. B., Raha, A. R., Yeap, S. K., Marlina, S.R. and Nishibuchi, M. (2009). Antibiotic resistance and plasmid profiling of *Vibrio parahaemolyticus* isolated from cockles in Padang, Indonesia. International Journal of Food Research **16**: 53-5.

APPENDIX A

Table 1: Triplicate analysis of COD and Temperature values for NGTW as detailed in Table 2.2 .

Weeks	Sampling points	COD 1	COD 2	COD 3	AVERAGE	STDEV	Temp 1	Temp 2	Temp 3	AVERAGE	STDEV
	UP	269	274	264	269.0	4.1	23	23	23	23	0
	BC	74	125	83	94.0	22.2	22	22	22	22	0
1	AC	308	309	310	309.0	0.8	21	21	21	21	0
	DS	257	307	312	292.0	24.8	22	22	22	22	0
	UP	38	42	31	37	4.5	18	18	18	18	0
	BC	75	61	80	72	8.0	23	23	23	23	0
2	AC	85	76	117	92.7	17.6	22	22	22	22	0
	DS	71	82	74	75.7	4.6	19	19	19	19	0
	UP	226	229	213	222.7	6.9	17	17	17	17	0
	BC	194	164	198	185.3	15.2	22	22	22	22	0
3	AC	105	94	103	100.7	4.8	22	22	22	22	0
	DS	145	244	172	187.0	41.8	18	18	18	18	0
	UP	214	279	194	229.0	36.3	15	15	15	15	0
	BC	165	214	184	187.7	20.2	20	20	20	20	0
4	AC	148	138	171	152.3	13.8	20	20	20	20	0
	DS	57	65	95	72.3	16.4	17	17	17	17	0
	UP	158	164	161	161.0	2.4	16	16	16	16	0
5	BC	226	216	206	216.0	8.2	20	20	20	20	0
	AC	172	168	170	170.0	1.6	20	20	20	20	0
	DS	292	282	287	287.0	4.1	15	15	15	15	0
	UP	303	303	304	303.3	0.5	21	21	21	21	0
6	BC	297	297	297	297.0	0.0	20	20	20	20	0
	AC	300	301	301	300.7	0.5	21	21	21	21	0
	DS	185	186	184	185.0	0.8	22	22	22	22	0

Table 2: Triplicate analysis of pH and TDS values for NGTW as detailed in Table 2.2.

Weeks	Sampling points	pH 1	pH 2	pH 3	AVERAGE	STDEV	TDS 1	TDS 2	TDS 3	AVERAGE	STDEV
	UP	8.69	8.76	8.82	8.8	0.1	429	430	430	429.7	0.5
	BC	7.22	7.16	7.18	7.2	0.0	541	541	540	540.7	0.5
1	**AC**	8.4	8.2	8.06	8.2	0.1	532	535	535	534.0	1.4
	DS	7.36	7.41	7.33	7.4	0.0	561	552	548	553.7	5.4
	UP	8.14	8.76	8.51	8.47	0.25	214.2	214.1	214	214.1	0.1
	BC	7.43	7.53	7.25	7.403	0.12	660	659	660	659.7	0.5
2	**AC**	7.69	7.56	7.43	7.56	0.11	477	476	476	476.3	0.5
	DS	8.15	8.39	8.43	8.32	0.12	375	375	375	375.0	0.0
	UP	8.03	8.03	8.03	8.0	0.000	197.2	197.5	197.5	197.4	0.14
	BC	8.09	8.09	8.09	8.09	0.000	772	773	772	772.3	0.47
3	**AC**	7.28	7.28	7.28	7.28	0.000	581	583	584	582.7	1.25
	DS	7.69	7.69	7.69	7.7	0.000	399	402	402	401	1.41
	UP	7.42	7.42	7.42	7.4	0.00	189.2	189.9	189.8	189.6	0.31
	BC	6.76	6.76	6.77	6.8	0.00	590	591	590	590.3	0.47
4	**AC**	6.77	6.8	6.75	6.8	0.02	577	577	576	576.7	0.47
	DS	7.13	7.13	7.13	7.1	0.00	433	432	432	432.3	0.47
	UP	6.66	6.65	6.67	6.7	0.01	283	283	282	282.7	0.47
5	**BC**	6.8	6.81	6.82	7.0	0.09	550	549	553	550.7	1.70
	AC	6.99	7.01	6.99	7.0	0.01	380	379	381	380.0	0.82
	DS	7.05	7.04	7.06	7.1	0.01	343	344	343	343.3	0.47
	UP	7.56	7.55	7.54	7.6	0.01	183.3	183.5	183.7	183.5	0.16
6	**BC**	7.35	7.32	7.31	7.0	0.17	619	619	620	619.3	0.47
	AC	7.48	7.38	7.35	7.4	0.06	480	479	482	480.3	1.25
	DS	7.5	7.46	7.41	7.5	0.04	465	466	466	465.7	0.47

Table 3: Triplicate turbidity values for NGTW as detailed in Table 2.2.

Weeks	Sampling points	Turbidity 1	Turbidity 2	Turbidity 3	AVERAGE	STDEV
	UP	13.15	13.14	13.16	13.2	0.0
	BC	21.1	21.2	21.2	21.2	0.0
1	**AC**	14.9	14.8	14.2	14.6	0.3
	DS	13.5	13.3	13.4	13.4	0.1
	UP	1.99	2.1	2.06	2.05	0.05
	BC	262	264	263	263	0.82
2	**AC**	1.25	1.26	1.24	1.25	0.01
	DS	39.1	35.7	36.1	37.0	1.52
	UP	3.29	3.3	3.28	3.29	0.01
	BC	284	282	276	280.7	3.40
3	**AC**	10.3	10.2	10.3	10.3	0.05
	DS	95.6	96	92.4	94.7	1.61
	UP	5.01	5.03	4.97	5.0	0.02
	BC	9.99	9.99	9.99	10.0	0.00
4	**AC**	34.3	34.5	34.7	34.5	0.16
	DS	55.3	52.9	51.4	53.2	1.61
	UP	21.2	22	21	21.4	0.43
	BC	19.6	19.6	19.6	19.6	0.00
5	**AC**	8.06	7.97	7.97	8.0	0.04
	DS	22	22.5	21.9	22.1	0.26
	UP	8.53	8.51	8.63	8.6	0.05
	BC	268	269	269	268.7	0.47
6	**AC**	94	91	95	93.3	1.70
	DS	452	451	453	452.0	0.82

Table 4: BOD values of the final treated effluent for NGTW as detailed in Table 2.2.

		BOD DAY 0					BOD DAY 5												
Week	**Sample dilution**	**1**	**2**	**3**	**AVG**	**SD**	**1**	**2**	**3**	**AVG**	**SD**	**p value**	**bod 1**	**bod 2**	**bod 3**	**avg**	**sd**	**FINAL**	**sd**
	UP 60	7.53	7.54	7.53	7.53	0.01	5.29	5.3	5	5.28	0.02	0.20	11.20	11.40	11.25	11.28	0.10	**7.31**	**0.16**
	UP 100	7.51	7.43	7.41	7.45	0.05	5.45	5.4	5	5.40	0.04	0.33	6.24	6.18	6.18	6.20	0.03		
	UP 150	7.81	7.8	7.83	7.81	0.02	5.68	5.7	5	5.59	0.15	0.50	4.26	4.24	4.82	4.44	0.33		
	BC 6	7.6	7.59	7.56	7.58	0.02	5.89	5.9	6	5.85	0.04	0.02	85.50	87.00	87.00	86.50	0.87	**91.39**	**1.61**
	BC 8	7.51	7.48	7.44	7.48	0.04	5.39	5.4	5	5.38	0.01	0.02	106.00	105.50	103.50	105.00	1.32		
	BC 10	7.46	7.44	7.4	7.43	0.03	4.92	4.9	5	4.95	0.05	0.03	84.67	83.67	79.67	82.67	2.65		
1	**AC 15**	7.29	7.31	7.31	7.30	0.01	6.27	6.2	6	6.24	0.03	0.05	20.40	22.00	21.40	21.27	0.81	**16.61**	**1.11**
	AC 20	7.53	7.52	7.5	7.52	0.02	6.05	6.2	6	6.15	0.09	0.06	24.67	22.17	21.33	22.72	1.73		
	AC 30	7.52	7.51	7.5	7.51	0.01	6.96	7	7	6.93	0.09	0.10	5.60	5.20	6.70	5.83	0.78		
	DS 60	6.34	6.35	6.32	6.34	0.02	5.69	5.7	6	5.68	0.01	0.20	3.25	3.40	3.25	3.30	0.09	**26.88**	**1.50**
	DS 100	5.62	5.61	5.59	5.61	0.02		4.5	5	4.51	0.02	0.03	36.67	37.33	36.00	36.67	0.67		
	DS 150	5.19	5.09	5.07	5.12	0.06	4.29	4.3	4	4.30	0.02	0.02	45.00	38.50	38.50	40.67	3.75		
	CONTROL	7.69	7.70	7.69	**7.69**	**0.01**	6.55	6.5	6	**6.51**	**0.04**	1.00	1.14	1.19	1.21	**1.18**	**0.04**		

Table 4 continued….

			BOD DAY 0				BOD DAY 5												
Week	**Sample dilution**	**1**	**2**	**3**	**AVG**	**SD**	**1**	**2**	**3**	**AVG**	**SD**	**p value**	**bod 1**	**bod 2**	**bod 3**	**avg**	**sd**	**FINAL**	**sd**
	UP 60	6.74	6.47	6.43	6.55	0.17	1.85	1.7	2	1.73	0.12	0.20	24.45	23.75	24.05	24.08	0.35	**13.74**	**0.44**
	UP 100	4.54	4.12	4.19	4.28	0.23	1.5	1.4	1	1.44	0.06	0.33	9.21	8.12	8.52	8.62	0.55		
	UP 150	5.72	5.33	5.29	5.45	0.24	1.23	1.2	1	1.19	0.04	0.50	8.98	8.28	8.28	8.51	0.40		
	BC 6	7.77	7.82	7.7	7.76	0.06	1.56	1.5	2	1.54	0.02	0.02	310.50	314.00	309.00	311.17	2.57	**282.96**	**2.51**
	BC 8	7.76	7.73	7.61	7.70	0.08	1.31	1.3	1	1.27	0.04	0.02	322.50	323.00	319.00	321.50	2.18		
2	**BC 10**	7.56	7.64	7.63	7.61	0.04	1.17	1.1	1	1.12	0.04	0.03	213.00	217.67	218.00	216.22	2.80		
	AC 15	7.85	7.8	7.81	7.82	0.03	1.1	1.1	1	1.07	0.04	0.05	135.00	134.40	135.80	135.07	0.70	**104.30**	**0.50**
	AC 20	7.89	7.85	7.78	7.84	0.06	1.4	1.4	1	1.38	0.03	0.06	108.17	107.83	107.17	107.72	0.51		
	AC 30	7.84	7.89	7.83	7.85	0.03	0.86	0.9	1	0.84	0.02	0.10	69.80	70.40	70.10	70.10	0.30		
	DS 60	7.9	7.16	7.15	7.40	0.43	1.61	1.6	2	1.59	0.02	0.20	31.45	27.90	27.80	29.05	2.08	**161.24**	**5.76**
	DS 100	7.86	7.17	7.04	7.36	0.44	1.22	1.2	1	1.19	0.03	0.03	221.33	199.67	196.00	205.67	13.69		
	DS 150	6.46	6.45	6.36	6.42	0.06	1.48	1.4	1	1.44	0.04	0.02	249.00	250.50	247.50	249.00	1.50		
	CONTROL	8.34	8.29	8.12	**8.25**	**0.12**	7.89	7.9	8	7.94	0.06	1.00	0.45	0.37	0.12	**0.31**	**0.17**		

Table 4 continued….

			BOD DAY 0				BOD DAY 7												
Week	**Sample dilution**	**1**	**2**	**3**	**AVG**	**SD**	**1**	**2**	**3**	**AVG**	**SD**	**p value**	**bod 1**	**bod 2**	**bod 3**	**avg**	**sd**	**FINAL**	**sd**
	UP 60	8.2	8.15	8.18	8.18	0.03	4.19	4.2	4	4.18	0.02	0.20	20.05	19.95	20.00	20.00	0.05	**13.38**	**0.05**
	UP 100	8.18	8.17	8.16	8.17	0.01	4.36	4.3	4	4.32	0.03	0.33	11.58	11.73	11.67	11.66	0.08		
	UP 150	8.31	8.29	8.25	8.28	0.03	4.07	4	4	4.05	0.02	0.50	8.48	8.50	8.44	8.47	0.03		
	BC 6	7.94	7.88	7.9	7.91	0.03	4.48	4.5	4	4.45	0.03	0.02	173.00	171.50	174.00	172.83	1.26	**159.31**	**1.34**
	BC 8	7.81	7.85	7.83	7.83	0.02	4.33	4.3	4	4.30	0.04	0.02	174.00	177.50	178.50	176.67	2.36		
3	**BC 10**	7.84	7.87	7.85	7.85	0.02	3.98	4	4	4.00	0.03	0.03	128.67	128.00	128.67	128.44	0.38		
	AC 15	7.98	7.95	7.97	7.97	0.02	6.72	6.7	7	6.71	0.01	0.05	25.20	24.80	25.40	25.13	0.31	**26.40**	**0.35**
	AC 20	7.96	8	8.01	7.99	0.03	6.04	6	6	6.03	0.01	0.06	32.00	33.00	33.17	32.72	0.63		
	AC 30	8	7.99	7.95	7.98	0.03	5.86	5.9	6	5.85	0.02	0.10	21.40	21.40	21.20	21.33	0.12		
	DS 60	7.08	7.11	7.12	7.10	0.02	4.15	4.1	4	4.13	0.02	0.20	14.65	14.95	15.05	14.88	0.21	**59.79**	**9.36**
	DS 100	6.69	6.91	6.81	6.80	0.11	4.99	4	5	4.66	0.56	0.03	56.67	96.33	61.00	71.33	21.76		
	DS 150	5.98	6	5.81	5.93	0.10	4.09	4	4	4.07	0.03	0.02	94.50	98.50	86.50	93.17	6.11		
	CONTROL	7.95	7.89	7.87	**7.90**	**0.04**	7.28	7.3	7	7.29	0.01	1.00	0.67	0.60	0.57	**0.61**	**0.05**		

Table 4 continued….

		BOD DAY 0					BOD DAY 5												
Week	**Sample dilution**	**1**	**2**	**3**	**AVG**	**SD**	**1**	**2**	**3**	**AVG**	**SD**	**p value**	**bod 1**	**bod 2**	**bod 3**	**avg**	**sd**	**FINAL**	**sd**
	UP 60	8.54	8.53	8.54	8.54	0.01	5.86	4.8	5	5.16	0.60	0.20	13.40	18.45	18.75	16.87	3.01	**11.27**	**1.20**
	UP 100	8.57	8.58	8.57	8.57	0.01	4.98	5	5	4.93	0.08	0.33	10.88	10.94	11.33	11.05	0.25		
	UP 150	8.63	8.64	8.63	8.63	0.01	5.88	5.6	6	5.69	0.17	0.50	5.50	6.02	6.16	5.89	0.35		
	BC 6	8.47	8.46	8.47	8.47	0.01	5.96	5.3	6	5.68	0.35	0.02	125.50	158.50	134.50	139.50	17.06	**123.28**	**12.11**
	BC 8	8.34	8.35	8.33	8.34	0.01	5.89	5.4	5	5.53	0.32	0.02	122.50	149.00	150.50	140.67	15.75		
	BC 10	8.29	8.28	8.29	8.29	0.01	5.49	5.7	6	5.60	0.10	0.03	93.33	86.33	89.33	89.67	3.51		
4	**AC 15**	8.4	8.41	8.4	8.40	0.01	6.18	6.3	6	6.32	0.15	0.05	44.40	42.00	38.60	41.67	2.91	**33.62**	**2.40**
	AC 20	8.37	8.38	8.38	8.38	0.01	6	6.1	6	6.01	0.11	0.06	39.50	37.50	41.17	39.39	1.84		
	AC 30	8.42	8.43	8.41	8.42	0.01	6.69	6.2	6	6.44	0.24	0.10	17.30	22.20	19.90	19.80	2.45		
	DS 60	6.95	6.93	6.91	6.93	0.02	4.02	4.2	4	4.10	0.07	0.20	14.65	13.85	13.90	14.13	0.45	**79.60**	**1.37**
	DS 100	6.84	6.86	6.84	6.85	0.01	3.98	4.1	4	4.00	0.05	0.03	95.33	93.67	96.00	95.00	1.20		
	DS 150	6.91	6.87	6.84	6.87	0.04	4.26	4.3	4	4.28	0.02	0.02	132.50	128.50	128.00	129.67	2.47		
	CONTROL	8.66	8.67	8.60	**8.64**	**0.04**	6.69	6.8	7	**6.76**	**0.06**	1.00	1.97	1.88	1.79	**1.88**	**0.09**		

Table 4 continued….

		BOD DAY 0					BOD DAY 5												
Week	**Sample dilution**	**1**	**2**	**3**	**AVG**	**SD**	**1**	**2**	**3**	**AVG**	**SD**	**p value**	**bod 1**	**bod 2**	**bod 3**	**avg**	**sd**	**FINAL**	**sd**
	UP 60	7.63	7.63	7.63	7.63	0.00	6.41	6.3	6	6.39	0.06	0.20	6.10	6.50	5.95	6.18	0.28	**4.35**	**0.29**
	UP 100	7.45	7.43	7.41	7.43	0.02	6.45	6.5	7	6.51	0.07	0.33	3.03	2.82	2.52	2.79	0.26		
	UP 150	7.69	7.56	7.63	7.63	0.07	5.68	5.7	5	5.59	0.15	0.50	4.02	3.76	4.42	4.07	0.33		
	BC 6	8.09	8.16	8.15	8.13	0.04	6.2	5.8	6	6.06	0.23	0.02	94.50	118.00	98.50	103.67	12.57	**73.41**	**12.53**
	BC 8	8.38	8.36	8.34	8.36	0.02	6.44	6.6	7	6.57	0.12	0.02	97.00	87.50	83.50	89.33	6.93		
	BC 10	8.16	8.1	8.13	8.13	0.03	7.97	7	7	7.31	0.57	0.03	6.33	37.33	38.00	27.22	18.09		
5	**AC 15**	8.42	8.43	8.41	8.42	0.01	6.18	6.3	6	6.32	0.15	0.05	44.80	42.40	38.80	42.00	3.02	**31.46**	**1.89**
	AC 20	8.37	8.38	8.38	8.38	0.01	6.01	6	6	6.05	0.10	0.06	39.33	40.00	36.83	38.72	1.67		
	AC 30	8.4	8.41	8.4	8.40	0.01	7.01	7	7	7.04	0.09	0.10	13.90	14.50	12.60	13.67	0.97		
	DS 60	6.84	6.79	6.83	6.82	0.03	5.25	5.3	5	5.27	0.02	0.20	7.95	7.60	7.75	7.77	0.18	**43.22**	**1.63**
	DS 100	6.92	6.93	6.95	6.93	0.02	5.41	5.5	6	5.47	0.05	0.03	50.33	48.00	48.33	48.89	1.26		
	DS 150	6.73	6.71	6.73	6.72	0.01	5.19	5.3	5	5.26	0.06	0.02	77.00	71.00	71.00	73.00	3.46		
	CONTROL	8.81	8.92	8.89	**8.87**	**0.06**	7.61	7.7	8	**7.68**	**0.06**	1.00	1.20	1.23	1.16	**1.20**	**0.04**		

Table 4 continued….

		BOD DAY 0					BOD DAY 5												
Week	**Sample dilution**	**1**	**2**	**3**	**AVG**	**SD**	**1**	**2**	**3**	**AVG**	**SD**	**p value**	**bod 1**	**bod 2**	**bod 3**	**avg**	**sd**	**FINAL**	**sd**
	UP 60	7.53	7.54	7.53	7.53	0.01	5.29	5.3	5	5.28	0.02	0.20	11.20	11.40	11.25	11.28	0.10	**7.31**	**0.16**
	UP 100	7.51	7.43	7.41	7.45	0.05	5.45	5.4	5	5.40	0.04	0.33	6.24	6.18	6.18	6.20	0.03		
	UP 150	7.81	7.8	7.83	7.81	0.02	5.68	5.7	5	5.59	0.15	0.50	4.26	4.24	4.82	4.44	0.33		
	BC 6	7.6	7.59	7.56	7.58	0.02	5.89	5.9	6	5.85	0.04	0.02	85.50	87.00	87.00	86.50	0.87	**91.39**	**1.61**
	BC 8	7.51	7.48	7.44	7.48	0.04	5.39	5.4	5	5.38	0.01	0.02	106.00	105.50	103.50	105.00	1.32		
	BC 10	7.46	7.44	7.4	7.43	0.03	4.92	4.9	5	4.95	0.05	0.03	84.67	83.67	79.67	82.67	2.65		
6	**AC 15**	7.29	7.31	7.31	7.30	0.01	6.27	6.2	6	6.24	0.03	0.05	20.40	22.00	21.40	21.27	0.81	**16.61**	**1.11**
	AC 20	7.53	7.52	7.5	7.52	0.02	6.05	6.2	6	6.15	0.09	0.06	24.67	22.17	21.33	22.72	1.73		
	AC 30	7.52	7.51	7.5	7.51	0.01	6.96	7	7	6.93	0.09	0.10	5.60	5.20	6.70	5.83	0.78		
	DS 60	6.34	6.35	6.32	6.34	0.02	5.69	5.7	6	5.68	0.01	0.20	3.25	3.40	3.25	3.30	0.09	**26.88**	**1.50**
	DS 100	5.62	5.61	5.59	5.61	0.02	4.52	4.5	5	4.51	0.02	0.03	36.67	37.33	36.00	36.67	0.67		
	DS 150	5.19	5.09	5.07	5.12	0.06	4.29	4.3	4	4.30	0.02	0.02	45.00	38.50	38.50	40.67	3.75		
	CONTROL	7.69	7.70	7.69	**7.69**	**0.01**	6.55	6.5	6	**6.51**	**0.04**	1.00	1.14	1.19	1.21	**1.18**	**0.04**		

Table 5: Enumeration of *Yersinia* species recovered from the treated effluent at NGTW over the sampling period as per figure 2.1.

			WK 1										WK 2					
Dilution	**UP**	**AVG**	**BC**	**AVG**	**AC**	**AVG**	**DS**	**AVG**			**UP**	**AVG**	**BC**	**AVG**	**AC**	**AVG**	**DS**	**AVG**
10^{-1}	TNTC		TNTC		TNTC		TNTC			10^{-1}	TNTC		TNTC		TNTC		TNTC	
cfu/ml	0		0		0		0			cfu/ml	0		0		0		0	
10^{-1}	TNTC		TNTC		TNTC		TNTC			10^{-1}	TNTC		TNTC		TNTC		TNTC	
cfu/ml	0	**0**	0	**0**	0	0	0	**0**		cfu/ml	0	**0**	0	**0**	0	**0**	0	**0**
10^{-2}	196		TNTC		93		TNTC			10^{-2}	215		TNTC		119		TNTC	
cfu/ml	2 x10^{5}		0		9 X10^{4}		0			cfu/ml	2 X10^{5}		0		1 X10^{5}		0	
10^{-2}	187		TNTC		82		TNTC			10^{-2}	204		TNTC		132		TNTC	
cfu/ml	2 x10^{5}	**2 x10^{5}**	0	**0**	8 X10^{4}	0	0	**0**		cfu/ml	2 X10^{5}	**2 X10^{5}**	0	**0**	1 X10^{5}	**1 X10^{5}**	0	**0**
10^{-3}	31		248		16		261			10^{-3}	32		161		11		169	
cfu/ml	3 x10^{5}		2 x10^{6}		2 x10^{5}		3 x10^{6}			cfu/ml	3 X10^{5}		2 X10^{6}		1 X10^{5}		2 X10^{6}	
10^{-3}	17		219		9		242			10^{-3}	19		142		16		172	
cfu/ml	5 x10^{5}	**4 x10^{5}**	2x10^{6}	**3 x10^{7}**	9 X10^{5}	**6 X10^{5}**	2 x10^{6}	**4 X10^{5}**		cfu/ml	2 X10^{5}	**3 X10^{5}**	1 X10^{6}	**2 X10^{6}**	2 X10^{5}	**2 X10^{5}**	2 X10^{6}	**2 X10^{6}**
10^{-4}	9		42		0		39			10^{-4}	7		18		0		18	
cfu/ml	9 x10^{5}		4 x10^{6}		0		4 x10^{6}			cfu/ml	7 X10^{5}		2 X10^{6}		0		2 X10^{6}	
10^{-4}	1		59		0		21			10^{-4}	1		9		0		12	
cfu/ml	1x10^{5}	**9 x10^{5}**	6 x10^{6}	**5 x10^{6}**	0	**0**	2 x10^{6}	**2 x10^{6}**		cfu/ml	1 X10^{5}	**4 x10^{5}**	9 X10^{6}	**6 X10^{6}**	0	**0**	1 X10^{6}	**2 X10^{6}**
10^{-5}	0		18		0		5			10^{-5}	0		2		0		1	
cfu/ml	0		2 x10^{7}		0		5 x10^{6}			cfu/ml	0		5 X10^{6}		0		1 X10^{6}	
10^{-5}	0		11		0		1			10^{-5}	0		0		0		0	
cfu/ml	0	**0**	1 x10^{7}	**2 x10^{7}**	0	**0**	1 x10^{6}	**3 x10^{6}**		cfu/ml	0	**0**	0	**5 X10^{6}**	0	**0**	0	**1 X10^{6}**

Table 5 continued….

			WK 3										WK 4					
Dilution	**UP**	**AVG**	**BC**	**AVG**	**AC**	**AVG**	**DS**	**AVG**		**Dilution**	**UP**	**AVG**	**BC**	**AVG**	**AC**	**AVG**	**DS**	**AVG**
10^{-1}	TNTC		TNTC		TNTC		TNTC			10^{-1}	TNTC		TNTC		TNTC		TNTC	
cfu/ml	0		0		0		0			cfu/ml	0		0		0		0	
10^{-1}	TNTC		TNTC		TNTC		TNTC			10^{-1}	TNTC		TNTC		TNTC		TNTC	
cfu/ml	0	**0**	0	**0**	0	**0**	0	**0**		cfu/ml	0	**0**	0	**0**	0	**0**	0	**0**
10^{-2}	TNTC		TNTC		163		TNTC			10^{-2}	TNTC		TNTC		193		TNTC	
cfu/ml	0		0		2 x10^5		0			cfu/ml	0		0		2 x10^5		0	
10^{-2}	TNTC		TNTC		141		TNTC			10^{-2}	TNTC		TNTC		171		TNTC	
cfu/ml	0	**0**	0	**0**	1 x10^5	**2 x10^5**	0	**0**		cfu/ml	0	**0**	0	**0**	2 x10^5	**2 x10^5**	0	**0**
10^{-3}	243		187		21		361			10^{-3}	218		167		21		216	
cfu/ml	2 x10^6		2 x10^6		2 x10^5		4 x10^6			cfu/ml	2 x10^5		2 x10^6		2 x10^5		2 x10^6	
10^{-3}	214		165		18		328			10^{-3}	214		152		19		208	
cfu/ml	2 x10^6	**2 x10^6**	2 x10^6	**2 x10^6**	2 x10^5	**2 x10^5**	3 x10^6	**4 x10^6**		cfu/ml	2 x10^5	**2 x10^5**	2 x10^6	**2 x10^6**	2 x10^5	**2 x10^5**	2 x10^6	**2 x10^6**
10^{-4}	31		18		4		59			10^{-4}	29		14		1		24	
cfu/ml	3 x10^6		2 x10^6		4 x10^5		6x10^6			cfu/ml	3 x10^6		1 x10^5		1 x10^5		2 x10^6	
10^{-4}	27		15		0		47			10^{-4}	18		9		1		19	
cfu/ml	3 x10^6	**3 x10^6**	2 x10^6	**2 x10^6**	0	**4 X10^{75}**	5x10^6	**6x10^6**		cfu/ml	2x10^6	**3 x10^6**	9 x10^5	**5 x10^5**	1 x10^5	**1 x10^5**	2 x10^6	**2 x10^6**
10^{-5}	4		1		0		11			10^{-5}	3		1		0		3	
cfu/ml	4 X10^6		1 X10^6		0		1 X10^6			cfu/ml	3 x10^6		1 x10^6		0		3 x10^6	
10^{-5}	1		1		0		4			10^{-5}	4		0		0		1	
cfu/ml	1 X10^6	**3 X10^6**	1 X10^6	**1 X10^6**	0	**0**	4 X10^6	**3 X10^6**		cfu/ml	4 X10^6	**4 X10^6**	0	**0**	0	**0**	1 x10^6	**2 x10^6**

Table 5 continued….

			WEEK 5										WEEK 6					
Dilution	**UP**	**AVG**	**BC**	**AVG**	**AC**	**AVG**	**DS**	**AVG**		**Dilution**	**UP**	**AVG**	**BC**	**AVG**	**AC**	**AVG**	**DS**	**AVG**
10^{-1}	TNTC		TNTC		TNTC		TNTC			10^{-1}	TNTC		TNTC		TNTC		TNTC	
cfu/ml	0		0		0		0			cfu/ml	0		0		0		0	
10^{-1}	TNTC		TNTC		TNTC		TNTC			10^{-1}	TNTC		TNTC		TNTC		TNTC	
cfu/ml	0	**0**	0	**0**	0	**0**	0	**0**		cfu/ml	0	**0**	0	**0**	0	**0**	0	**0**
10^{-2}	TNTC		TNTC		217		TNTC			10^{-2}	TNTC		TNTC		49		TNTC	
cfu/ml	0		0		2.2 x10^5		0			cfu/ml	0		0		1.5 x10^3		0	
10^{-2}	TNTC		TNTC		189		TNTC			10^{-2}	TNTC		TNTC		40		TNTC	
cfu/ml	0	**0**	0	**0**	1.9 x10^5	**2 x10^5**	0	**0**		cfu/ml	0	**0**	0	**0**	1.9 x10^5	**2 x10^5**	0	**0**
10^{-3}	TNTC		243		44		158			10^{-3}	186		288		11		181	
cfu/ml	0		2.4 x10^6		4.4 x10^5		1.6 x10^6			cfu/ml	1.8x10^7		2.8x10^6		1.1 x10^5		1.8 x10^6	
10^{-3}	TNTC		316		21		139			10^{-3}	141		263		10		143	
cfu/ml	0	**0**	3.1 x10^6	**2.8 x10^6**	2.1 x10^5	**3.3 x10^5**	2 x10^6	**1.8 x10^6**		cfu/ml	1.4x10^7	**1.6 x10^7**	2.6x10^6	**2.7x10^6**	1.0x10^5	**1.1x10^5**	1.4 x10^6	**1.6 x10^6**
10^{-4}	187		49		5		47			10^{-4}	76		50		2		32	
cfu/ml	1.9 x10^7		5x10^6		5x10^5		4.7x10^6			cfu/ml	7.6x10^7		5 x10^6		2x10^5		3.2 x10^6	
10^{-4}	193		78		6		26			10^{-4}	61		48		0		41	
cfu/ml	1.9x10^7	**1.9 x10^7**	7.8 x10^6	**6.4 x10^6**	6 x10^5	**5.5 x10^5**	2.6 x10^6	**3.6 x10^6**		cfu/ml	6.1 X10^7	**6.8 X10^7**	4.8 x10^6	**4.9 x10^6**	0	**2 x10^5**	4.1 x10^6	**3.6 x10^6**
10^{-5}	24		7		0		4			10^{-5}	9		5		0		3	
cfu/ml	2.4 x10^7		7 x10^6		0		4 x10^6			cfu/ml	9 x10^6		5 x10^6		0		3x10^6	
10^{-5}	18		12		0		1			10^{-5}	7		4		0		8	
cfu/ml	1.8 X10^7	**2 X10^7**	1.2 x10^6	**4.1 x10^6**	0	**0**	1 x10^6	**2.5 x10^6**		cfu/ml	7 X10^6	**8 X10^6**	4 x10^6	**4.5 x10^6**	0	**0**	8 x10^6	**5.5 x10^6**
10^{-6}	3		0		0		0			10^{-6}	2		0		0		0	
cfu/ml	3x10^7		0		0		0			cfu/ml	2 x10^7		0		0		0	
10^{-6}	1		1		0		0			10^{-6}	2		1		0		1	
cfu/ml	1 X10^7	**2 X10^7**	0	**1 X10^7**	0	**0**	0	**0**		cfu/ml	2 X10^7	**2 X10^7**	0	**1 X10^7**	0	**0**	0	**1 X10^7**

Table 6: Triplicate analysis COD and Temperature values for NWWTP as detailed in Table 2.3.

Weeks	Sampling points	COD 1	COD 2	COD 3	AVERAGE	STDEV	Temp 1	Temp 2	Temp 3	AVERAGE	STDEV
	UP	151	151	155	152.3	1.9	22	22	22	22	0
	BC	56	58	61	58.3	2.1	22	22	22	22	0
1	AC	290	301	286	292.3	6.3	21	21	21	21	0
	DS	134	129	153	138.7	10.3	22	22	22	22	0
	UP	239	253	272	254.7	13.52	19	19	19	19	0
	BC	31	56	71	52.7	16.50	19	19	19	19	0
2	AC	131	75	82	96	24.91	20	20	20	20	0
	DS	36	61	72	56.3	15.06	19	19	19	19	0
	UP	44	71	87	67.3	17.7	19	19	19	19	0
	BC	176	179	172	175.7	2.9	19	19	19	19	0
3	AC	114	136	120	123.3	9.3	18	18	18	18	0
	DS	102	71	89	87.3	12.7	19	19	19	19	0
	UP	81	95	76	84.0	8.0	17	17	17	17	0
	BC	184	179	181	181.3	2.1	18	18	18	18	0
4	AC	99	76	81	85.3	9.9	17	17	17	17	0
	DS	57	65	108	76.7	22.4	17	17	17	17	0
	UP	164	154	159	159.0	4.1	21	21	21	21	0
	BC	271	263	267	267.0	3.3	21	21	21	21	0
5	AC	275	261	268	268.0	5.7	21	21	21	21	0
	DS	115	123	119	119.0	3.3	21	21	21	21	0
	UP	161	148	159	156.0	5.7	23	23	23	23	0
	BC	189	201	204	198.0	6.5	23	23	23	23	0
6	AC	149	151	158	152.7	3.9	22	22	22	22	0
	DS	199	199	187	195.0	5.7	24	24	24	24	0

Table 7: Triplicate analysis of pH and TDS values for NWWTP as detailed in Table 2.3.

Weeks	Sampling points	pH 1	pH 2	pH 3	AVERAGE	STDEV	TDS 1	TDS 2	TDS 3	AVERAGE	STDEV
1	**UP**	7.51	7.19	7.21	7.30	0.15	294	293	294	293.7	0.5
	BC	7.33	5.95	6.82	6.70	0.57	344	343	345	344.0	0.8
	AC	7.25	8.26	7.61	7.71	0.42	344	345	345	344.7	0.5
	DS	7.17	7.75	7.15	7.36	0.28	346	346	346	346.0	0.0
2	**UP**	9	9.1	8.9	9	0.082	294	295	294	294.3	0.5
	BC	8.9	8.9	8.9	8.9	0.000	368	369	370	369.0	0.8
	AC	8.3	8.2	8.1	8.2	0.082	375	374	374	374.3	0.5
	DS	7.6	7.6	7.8	7.7	0.094	2155	2156	2157	2156.0	0.8
3	**UP**	7.51	7.69	7.41	7.5	0.116	295	293	293	294	0.94
	BC	7.89	7.46	7.44	7.6	0.208	331	344	340	338	5.44
	AC	7.71	7.65	7.64	7.7	0.031	345	345	346	345	0.47
	DS	7.17	7.22	7.18	7.2	0.022	225	226	228	226	1.25
4	**UP**	7.15	7.15	7.15	7.2	0.00	289	293	295	292.3	2.49
	BC	7.1	7.1	7.1	7.1	0.00	353	353	355	353.7	0.94
	AC	7.15	7.15	7.15	7.2	0.00	355	356	356	355.7	0.47
	DS	7.38	7.38	7.38	7.4	0.00	1739	1742	1753	1744.7	6.02
5	**UP**	7.24	7.15	7.08	7.2	0.07	1136	1134	1131	1133.7	2.05
	BC	7.27	7.25	7.25	7.3	0.01	367	365	366	366.0	0.82
	AC	7.19	7.2	7.2	7.2	0.00	370	371	371	370.7	0.47
	DS	7.2	7.21	7.22	7.2	0.01	326	326	327	326.3	0.47
6	**UP**	7.33	7.31	7.29	7.3	0.02	321	322	321	321.3	0.47
	BC	7.07	7.09	7.08	7.0	0.05	369	368	368	368.3	0.47
	AC	7.16	7.14	7.11	7.1	0.02	373	369	373	371.7	1.89
	DS	7.62	7.61	7.59	7.6	0.01	264	266	265	265.0	0.82

Table 8: Triplicate analysis of turbidity values for NWWTP as detailed in Table 2.3.

Weeks	Sampling point	Turbidity 1	Turbidity 2	Turbidity 3	AVERAGE	STDEV
1	**UP**	9.2	9.18	9.14	9.17	0.02
	BC	2.88	2.87	2.88	2.88	0.00
	AC	2.39	2.4	2.43	2.41	0.02
	DS	9.19	9.21	9.2	9.20	0.01
2	**UP**	16.3	16.1	16.2	16.2	0.08
	BC	13.8	14	14.1	14.0	0.12
	AC	11	11.1	11.1	11.1	0.05
	DS	7.13	7.18	7.14	7.15	0.02
3	**UP**	2.12	2.84	3.06	2.67	0.40
	BC	2.33	2.3	2.06	2.2	0.12
	AC	2.4	2.42	2.35	2.4	0.03
	DS	9.83	9.87	9.88	9.9	0.02
4	**UP**	2.41	2.45	2.81	2.6	0.18
	BC	4.7	4.59	4.8	4.7	0.09
	AC	4.97	4.91	5.16	5.0	0.11
	DS	6.35	6.11	6.09	6.2	0.12
5	**UP**	4.87	4.98	5.01	5.0	0.06
	BC	13	13.2	14.1	13.4	0.48
	AC	9.99	9.99	9.99	10.0	0.00
	DS	4.78	4.93	4.95	4.9	0.08
6	**UP**	3.98	4.59	4.21	4.3	0.25
	BC	5.19	5.16	5.21	5.2	0.02
	AC	4.91	4.89	4.93	4.9	0.02
	DS	5.11	5.09	5.14	5.1	0.02

Table 9: BOD values of the final treated effluent for NWWTP as detailed in Table 2.3.

		BOD DAY 0					BOD DAY 5												
Weeks	**Sample dilution**	**1**	**2**	**3**	**AVG**	**SD**	**1**	**2**	**3**	**AVG**	**SD**	**p value**	**bod 1**	**bod 2**	**bod 3**	**avg**	**sd**	**FINAL**	**sd**
	UP 60	7.74	7.24	7.5	7.51	0.25	0.57	0.55	1	0.57	0.02	0.20	35.85	33.45	34.80	34.70	1.20	**22.67**	**0.42**
	UP 100	7.54	7.55	7.5	7.53	0.02	0.73	0.74	1	0.73	0.01	0.33	20.64	20.64	20.58	20.62	0.03		
	UP 150	7.2	7.17	7.2	7.19	0.02	0.85	0.85	1	0.85	0.00	0.50	12.70	12.64	12.70	12.68	0.03		
	BC 6	8.13	8.19	8.2	8.16	0.03	7.25	7.26	7	7.25	0.01	0.02	44.00	46.50	45.00	45.17	1.26	**40.65**	**5.04**
	BC 8	8.17	7.74	8.1	8.00	0.23	7.14	7.12	7	7.14	0.02	0.02	51.50	31.00	47.50	43.33	10.87		
	BC 10	8.14	7.89	8	8.01	0.13	7.05	6.98	7	7.01	0.04	0.03	36.33	30.33	33.67	33.44	3.01		
1	**AC 15**	8.01	8.05	8	8.02	0.03	1.02	0.98	1	1.00	0.02	0.05	139.80	141.40	140.00	140.40	0.87	**109.96**	**0.92**
	AC 20	8.06	7.98	8	8.01	0.04	0.67	0.45	1	0.54	0.11	0.06	123.17	125.50	124.83	124.50	1.20		
	AC 30	7.54	7.5	7.4	7.49	0.06	0.99	0.98	1	0.99	0.01	0.10	65.50	65.20	64.20	64.97	0.68		
	DS 60	8.2	8.17	8.2	8.19	0.02	0.8	0.88	1	0.84	0.04	0.20	37.00	36.45	36.80	36.75	0.28	**205.10**	**3.82**
	DS 100	8.03	7.92	8.1	8.00	0.07	0.93	0.9	1	0.91	0.02	0.03	236.67	234.00	239.00	236.56	2.50		
	DS 150	7.84	8.01	7.7	7.85	0.16	1.04	0.98	1	1.01	0.03	0.02	340.00	351.50	334.50	342.00	8.67		
	CONTROL	8.04	8.03	8.02	**8.03**	**0.01**	6.89	6.87	7	6.86	0.04	1.00	1.15	1.16	1.21	**1.17**	**0.03**		

Table 9 continued….

		BOD DAY 0					BOD DAY 7												
Weeks	**Sample dilution**	**1**	**2**	**3**	**AVG**	**SD**	**1**	**2**	**3**	**AVG**	**SD**	**p value**	**bod 1**	**bod 2**	**bod 3**	**avg**	**sd**	**FINAL**	**sd**
	UP 60	8.08	8.07	8.1	8.07	0.01	1.45	1.44	1	1.42	0.04	0.20	33.15	33.15	33.45	33.25	0.17	**21.47**	**0.09**
	UP 100	8	7.99	8	7.99	0.02	1.77	1.75	2	1.77	0.02	0.33	18.88	18.91	18.76	18.85	0.08		
	UP 150	7.76	7.75	7.7	7.75	0.02	1.61	1.59	2	1.59	0.02	0.50	12.30	12.32	12.32	12.31	0.01		
	BC 6	8.28	8.27	8.3	8.27	0.01	6.62	6.61	7	6.61	0.01	0.02	83.00	83.00	83.00	83.00	0.00	**91.09**	**0.06**
	BC 8	8.27	8.26	8.3	8.26	0.01	6	5.99	6	5.99	0.01	0.02	113.50	113.50	113.50	113.50	0.00		
	BC 10	8.24	8.23	8.2	8.23	0.01	5.93	5.93	6	5.93	0.01	0.03	77.00	76.67	76.67	76.78	0.19		
2	**AC 15**	8.3	8.29	8.3	8.29	0.01	7.52	7.53	8	7.52	0.01	0.05	15.60	15.20	15.40	15.40	0.20	**12.17**	**0.07**
	AC 20	8.27	8.26	8.3	8.26	0.01	7.58	7.57	8	7.57	0.01	0.06	11.50	11.50	11.50	11.50	0.00		
	AC 30	8.3	8.29	8.3	8.29	0.01	7.34	7.33	7	7.33	0.01	0.10	9.60	9.60	9.60	9.60	0.00		
	DS 60	8.02	8.01	8	8.01	0.02	1.25	1.24	1	1.27	0.04	0.20	33.85	33.85	33.40	33.70	0.26	**178.86**	**0.83**
	DS 100	7.58	7.57	7.6	7.57	0.02	1.27	1.15	1	1.19	0.07	0.03	210.33	214.00	213.33	212.56	1.95		
	DS 150	7.41	7.4	7.4	7.40	0.02	1.61	1.59	2	1.59	0.02	0.02	290.00	290.50	290.50	290.33	0.29		
	CONTROL	8.34	8.29	8.12	**8.25**	**0.12**	7.89	7.92	8	7.94	0.06	1.00	0.45	0.37	0.12	**0.31**	**0.17**		

Table 9 continued….

		BOD DAY 0					BOD DAY 7												
Weeks	**Sample dilution**	**1**	**2**	**3**	**AVG**	**SD**	**1**	**2**	**3**	**AVG**	**SD**	**p value**	**bod 1**	**bod 2**	**bod 3**	**avg**	**sd**	**FINAL**	**sd**
	UP 60	8.01	7.93	7.97	7.97	0.04	4.07	4.09	4.1	4.08	0.01	0.20	19.70	19.20	19.50	19.47	0.25	**12.69**	**0.15**
	UP 100	7.98	8.03	8	8.00	0.03	4.16	4.18	4.2	4.16	0.02	0.33	11.58	11.67	11.67	11.64	0.05		
	UP 150	8.08	8.02	8.02	8.04	0.03	4.57	4.62	4.5	4.56	0.07	0.50	7.02	6.80	7.06	6.96	0.14		
	BC 6	7.96	7.97	7.97	7.97	0.01	5.89	5.88	5.9	5.88	0.02	0.02	103.50	104.50	105.50	104.50	1.00	**111.28**	**0.83**
	BC 8	7.98	7.98	7.96	7.97	0.01	5.22	5.21	5.2	5.21	0.02	0.02	138.00	138.50	138.50	138.33	0.29		
	BC 10	7.93	7.94	7.96	7.94	0.02	5.23	5.22	5.2	5.21	0.02	0.03	90.00	90.67	92.33	91.00	1.20		
3	**AC 15**	7.94	7.96	7.93	7.94	0.02	3.97	3.95	3.9	3.95	0.02	0.05	79.40	80.20	79.80	79.80	0.40	**67.36**	**0.61**
	AC 20	7.93	7.97	8	7.97	0.04	3.07	3.03	3	3.04	0.03	0.06	81.00	82.33	83.00	82.11	1.02		
	AC 30	8	7.94	7.99	7.98	0.03	3.99	3.96	3.9	3.96	0.03	0.10	40.10	39.80	40.60	40.17	0.40		
	DS 60	7.91	7.94	7.92	7.92	0.02	4.16	4.12	4	4.09	0.09	0.20	18.75	19.10	19.70	19.18	0.48	**81.10**	**6.79**
	DS 100	6.98	6.89	6.93	6.93	0.05	4.02	3.98	3.9	3.97	0.06	0.03	98.67	97.00	100.67	98.78	1.84		
	DS 150	7	6.99	7.02	7.00	0.02	4.91	4.27	4.3	4.50	0.36	0.02	104.50	136.00	135.50	125.33	18.04		
	CONTROL	7.95	7.89	7.87	**7.90**	**0.04**	7.28	7.29	7.3	7.29	0.01	1.00	0.67	0.60	0.57	**0.61**	**0.05**		

Table 9 continued….

		BOD DAY 0					BOD DAY 7												
Weeks	**Sample dilution**	**1**	**2**	**3**	**AVG**	**SD**	**1**	**2**	**3**	**AVG**	**SD**	**p value**	**bod 1**	**bod 2**	**bod 3**	**avg**	**sd**	**FINAL**	**sd**
	UP 60	8.22	8.09	8.03	8.11	0.10	4.72	4.79	4.8	4.76	0.04	0.20	17.50	16.50	16.25	16.75	0.66	**10.80**	**0.30**
	UP 100	8.02	7.82	7.81	7.88	0.12	4.89	4.72	4.8	4.81	0.09	0.33	9.48	9.39	9.09	9.32	0.21		
	UP 150	7.49	7.48	7.47	7.48	0.01	4.32	4.31	4.3	4.32	0.01	0.50	6.34	6.34	6.28	6.32	0.03		
	BC 6	8.28	8.27	8.29	8.28	0.01	4.89	4.71	4.9	4.84	0.11	0.02	169.50	178.00	169.00	172.17	5.06	**158.52**	**3.54**
	BC 8	8.37	8.36	8.35	8.36	0.01	4.69	4.71	4.8	4.72	0.03	0.02	184.00	182.50	180.00	182.17	2.02		
	BC 10	8.36	8.37	8.35	8.36	0.01	4.61	4.75	4.8	4.72	0.10	0.03	125.00	120.67	118.00	121.22	3.53		
4	**AC 15**	8.42	8.41	8.41	8.41	0.01	3.92	3.77	3.8	3.83	0.08	0.05	90.00	92.80	92.00	91.60	1.44	**70.71**	**0.84**
	AC 20	8.41	8.41	8.42	8.41	0.01	3.89	3.79	3.9	3.85	0.05	0.06	75.33	77.00	75.83	76.06	0.86		
	AC 30	8.44	8.42	8.43	8.43	0.01	4.01	3.98	4	3.98	0.03	0.10	44.30	44.40	44.70	44.47	0.21		
	DS 60	7.8	7.79	7.8	7.80	0.01	5.96	5.81	5.9	5.90	0.08	0.20	9.20	9.90	9.40	9.50	0.36	**55.85**	**1.49**
	DS 100	7.32	7.3	7.28	7.30	0.02	4.91	4.84	4.8	4.85	0.05	0.03	80.33	82.00	82.33	81.56	1.07		
	DS 150	6.37	6.36	6.36	6.36	0.01	4.87	4.76	4.9	4.83	0.06	0.02	75.00	80.00	74.50	76.50	3.04		
	CONTROL	8.66	8.67	8.64	**8.66**	**0.02**	6.69	6.79	6.8	6.76	0.06	1.00	1.97	1.88	1.83	**1.89**	**0.07**		

Table 9 continued….

		BOD DAY 0					BOD DAY 7												
Weeks	**SAMPLE**	**1**	**2**	**3**	**AVG**	**SD**	**1**	**2**	**3**	**AVG**	**SD**	**p value**	**bod 1**	**bod 2**	**bod 3**	**avg**	**sd**	**FINAL**	**sd**
	UP 60	8.52	8.53	8.52	8.52	0.01	4.37	4.49	4.3	4.40	0.08	0.20	20.75	20.20	20.90	20.62	0.37	**11.65**	**0.50**
	UP 100	8.42	8.5	8.5	8.47	0.05	5.63	5.55	5.8	5.67	0.14	0.33	8.45	8.94	8.09	8.49	0.43		
	UP 150	8.51	8.53	8.53	8.52	0.01	5.96	5.28	5.6	5.61	0.34	0.50	5.10	6.50	5.88	5.83	0.70		
	BC 6	7.85	7.76	7.46	7.69	0.20	7.01	7.04	7	7.01	0.03	0.02	42.00	36.00	23.50	33.83	9.44	**33.35**	**4.16**
	BC 8	7.94	7.97	7.94	7.95	0.02	6.98	7.03	7.1	7.02	0.04	0.02	48.00	47.00	44.00	46.33	2.08		
	BC 10	8.06	8.05	8.07	8.06	0.01	7.43	7.47	7.5	7.46	0.03	0.03	21.00	19.33	19.33	19.89	0.96		
5	**AC 15**	8.13	8.21	8.15	8.16	0.04	6.74	6.81	6.8	6.78	0.04	0.05	27.80	28.00	27.40	27.73	0.31	**23.48**	**0.86**
	AC 20	8.41	8.29	8.36	8.35	0.06	6.69	6.42	6.4	6.52	0.15	0.06	28.67	31.17	32.00	30.61	1.73		
	AC 30	8.06	8.14	8.18	8.13	0.06	6.91	6.89	7	6.92	0.03	0.10	11.50	12.50	12.30	12.10	0.53		
	DS 60	7.24	7.16	7.17	7.19	0.04	5.72	5.63	5.6	5.64	0.08	0.20	7.60	7.65	8.00	7.75	0.22	**60.14**	**1.13**
	DS 100	7.49	7.29	7.31	7.36	0.11	5.29	5.19	5.2	5.21	0.07	0.03	73.33	70.00	71.67	71.67	1.67		
	DS 150	7.08	7.15	7.19	7.14	0.06	5.09	5.13	5.1	5.12	0.03	0.02	99.50	101.00	102.50	101.00	1.50		
	CONTROL	8.81	8.92	8.89	**8.87**	**0.06**	7.61	7.69	7.7	7.68	0.06	1.00	1.20	1.23	1.16	**1.20**	**0.04**		

Table 9 continued….

		DAY 0 BOD					DAY 7 BOD												
Weeks	**SAMPLE**	**1**	**2**	**3**	**AVG**	**SD**	**1**	**2**	**3**	**AVG**	**SD**	**p value**	**bod 1**	**bod 2**	**bod 3**	**avg**	**sd**	**FINAL**	**sd**
	UP 60	7.89	7.9	7.53	7.77	0.21	4.61	4.59	4.6	4.61	0.02	0.20	16.40	16.55	14.55	15.83	1.11	**10.69**	**0.41**
	UP 100	7.88	7.89	7.9	7.89	0.01	4.81	4.77	4.8	4.79	0.02	0.33	9.30	9.45	9.42	9.39	0.08		
	UP 150	7.9	7.91	7.92	7.91	0.01	4.46	4.48	4.5	4.48	0.03	0.50	6.88	6.86	6.82	6.85	0.03		
	BC 6	7.92	7.91	7.92	7.92	0.01	6.79	6.61	6.6	6.66	0.11	0.02	56.50	65.00	66.50	62.67	5.39	**23.48**	**71.51**
	BC 8	7.9	7.89	0.9	5.56	4.04	5.91	6.17	6.2	6.09	0.16	0.02	99.50	86.00	-264.50	-26.33	206.37		
	BC 10	7.87	7.86	7.86	7.86	0.01	6.94	6.81	6.8	6.84	0.09	0.03	31.00	35.00	36.33	34.11	2.78		
6	**AC 15**	8.04	8.03	8.02	8.03	0.01	5.39	5.18	5.2	5.26	0.11	0.05	53.00	57.00	56.20	55.40	2.12	**34.15**	**2.76**
	AC 20	7.97	7.99	7.98	7.98	0.01	6.15	5.69	6.1	5.98	0.25	0.06	30.33	38.33	31.17	33.28	4.40		
	AC 30	7.96	7.98	7.99	7.98	0.02	6.42	6.79	6.6	6.60	0.19	0.10	15.40	11.90	14.00	13.77	1.76		
	DS 60	7.89	7.99	7.99	7.96	0.06	6.2	6.19	6.2	6.19	0.01	0.20	8.45	9.00	9.05	8.83	0.33	**37.41**	**4.87**
	DS 100	7.99	8.01	8.03	8.01	0.02	6.91	6.89	6.4	6.74	0.27	0.03	36.00	37.33	53.33	42.22	9.65		
	DS 150	8.16	8.17	8.17	8.17	0.01	7.04	6.92	6.9	6.94	0.09	0.02	56.00	62.50	65.00	61.17	4.65		
	CONTROL	7.90	7.91	7.92	**7.91**	**0.01**	6.88	7.25	7.2	**7.09**	**0.19**	1.00	1.02	0.66	0.77	**0.82**	**0.18**		

Table 10: Enumeration of *Yersinia* species recovered from the treated effluent at NWWTP over the sampling period as per figure 2.2.

WEEK 1									WEEK 2								
Dilution	**UP**	**AVG**	**BC**	**AVG**	**AC**	**AVG**	**DS**	**AVG**	**Dilution**	**UP**	**AVG**	**BC**	**AVG**	**AC**	**AVG**	**DS**	**AVG**
10^{-1}	TNTC		TNTC		214		TNTC		10^{-1}	TNTC		TNTC		127		TNTC	
cfu/ml	0		0		2 X10^{4}		0		cfu/ml	0		0		1 x10^{4}		0	
10^{-1}	TNTC		TNTC		169		TNTC		10^{-1}	TNTC		TNTC		111		TNTC	
cfu/ml	0	**0**	0	**0**	2 X10^{4}	2 X10^{4}	0	**0**	cfu/ml	0	**0**	0	**0**	1 x10^{4}	**1 x10^{4}**	0	**0**
10^{-2}	361		TNTC		39		184		10^{-2}	218		TNTC		15		316	
cfu/ml	4 x10^{5}		0		4 X10^{4}		2 x10^{5}		cfu/ml	2 x10^{5}		0		2 x10^{4}		3 x10^{5}	
10^{-2}	312		TNTC		15		91		10^{-2}	309		TNTC		22		298	
cfu/ml	3 x10^{5}	**4 x10^{5}**	0	**0**	2 X10^{4}	3 X10^{4}	9 x10^{5}	**0**	cfu/ml	3 x10^{5}	**3 x10^{5}**	0	**0**	2 x10^{4}	**2 x10^{4}**	3 x10^{5}	**3 x10^{5}**
10^{-3}	89		182		1		17		10^{-3}	62		114		0		48	
cfu/ml	9 x10^{5}		2 x10^{6}		1 X10^{4}		2 x10^{5}		cfu/ml	6 x10^{5}		1 x10^{6}		0		5 x10^{5}	
10^{-3}	51		102		0		5		10^{-3}	49		179		1		32	
cfu/ml	5 x10^{5}	**7 x10^{5}**	1x10^{6}	**2 x10^{6}**	0	**1 X10^{4}**	5 x10^{5}	**4 X10^{5}**	cfu/ml	5 x10^{5}	**6 x10^{5}**	2 x10^{6}	**2 x10^{6}**	1 X10^{4}	**1 X10^{4}**	3x10^{5}	**4 x10^{5}**
10^{-4}	2		19		0		0		10^{-4}	9		4		0		7	
cfu/ml	2 x10^{5}		2 x10^{6}		0		0		cfu/ml	9 X10^{5}		4 x10^{5}		0		7 x10^{5}	
10^{-4}	0		7		0		0		10^{-4}	1		11		0		4	
cfu/ml	0	**2 x10^{5}**	7 x10^{6}	**5 x10^{6}**	0	**0**	0	**0**	cfu/ml	1 x10^{5}	**5 x10^{5}**	1 x10^{5}	**3 x10^{5}**	0	**0**	4X10^{5}	**6 x10^{5}**
10^{-5}	0		2		0		0		10^{-5}	0		0		0		0	
cfu/ml	0		2 x10^{6}		0		0		cfu/ml	0		0		0		0	
10^{-5}	0		0		0		0		10^{-5}	0		1		0		0	
cfu/ml	0	**0**	0	**2 x10^{6}**	0	**0**	0	**0**	cfu/ml	0	**0**	1 X10^{6}	**1 X10^{6}**	0	**0**	0	**0**

Table 10 continued….

WEEK 3								
Dilution	**UP**	**AVG**	**BC**	**AVG**	**AC**	**AVG**	**DS**	**AVG**
10^{-1}	TNTC		TNTC		168		TNTC	
cfu/ml	0		0		2×10^4		0	
10^{-1}	TNTC		TNTC		144		TNTC	
cfu/ml	0	**0**	0	**0**	1×10^4	**2×10^4**	0	**0**
10^{-2}	181		TNTC		27		216	
cfu/ml	2×10^5		0		3×10^4		2×10^5	
10^{-2}	178		TNTC		16		193	
cfu/ml	2×10^5	**2×10^5**	0	**0**	2×10^4	**3×10^4**	2×10^5	**2×10^5**
10^{-3}	11		219		2		47	
cfu/ml	1×10^5		2×10^6		2×10^4		5×10^5	
10^{-3}	8		252		0		17	
cfu/ml	1×10^5	**1×10^5**	3×10^6	**3×10^6**	0	**2×10^4**	2×10^5	**4×10^5**
10^{-4}	0		37		0		9	
cfu/ml	0		4×10^6		0		9×10^5	
10^{-4}	0		29		0		0	
cfu/ml	0	**0**	3×10^6	**4×10^6**	0	**0**	0	**9×10^5**
10^{-5}	0		4		0		0	
cfu/ml	0		4×10^6		0		0	
10^{-5}	0		1		0		0	
cfu/ml	0	**0**	1×10^6	**3×10^6**	0	**0**	0	**0**

WEEK 4								
	UP	**AVG**	**BC**	**AVG**	**AC**	**AVG**	**DS**	**AVG**
10^{-1}	TNTC		TNTC		113		TNTC	
cfu/ml	0		0		1×10^4		0	
10^{-1}	TNTC		TNTC		98		TNTC	
cfu/ml	0	**0**	0	**0**	1×10^4	**1×10^4**	0	**0**
10^{-2}	219		TNTC		34		181	
cfu/ml	2×10^5		0		3×10^4		2×10^5	
10^{-2}	212		TNTC		7		165	
cfu/ml	2×10^5	**2×10^5**	0	**0**	7×10^4	**5×10^4**	2×10^5	**2×10^5**
10^{-3}	26		198		2		29	
cfu/ml	3×10^5		2×10^6		2×10^4		3×10^5	
10^{-3}	18		243		0		41	
cfu/ml	2×10^5	**3×10^5**	2×10^6	**2×10^6**	0	**2×10^4**	4×10^5	**4×10^5**
10^{-4}	1		17		0		1	
cfu/ml	1×10^5		2×10^6		0		1×10^5	
10^{-4}	0		36		0		7	
cfu/ml	0	**1×10^5**	4×10^6	**3×10^6**	0	0	7×10^5	**4×10^5**
10^{-5}	0		4		0		0	
cfu/ml	0		4×10^6		0		0	
10^{-5}	0		1		0		0	
cfu/ml	0	**0**	1×10^6	**3×10^6**	0	**0**	0	**0**

Table 10 continued….

WEEK 5									WEEK 6								
Dilution	**UP**	**AVG**	**BC**	**AVG**	**AC**	**AVG**	**DS**	**AVG**	**Dilution**	**UP**	**AVG**	**BC**	**AVG**	**AC**	**AVG**	**DS**	**AVG**
10^{-1}	TNTC		TNTC		TNTC		TNTC		10^{-1}	TNTC		TNTC		TNTC		TNTC	
cfu/ml	0		0		0		0		cfu/ml	0		0		0		0	
10^{-1}	TNTC		TNTC		TNTC		TNTC		10^{-1}	TNTC		TNTC		TNTC		TNTC	
cfu/ml	0	**0**	0	**0**	0	**0**	0	**0**	cfu/ml	0	**0**	0	**0**	0	**0**	0	**0**
10^{-2}	TNTC		TNTC		157		TNTC		10^{-2}	TNTC		TNTC		218		TNTC	
cfu/ml	0		0		1.6 x10^{5}		0		cfu/ml	0		0		2.2 x10^{5}		0	
10^{-2}	TNTC		TNTC		171		TNTC		10^{-2}	TNTC		TNTC		183		TNTC	
cfu/ml	0	**0**	0	**0**	1.7 x10^{5}	**1.7 x10^{5}**	0	**0**	cfu/ml	0	**0**	0	**0**	1.8 x10^{5}	**2 x10^{5}**	0	**0**
10^{-3}	216		184		44		189		10^{-3}	208		311		59		167	
cfu/ml	2.2 x10^{6}		1.8 X10^{6}		4.4 X10^{5}		1.9 x10^{6}		cfu/ml	2 x10^{7}		3.1 X10^{6}		5.9 X10^{5}		1.7 x10^{6}	
10^{-3}	229		169		69		146		10^{-3}	217		296		31		153	
cfu/ml	2.3 x10^{6}	**2.3 x10^{6}**	1.7X10^{6}	**1.8 X10^{6}**	6.9 X10^{5}	**5.7 X10^{5}**	1.5 x10^{6}	**1.7 x10^{6}**	cfu/ml	2.2 x10^{7}	**2.1 x10^{7}**	2.9X10^{6}	**3 X10^{6}**	3.1 X10^{5}	**4.5 X10^{5}**	1.5 x10^{6}	**1.6 x10^{6}**
10^{-4}	60		57		8		62		10^{-4}	49		61		8		39	
cfu/ml	6 x10^{6}		5.7 X10^{6}		8 X10^{5}		6.2 x10^{6}		cfu/ml	4.9 x10^{7}		6.1 X10^{6}		8 X10^{5}		3.9 x10^{6}	
10^{-4}	44		31		15		41		10^{-4}	58		52		4		28	
cfu/ml	4.4 x10^{6}	**5.2 x10^{6}**	3.1 X10^{6}	**4.4 X10^{6}**	1.5 X10^{5}	4.8 X10^{5}	4.1 x10^{6}	**5.2 x10^{6}**	cfu/ml	5.8 x10^{7}	**5.4 x10^{7}**	5.2 X10^{6}	**5.7 X10^{6}**	4 X10^{5}	6 X10^{5}	2.8 x10^{6}	**3.4 x10^{6}**
10^{-5}	13		9		1		11		10^{-5}	15		19		1		3	
cfu/ml	1.3 x10^{7}		9 X10^{6}		1 X10^{6}		1.1 x10^{6}		cfu/ml	1.5 x10^{7}		1.9 X10^{7}		1 X10^{6}		3 x10^{6}	
10^{-5}	7		15		4		2		10^{-5}	21		11		0		1	
cfu/ml	7 x10^{7}	**4.2 x10^{6}**	1.5 X10^{6}	**5.3 X10^{6}**	4 X10^{6}	**2.5 X10^{6}**	2 x10^{6}	**1.6 x10^{6}**	cfu/ml	2.1 x10^{7}	**5.4 x10^{7}**	1.1 X10^{7}	**1.5 X10^{7}**	0	**1 X10^{6}**	1 x10^{6}	**2 x10^{6}**
10^{-6}	1		0		0		0		10^{-6}	0		2		0		0	
cfu/ml	1 x10^{7}		0		0		0		cfu/ml	0		2 x10^{7}		0		0	
10^{-6}	0		2		0		0		10^{-6}	1		1		0		0	
cfu/ml	0	**1 x10^{7}**	1 X10^{7}	**1X10^{7}**	0	**0**	0	**0**	cfu/ml	1 x10^{7}	**1 x10^{7}**	1 X10^{7}	**1.5 X10^{7}**	0	**0**	0	**0**

Table 11: Statistical analysis of the physicochemical parameters and microbial counts at NGTW as per table 2.4.

(UP)

Week	COD (mg/ml)	Temp (T˚C)	Turb (NTU)	TDS (mg/l)	pH	BOD (mg/ml)	YERS
1	269.0	23	13.2	429.7	8.8	23.20	5.6
2	37.0	18	2.1	214.1	8.5	13.70	5.4
3	222.7	17	3.2	197.4	8.0	13.40	4.3
4	229.0	15	5.0	189.6	7.4	11.30	5.3
5	161.0	16	21.4	282.7	6.7	4.40	7.2
6	303.3	21	8.6	183.5	7.5	7.30	7.8

Correlations

		CODRlog10	pHRlog10	Temp	BOD	Turbidity	TDS	*Yersinia*
CODRlog10	Pearson Correlation	1	-.254	.248	.016	.308	.174	.205
	Sig. (2-tailed)		.627	.636	.976	.553	.741	.697
	N	6	6	6	6	6	6	6
pHRlog10	Pearson Correlation	-.254	1	.602	.898	-.517	.379	-.550
	Sig. (2-tailed)	.627		.206	.015	.294	.459	.258
	N	6	6	6	6	6	6	6
Temp	Pearson Correlation	.248	.602	1	.575	.115	.592	.245
	Sig. (2-tailed)	.636	.206		.233	.828	.216	.640
	N	6	6	6	6	6	6	6
BOD	Pearson Correlation	.016	.898	.575	1	-.262	.642	-.587
	Sig. (2-tailed)	.976	.015	.233		.616	.170	.221
	N	6	6	6	6	6	6	6
Turbidity	Pearson Correlation	.308	-.517	.115	-.262	1	.567	.610
	Sig. (2-tailed)	.553	.294	.828	.616		.241	.199
	N	6	6	6	6	6	6	6
TDS	Pearson Correlation	.174	.379	.592	.642	.567	1	.001
	Sig. (2-tailed)	.741	.459	.216	.170	.241		.998
	N	6	6	6	6	6	6	6
Yersinia	Pearson Correlation	.205	-.550	.245	-.587	.610	.001	1
	Sig. (2-tailed)	.697	.258	.640	.221	.199	.998	
	N	6	6	6	6	6	6	6

(BC) Table 11 continued….

Week	COD (mg/ml)	Temp (T˚C)	Turb (NTU)	TDS (mg/l)	pH	BOD (mg/ml)	YERS
1	94.0	22	21.2	540.7	7.2	314.00	7.4
2	72.0	23	263.0	659.7	7.4	282.90	6.3
3	185.3	22	280.7	772.3	8.1	159.30	5.3
4	187.7	20	10.0	590.3	6.8	123.30	6.3
5	216.0	20	19.6	550.7	6.9	73.40	6.8
6	297.0	20	268.7	619.3	6.9	91.40	6.6

Correlations

		COD	pH	Temp	BODRlog10	Turbidity	TDS	*Yersinia*Rlog10
COD	Pearson Correlation	1	-.325	-.826	-.890	.131	.017	-.132
	Sig. (2-tailed)		.530	.043	.018	.805	.975	.803
	N	6	6	6	6	6	6	6
pH	Pearson Correlation	-.325	1	.705	.432	.590	.851	-.717
	Sig. (2-tailed)	.530		.118	.393	.217	.032	.109
	N	6	6	6	6	6	6	6
Temp	Pearson Correlation	-.826	.705	1	.883	.417	.439	-.208
	Sig. (2-tailed)	.043	.118		.020	.411	.384	.693
	N	6	6	6	6	6	6	6
BODRlog10	Pearson Correlation	-.890	.432	.883	1	.113	.124	.092
	Sig. (2-tailed)	.018	.393	.020		.832	.815	.862
	N	6	6	6	6	6	6	6
Turbidity	Pearson Correlation	.131	.590	.417	.113	1	.801	-.609
	Sig. (2-tailed)	.805	.217	.411	.832		.055	.200
	N	6	6	6	6	6	6	6
TDS	Pearson Correlation	.017	.851	.439	.124	.801	1	-.938
	Sig. (2-tailed)	.975	.032	.384	.815	.055		.006
	N	6	6	6	6	6	6	6
*Yersinia*Rlog10	Pearson Correlation	-.132	-.717	-.208	.092	-.609	-.938	1
	Sig. (2-tailed)	.803	.109	.693	.862	.200	.006	
	N	6	6	6	6	6	6	6

Table 11 continued….

(AC)

Week	COD (mg/ml)	Temp (T˚C)	Turb (NTU)	TDS (mg/l)	pH	BOD (mg/ml)	YERS
1	309.0	21	14.6	534.0	8.2	105.50	5.7
2	92.7	22	1.3	476.3	7.6	104.30	5.3
3	100.7	22	10.2	582.6	7.3	26.40	4.3
4	152.4	20	34.5	576.7	6.8	33.60	5.3
5	170.0	20	8.0	380.0	7.0	31.50	5.7
6	300.6	21	93.3	480.3	7.4	16.60	5.3

Correlations

		CODT	pHT	TempT	BODT	TurbidityT	TDST	*Yersinia*T
CODT	Pearson Correlation	1	.361	-.430	-.134	.699	-.118	.565
	Sig. (2-tailed)		.482	.395	.800	.122	.824	.242
	N	6	6	6	6	6	6	6
pHT	Pearson Correlation	.361	1	.530	.640	-.214	.132	.170
	Sig. (2-tailed)	.482		.279	.171	.684	.803	.747
	N	6	6	6	6	6	6	6
TempT	Pearson Correlation	-.430	.530	1	.282	-.462	.337	-.602
	Sig. (2-tailed)	.395	.279		.589	.356	.514	.206
	N	6	6	6	6	6	6	6
BODT	Pearson Correlation	-.134	.640	.282	1	-.689	.053	.359
	Sig. (2-tailed)	.800	.171	.589		.130	.921	.485
	N	6	6	6	6	6	6	6
TurbidityT	Pearson Correlation	.699	-.214	-.462	-.689	1	.239	.036
	Sig. (2-tailed)	.122	.684	.356	.130		.649	.946
	N	6	6	6	6	6	6	6
TDST	Pearson Correlation	-.118	.132	.337	.053	.239	1	-.563
	Sig. (2-tailed)	.824	.803	.514	.921	.649		.245
	N	6	6	6	6	6	6	6
*Yersinia*T	Pearson Correlation	.565	.170	-.602	.359	.036	-.563	1
	Sig. (2-tailed)	.242	.747	.206	.485	.946	.245	
	N	6	6	6	6	6	6	6

Table 11 continued….

(DS)

Week	COD (mg/ml)	Temp (T˚C)	Turb (NTU)	TDS (mg/l)	pH	BOD (mg/ml)	YERS
1	292.0	22	13.4	553.7	7.4	162.10	5.6
2	75.7	19	36.9	375.0	8.3	161.20	6.3
3	187.0	18	94.6	401.0	7.7	59.80	6.6
4	72.3	17	53.2	432.0	7.1	79.60	6.3
5	287.0	15	22.1	343.3	7.1	43.20	6.5
6	185.0	22	452.0	465.6	7.4	26.80	6.5

Correlations

		CODRlog10	pH	Temp	BOD	Turbidity	TDS	*Yersinia*Rlog10
CODRlog10	Pearson Correlation	1	-.397	.153	-.233	.072	.274	-.219
	Sig. (2-tailed)		.436	.772	.656	.893	.599	.677
	N	6	6	6	6	6	6	6
pH	Pearson Correlation	-.397	1	.254	.546	-.074	-.189	.040
	Sig. (2-tailed)	.436		.627	.262	.889	.720	.940
	N	6	6	6	6	6	6	6
Temp	Pearson Correlation	.153	.254	1	.320	.518	.814	-.518
	Sig. (2-tailed)	.772	.627		.536	.293	.049	.293
	N	6	6	6	6	6	6	6
BOD	Pearson Correlation	-.233	.546	.320	1	-.573	.346	-.753
	Sig. (2-tailed)	.656	.262	.536		.235	.501	.084
	N	6	6	6	6	6	6	6
Turbidity	Pearson Correlation	.072	-.074	.518	-.573	1	.193	.367
	Sig. (2-tailed)	.893	.889	.293	.235		.713	.474
	N	6	6	6	6	6	6	6
TDS	Pearson Correlation	.274	-.189	.814	.346	.193	1	-.782
	Sig. (2-tailed)	.599	.720	.049	.501	.713		.066
	N	6	6	6	6	6	6	6
YersiniaRlog10	Pearson Correlation	-.219	.040	-.518	-.753	.367	-.782	1
	Sig. (2-tailed)	.677	.940	.293	.084	.474	.066	
	N	6	6	6	6	6	6	6

Table 12: Statistical analysis of the physicochemical parameters and microbial counts at NWWTP as per table 2.5. (UP)

Weeks	COD (mg/ml)	Temp (T˚C)	Turb (NTU)	TDS (mg/l)	pH	BOD (mg/ml)	YERS
1	269.0	23	13.2	429.7	8.8	23.20	5.84
2	37.0	18	2.1	214.1	8.5	13.70	5.8
3	222.7	17	3.2	197.4	8.0	13.40	5
4	229.0	15	5.0	189.6	7.4	11.30	5.5
5	161.0	16	21.4	282.7	6.7	4.40	6.3
6	156.0	23	4.3	321.3	7.3	10.60	7.3

Correlations

		CODRlog10	pHRlog10	Temp	BOD	Turbidity	TDS	*Yersinia*
CODRlog10	Pearson Correlation	1	1.000	.409	.908	-.419	.244	-.419
	Sig. (2-tailed)		.000	.421	.012	.408	.641	.408
	N	6	6	6	6	6	6	6
pHRlog10	Pearson Correlation	1.000	1	.409	.908	-.419	.244	-.419
	Sig. (2-tailed)	.000		.421	.012	.408	.641	.408
	N	6	6	6	6	6	6	6
Temp	Pearson Correlation	.409	.409	1	.577	-.047	.820	.565
	Sig. (2-tailed)	.421	.421		.230	.929	.046	.243
	N	6	6	6	6	6	6	6
BOD	Pearson Correlation	.908	.908	.577	1	-.233	.517	-.295
	Sig. (2-tailed)	.012	.012	.230		.657	.293	.570
	N	6	6	6	6	6	6	6
Turbidity	Pearson Correlation	-.419	-.419	-.047	-.233	1	.491	.199
	Sig. (2-tailed)	.408	.408	.929	.657		.323	.706
	N	6	6	6	6	6	6	6
TDS	Pearson Correlation	.244	.244	.820	.517	.491	1	.465
	Sig. (2-tailed)	.641	.641	.046	.293	.323		.353
	N	6	6	6	6	6	6	6
Yersinia	Pearson Correlation	-.419	-.419	.565	-.295	.199	.465	1
	Sig. (2-tailed)	.408	.408	.243	.570	.706	.353	
	N	6	6	6	6	6	6	6

Table 12 continued….

(BC)

Weeks	COD (mg/ml)	Temp (T˚C)	Turb (NTU)	TDS (mg/l)	pH	BOD (mg/ml)	YERS
1	94.0	22	21.2	540.7	7.2	314.00	5.30
2	72.0	23	26.0	659.7	7.4	282.90	6.3
3	185.3	22	280.7	772.3	8.1	159.30	6.4
4	187.7	20	10.0	590.3	6.8	123.30	6.3
5	216.0	20	19.6	550.7	6.9	73.40	6.2
6	198.0	23	5.2	368.3	7.0	23.50	6.4

Correlations

		CODRlog10	pH	TempRlog10	BOD	Turbidity	TDSRlog10	*Yersinia*Rlog10
CODRlog10	Pearson Correlation	1	-.189	-.534	-.905	.195	-.254	.474
	Sig. (2-tailed)		.720	.275	.013	.711	.628	.342
	N	6	6	6	6	6	6	6
pH	Pearson Correlation	-.189	1	.461	.333	.912	.655	.122
	Sig. (2-tailed)	.720		.357	.518	.011	.158	.819
	N	6	6	6	6	6	6	6
TempRlog10	Pearson Correlation	-.534	.461	1	.311	.136	-.172	-.019
	Sig. (2-tailed)	.275	.357		.549	.797	.745	.971
	N	6	6	6	6	6	6	6
BOD	Pearson Correlation	-.905	.333	.311	1	.040	.496	-.643
	Sig. (2-tailed)	.013	.518	.549		.940	.317	.168
	N	6	6	6	6	6	6	6
Turbidity	Pearson Correlation	.195	.912	.136	.040	1	.652	.255
	Sig. (2-tailed)	.711	.011	.797	.940		.161	.626
	N	6	6	6	6	6	6	6
TDSRlog10	Pearson Correlation	-.254	.655	-.172	.496	.652	1	.072
	Sig. (2-tailed)	.628	.158	.745	.317	.161		.892
	N	6	6	6	6	6	6	6
*Yersinia*Rlog10	Pearson Correlation	.474	.122	-.019	-.643	.255	.072	1
	Sig. (2-tailed)	.342	.819	.971	.168	.626	.892	
	N	6	6	6	6	6	6	6

Table 12 continued….

(AC)

Weeks	COD (mg/ml)	Temp (T˚C)	Turb (NTU)	TDS (mg/l)	pH	BOD (mg/ml)	YERS
1	309.0	21	14.6	534.0	8.2	105.50	4
2	92.7	22	1.3	476.3	7.6	104.30	4
3	100.7	22	10.2	582.6	7.3	26.40	4.3
4	152.4	20	34.5	576.7	6.8	33.60	4.3
5	170.0	20	8.0	380.0	7.0	31.50	5.7
6	152.7	22	4.9	371.7	7.1	34.20	5.6

Correlations

		COD	pH	TempRlog10	BOD	Turbidity	TDSRlog10	*Yersinia*
COD	Pearson Correlation	1	.587	-.354	.402	.228	.039	-.085
	Sig. (2-tailed)		.220	.491	.429	.664	.941	.873
	N	6	6	6	6	6	6	6
pH	Pearson Correlation	.587	1	.358	.854	-.321	.243	-.559
	Sig. (2-tailed)	.220		.486	.030	.536	.642	.249
	N	6	6	6	6	6	6	6
TempRlog10	Pearson Correlation	-.354	.358	1	.241	-.660	-.015	-.198
	Sig. (2-tailed)	.491	.486		.645	.153	.977	.707
	N	6	6	6	6	6	6	6
BOD	Pearson Correlation	.402	.854	.241	1	-.261	.166	-.613
	Sig. (2-tailed)	.429	.030	.645		.617	.753	.195
	N	6	6	6	6	6	6	6
Turbidity	Pearson Correlation	.228	-.321	-.660	-.261	1	.580	-.278
	Sig. (2-tailed)	.664	.536	.153	.617		.227	.593
	N	6	6	6	6	6	6	6
TDSRlog10	Pearson Correlation	.039	.243	-.015	.166	.580	1	-.865
	Sig. (2-tailed)	.941	.642	.977	.753	.227		.026
	N	6	6	6	6	6	6	6
Yersinia	Pearson Correlation	-.085	-.559	-.198	-.613	-.278	-.865	1
	Sig. (2-tailed)	.873	.249	.707	.195	.593	.026	
	N	6	6	6	6	6	6	6

Table 11 continued….

(DS)

Weeks	COD (mg/ml)	Temp (T˚C)	Turb (NTU)	TDS (mg/l)	pH	BOD (mg/ml)	YERS
1	292.0	22	13.4	553.7	7.4	162.10	5.6
2	75.7	19	36.9	375.0	8.3	161.20	4
3	187.0	18	94.6	401.0	7.7	59.80	5.6
4	72.3	17	53.2	432.0	7.1	79.60	5.6
5	287.0	15	22.1	343.3	7.1	43.20	6.2
6	195.0	24	5.1	265.0	7.6	37.40	6.2

Correlations

		CODRlog10	pH	Temp	BOD	Turbidity	TDS	*Yersinia*Rlog10
CODRlog10	Pearson Correlation	1	-.372	.182	-.247	-.322	.086	.654
	Sig. (2-tailed)		.468	.729	.637	.534	.871	.159
	N	6	6	6	6	6	6	6
pH	Pearson Correlation	-.372	1	.317	.477	.135	-.174	-.810
	Sig. (2-tailed)	.468		.540	.339	.798	.742	.050
	N	6	6	6	6	6	6	6
Temp	Pearson Correlation	.182	.317	1	.202	-.481	-.029	.046
	Sig. (2-tailed)	.729	.540		.701	.334	.956	.931
	N	6	6	6	6	6	6	6
BOD	Pearson Correlation	-.247	.477	.202	1	-.133	.680	-.737
	Sig. (2-tailed)	.637	.339	.701		.802	.137	.095
	N	6	6	6	6	6	6	6
Turbidity	Pearson Correlation	-.322	.135	-.481	-.133	1	.134	-.175
	Sig. (2-tailed)	.534	.798	.334	.802		.800	.739
	N	6	6	6	6	6	6	6
TDS	Pearson Correlation	.086	-.174	-.029	.680	.134	1	-.151
	Sig. (2-tailed)	.871	.742	.956	.137	.800		.776
	N	6	6	6	6	6	6	6
*Yersinia*Rlog10	Pearson Correlation	.654	-.810	.046	-.737	-.175	-.151	1
	Sig. (2-tailed)	.159	.050	.931	.095	.739	.776	
	N	6	6	6	6	6	6	6

Table 13: Antibiotic susceptibility of *Yersinia* spprecovered from the treated effluent at NGTW and NWWTP as per table 3.4.

Isolate	AMC (30)	AMP (10)	CRO (30)	CXM (30)	C (30)	KF (30)	CIP(5)	CN (10)	K (30)	S(10)	TE (30)	W (5)	MAR
YE- CONTROL	**R**	**R**	**S**	**R**	**I**	**R**	**S**	**S**	**I**	**R**	**R**	**R**	**0.58**
43	I	R	S	R	R	R	S	S	S	I	R	R	**0.5**
206	I	R	S	R	I	R	S	S	S	I	R	R	**0.4**
33	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
81	R	R	S	R	R	R	S	S	I	R	R	R	**0.7**
253	R	R	S	R	R	R	S	S	I	R	R	R	**0.7**
62	R	R	S	R	R	R	S	S	S	I	R	R	**0.58**
150	R	R	S	R	R	R	S	S	S	I	R	R	**0.58**
302	R	I	R	S	R	R	S	S	I	R	R	R	**0.58**
11	R	R	S	R	I	R	S	S	I	R	R	R	**0.58**
286	I	R	S	R	R	R	S	S	R	R	R	R	**0.7**
295	I	R	S	R	I	R	S	S	S	I	R	R	**0.4**
282	R	R	S	R	I	R	S	S	S	R	R	R	**0.58**
79	R	R	S	R	I	R	S	S	I	R	R	R	**0.58**
208	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
104	R	R	S	R	R	S	S	S	R	I	R	R	**0.58**
48	R	R	S	R	R	R	S	S	R	R	R	R	**0.7**
68	R	R	S	R	I	R	S	S	S	R	R	R	**0.5**
289	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
254	R	R	S	R	R	R	S	S	I	R	R	R	**0.7**
75	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
84	R	R	S	R	I	R	S	S	S	R	R	R	**0.58**
112	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
54	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
259	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
49	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
77	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
103	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
260	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
8	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
207	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
294	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
280	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
113	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**

R- resistant, S- susceptible, I- intermediate

Table 13 continued….

47	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
118	R	R	S	R	R	R	S	S	R	R	R	R	**0.58**
191	R	R	S	R	R	R	S	S	R	R	R	R	**0.58**
192	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
281	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
218	R	R	S	R	I	R	S	S	S	R	R	R	**0.7**
190	R	R	S	R	R	R	S	S	S	I	R	R	**0.58**
151	R	R	S	I	R	R	S	S	S	R	R	R	**0.58**
285	R	R	S	I	R	R	S	S	S	R	R	R	**0.58**
262	R	R	S	R	I	R	S	S	S	R	R	R	**0.5**
55	R	R	S	R	R	R	S	S	S	I	R	R	**0.7**
303	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
120	R	R	S	R	R	R	S	S	S	I	R	R	**0.5**
86	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
53	I	R	S	R	R	R	S	S	S	R	R	R	**0.7**
40	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
135	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
252	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
189	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
253	I	R	S	R	R	R	S	S	S	I	R	R	**0.5**
37	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
261	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
45	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
129	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
34	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
185	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
72	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
78	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
80	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
136	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
230	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
197	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
198	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
215	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
186	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
187	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
188	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**

R- resistant, S- susceptible, I- intermediate

Table 13 continued….

199	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
9	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
7	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
57	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
46	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
88	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
35	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
36	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
38	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
44	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
51	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
85	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
106	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
108	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
130	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
131	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
137	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
140	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
142	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
143	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
147	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
148	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
146	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
223	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
216	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
217	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
205	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
153	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
157	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
213	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
183	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
184	R	I	S	R	I	R	S	S	S	R	R	R	**0.5**
263	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
264	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
240	R	R	S	R	I	R	S	S	S	R	R	R	**0.58**
236	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
279	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**

R- resistant, S- susceptible, I- intermediate

Table 13 continued….

329	R	R	S	R	I	R	S	S	S	I	R	R	**0.5**
433	R	R	S	R	I	R	S	S	S	I	R	R	**0.5**
340	R	R	S	R	I	R	S	S	S	R	R	R	**0.58**
318	R	R	S	I	R	R	S	S	S	R	R	R	**0.58**
362	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
359	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
358	R	R	S	R	I	R	S	S	S	R	R	R	**0.58**
334	R	R	S	I	I	R	S	S	S	R	R	R	**0.58**
395	R	R	S	I	I	R	S	S	S	R	R	R	**0.5**
399	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
403	R	R	S	R	I	R	S	S	S	R	R	R	**0.58**
402	R	R	S	I	R	R	S	S	S	R	R	R	**0.58**
356	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
391	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
393	R	R	S	I	R	R	S	S	S	I	R	R	**0.5**
394	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
308	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
370	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
352	R	R	S	R	I	R	S	S	S	R	R	R	**0.58**
346	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
426	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
364	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
375	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
377	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
383	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
336	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
325	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
314	R	R	S	R	I	R	S	S	S	R	R	R	**0.58**
466	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
390	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
315	R	R	S	R	I	R	S	S	S	R	R	R	**0.58**
319	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
320	R	R	S	R	I	R	S	S	S	R	R	R	**0.58**
344	R	R	S	R	I	R	S	S	S	R	R	R	**0.58**
345	R	R	S	R	I	R	S	S	S	R	R	R	**0.58**
347	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
326	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**

R- resistant, S- susceptible, I- intermediate

Table 13 continued….

409	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
422	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
415	R	R	S	R	I	R	S	S	S	R	R	R	**0.58**
416	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
418	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
419	R	R	S	R	I	R	S	S	S	I	R	R	**0.5**
424	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
428	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
436	R	R	S	R	I	R	S	S	S	R	R	R	**0.58**
439	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
440	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
441	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
442	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
447	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
450	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
451	I	R	S	R	R	R	S	S	S	R	R	R	**0.58**
453	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
454	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
455	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
459	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
462	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
467	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
468	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
469	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
470	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
472	R	R	S	R	R	R	S	S	S	R	R	R	**0.7**
408	R	R	S	R	I	R	S	S	S	I	R	R	**0.5**

R- resistant, S- susceptible, I- intermediate

Table 14: Summary of genotypic and phenotypic assays of *Yersinia* spp recovered from treated effluent.

			Antibiotic Profile										Integrons			Virulence genes				Assays	
	No.	Code									MAR index	Phenotype	Int 1	Int 2	Int 3	*ail* 425 bp	*ystA* 79 bp	*Vir/Lcr* 561 bp	*YadA* 849 bp	Crystal violet	MBL
UP-NG	308	YE 27	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	+	–	–	–	–	–
UP-NG	55	YE 25	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	+	–	–	+	+	–	–	+	–
DS-NG	40	YE 26	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	–	+	–	–	–	–
UP-N	441	YE 29	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	+	–	–	–	–	–	–	–	–
DS-NG	428	YE 22	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	+	+	–	–	–	–	–	–	–
AC-N	188	YE 41	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	–	–	+	+	–	–	–	–
BC-NG	207	YE 23	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	+	–	–	+	–	–	–	–	–
DS-NG	103	YE 30	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	+	–	–	–	+	–	–	+	–
UP-NG	252	YE 20	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	–	+	–	–	+	–
BC-NG	259	YE 43	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	+	+	–	–	–	–	–	–	–
DS-NG	428	YE 22	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	+	+	–	–	–	–	–	–	–
AC-NG	80	YE 21	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	–	+	–	–	–	–
UP-N	112	YE 31	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	–	+	–	–	–	–
DS-N	303	YE 33	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	–	–	–	–	–	–
BC-N	453	YE 35	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	+	+	–	+	–	–	–	–	–
DS-N	472	YE 37	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	+	–	–	+	–	–	–	+	–
AC-N	459	YE 39	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	+	–	–	+	–	–	–	–	–
AC-NG	78	YE 40	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	–	–	+	+	–	–	–	–
DS-N	302	YE 34	AMC	CXM	CRO	C	KF	S	TE	W	**0.66**	**A**	–	–	–	–	+	–	+	+	–
AC-N	136	YE 36	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	+	+	–	–	–	–
AC-N	185	YE 38	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	+	+	–	–	–	–
AC-N	462	YE 32	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	–	–	–	–	–	–
BC-NG	318	YE 2	AMC	AMP	C	KF	S	TE	W		**0.58**	**E**	+	–	–	+	–	–	–	–	–
BC-NG	213	YE 42	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	+	–	–	–	–	–	–	–	–
DS-NG	428	YE 22	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	+	+	–	–	–	–	–	–	–

AC-NG	80	YE 21	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	–	+	–	–	–	–
DS-NG	40	YE 26	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	–	+	–	–	–	–
UP-NG	55	YE 25	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	+	–	–	+	+	–	–	+	–
BC-NG	72	YE 4	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	–	+	–	–	–	–
BC-NG	419	YE 70	AMC	AMP	CXM	KF	TE	W			**0.5**	**J**	+	–	–	–	–	–	–	–	–
AC-NG	33	YE 7	AMC	AMP	CXM,	C	KF	S	TE	W	**0.66**	**A**	–	+	–	–	+	–	+	+	–
DS-NG	344	YE 66	AMC	AMP	CXM	C	S	TE	W		**0.58**	**F**	+	–	–	–	+	–	–	–	–
UP-N	442	YE 10	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	+	+	–	–	–	–	–	–	–
DS- NG	104	YE 63	AMC	AMP	CXM	C	K	TE	W		**0.58**	**C**	–	+	–	–	+	–	–	–	–
AC-NG	79	YE 61	AMC	AMP	CXM	KF	S	TE	W		**0.58**	**B**	–	+	–	+	+	–	–	–	–
UP-NG	253	YE 14	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	–	+	–	–	–	–
BC-NG	415	YE 62	AMC	AMP	CXM	KF	S	TE	W		**0.58**	**B**	+	–	–	–	–	–	–	–	–
BC-NG	264	YE 13	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	+	–	–	–	–	–	–	–	–
DS-N	467	YE 5	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	–	–	–	+	–	–	–	–
DS-NG	48	YE 11	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	–	+	–	–	–	–
DS-N	197	YE 15	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	–	+	–	–	+	–
UP-N	118	YE 16	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	+	+	–	–	–	–
UP-NG	409	YE 3	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	+	–	–	–	–	–
UP-NG	55	YE 25	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	+	–	–	+	+	–	–	+	–
BC-NG	72	YE 4	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	–	+	–	–	–	–
AC-NG	33	YE 7	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	–	+	–	+	+	–
DS-NG	344	YE 66	AMC	AMP	CXM	C	S	TE	W		**0.58**	**F**	+	–	–	–	+	–	–	–	–
UP-N	442	YE 10	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	+	+	–	–	–	–	–	–	–
DS- NG	104	YE 63	AMC	AMP	CXM	C	K	TE	W		**0.58**	**C**	–	+	–	–	+	–	–	–	–
AC-NG	79	YE 61	AMC	AMP	CXM	KF	S	TE	W		**0.58**	**B**	–	+	–	+	+	–	–	–	–
UP-NG	253	YE 14	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	–	+	–	–	–	–
BC-NG	415	YE 62	AMC	AMP	CXM	KF	S	TE	W		**0.58**	**B**	+	–	–	–	–	–	–	–	–
BC-NG	264	YE 13	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	+	–	–	–	–	–	–	–	–
DS-N	467	YE 5	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	–	–	–	+	–	–	–	–
DS-NG	48	YE 11	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	–	+	–	–	–	–
DS-N	197	YE 15	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	–	+	–	–	+	–

UP-N	118	YE 16	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	+	+	–	–	–	–
UP-NG	409	YE 3	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	+	–	–	–	–	–
UP-NG	254	YE 6	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	–	+	–	–	–	–
AC-N	191	YE 17	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	–	+	–	–	–	–
DS-N	88	YE 18	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	–	+	–	–	–	–	+	–
DS- NG	43	YE 67	AMP	CXM	C	KF	TE	W			**0.5**	**G**	–	–	+	+	–	–	–	+	–
DS-N	468	YE 44	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	–	–	+	+	+	–	+	–
AC-NG	216	YE 9	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	–	–	–	–	–	–
DS-N	469	YE 12	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	+	+	–	–	–	–	–	–	–
UP-N	150	YE 64	AMC	AMP	CXM	C	KF	TE	W		**0.58**	**D**	–	–	+	+	–	–	–	+	–
Control		**YE-Control**	AMC	AMP	CXM	C	KF	S	TE	W	**0.66**	**A**	–	+	–	+	–	–	–	–	–

Table 15: Percentage score similarity generated from GelCompare software for *Yersinia* s spp. bands on the RAPD profiles.

Isolate code	Matrix
YE 28	100
YE 35	88.89 100.00
YE 2	44.45 42.11 100.00
YE 38	13.33 12.50 22.73 100.00
YE 42	45.46 30.77 36.85 70.00 100.00
YE 34	7.69 7.15 14.29 50.00 40.00 100.00
YE 36	25.00 14.29 19.05 60.00 36.37 85.72 100.00
YE 35	16.67 15.39 14.29 7.15 7.69 9.09 8.33 100.00
YE 37	9.09 8.33 4.77 18.19 0.00 11.11 10.00 66.67 100.00
YE 39	9.09 8.33 4.77 18.19 9.09 11.11 10.00 25.00 60.00 100.00
YE 40	0.00 9.09 5.00 20.00 22.23 0.00 0.00 12.50 40.00 75.00 100.00
YE 43	7.69 7.15 4.35 15.39 7.69 20.00 8.33 50.00 42.86 11.11 12.50 100.00
YE 33	20.00 18.19 10.00 0.00 9.09 11.11 10.00 66.67 60.00 33.33 16.67 66.67 100.00
YE 20	9.09 8.33 4.77 8.33 9.09 0.00 10.00 25.00 33.33 14.29 16.67 66.67 60.00 100.00
YE 31	9.09 8.33 4.77 18.19 9.09 0.00 22.23 25.00 33.33 0.00 16.67 66.67 100.00 100.00 100.00
YE 21	18.19 16.67 15.00 7.69 8.33 10.00 9.09 22.23 12.50 12.50 14.29 22.23 28.57 80.01 80.01 100.00
YE 22	25.00 23.08 19.05 23.08 25.00 18.19 27.27 18.19 10.00 10.00 11.11 18.19 22.23 37.50 37.50 71.43 100.00
YE 26	0.00 0.00 4.55 16.67 8.33 10.00 9.09 0.00 0.00 0.00 0.00 10.00 0.00 12.50 0.00 25.00 20.00 100.00
YE 23	9.09 18.19 4.77 8.33 9.09 11.11 10.00 11.11 0.00 0.00 0.00 11.11 14.29 33.33 14.29 50.00 37.50 50.00 100.00
YE 29	0.00 0.00 4.55 7.69 0.00 10.00 9.09 0.00 0.00 12.50 0.00 10.00 0.00 28.57 0.00 25.00 20.00 42.86 28.57 100.00
YE 27	0.00 0.00 0.00 30.00 9.09 25.00 22.23 0.00 0.00 0.00 0.00 11.11 0.00 14.29 0.00 12.50 22.23 50.00 33.33 50.00 100.00
YE 24	6.25 5.89 8.00 20.00 21.43 25.00 14.29 7.15 0.00 0.00 0.00 7.15 0.00 8.33 8.33 27.27 33.33 40.00 30.00 27.27 30.00 100.00
YE 41	11.77 17.65 16.00 11.11 5.56 6.25 5.89 6.25 0.00 7.15 0.00 0.00 0.00 7.15 0.00 14.29 12.50 23.08 15.39 23.08 15.39 17.65 100.00
YE 30	0.00 0.00 4.77 18.19 0.00 11.11 10.00 0.00 0.00 0.00 0.00 0.00 0.00 14.29 14.29 12.50 10.00 12.50 14.29 12.50 14.29 18.19 7.15 100.00
YE 25	0.00 8.33 10.00 8.33 20.00 0.00 0.00 0.00 0.00 0.00 16.67 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 15.39 0.00 100.00

Table 16: Percentage score similarity generated from GelCompare software for *Yersinia* s spp. bands on the RAPD profiles.

Isolate code	Matrix
YE 70	100
YE 10	83.33 100.00
YE 62	63.64 50.00 100.00
YE 5	69.23 29.41 50.00 100.00
YE 19	31.25 50.00 54.55 23.53 100.00
YE 61	36.85 30.00 15.79 30.00 25.00 100.00
YE 17	12.50 12.50 7.69 12.50 13.33 22.23 100.00
YE 18	30.77 13.33 8.33 6.25 23.08 31.25 85.72 100.00
YE 12	18.75 26.67 15.39 26.67 38.47 21.05 36.37 55.56 100.00
YE 66	10.53 10.53 21.43 31.25 17.65 13.64 13.33 6.67 20.00 100.00
YE 7	18.75 5.56 25.00 46.15 28.57 15.00 25.00 16.67 33.33 50.00 100.00
YE 9	18.75 11.77 15.39 11.77 12.50 9.53 0.00 0.00 0.00 28.57 14.29 100.00
YE 15	6.67 23.08 0.00 0.00 25.00 5.27 9.09 0.00 8.33 7.15 0.00 8.33 100.00
YE 16	6.67 14.29 0.00 6.67 36.37 11.11 20.00 10.00 0.00 7.15 0.00 8.33 66.67 100.00
YE 13	11.77 58.33 15.39 18.75 38.47 9.53 15.39 7.69 23.08 5.89 14.29 6.67 44.45 44.45 100.00
YE 14	17.65 25.00 6.67 5.27 35.72 14.29 14.29 15.39 21.43 5.56 6.25 6.25 40.00 27.27 70.00 100.00
YE 11	31.58 47.06 16.67 19.05 33.33 11.54 31.25 33.33 22.23 20.00 22.23 10.00 18.75 26.67 29.41 43.75 100.00
YE 67	13.33 6.25 8.33 6.25 14.29 16.67 18.19 9.09 16.67 14.29 7.69 7.69 10.00 37.50 16.67 15.39 17.65 100.00
YE 44	12.50 5.89 7.69 5.89 13.33 15.79 16.67 8.33 15.39 21.43 7.15 15.39 20.00 33.33 25.00 23.08 23.53 85.72 100.00
YE 6	26.32 20.00 17.65 20.00 27.78 21.74 5.27 11.77 10.53 21.05 16.67 16.67 0.00 5.89 10.53 29.41 17.39 26.67 33.33 100.00
YE 65	13.05 8.33 4.77 23.81 13.64 25.00 15.79 10.53 9.53 25.00 15.00 21.05 5.27 5.27 9.53 14.29 16.00 10.53 15.79 27.27 100.00
YE 69	14.29 4.35 5.27 14.29 15.00 27.27 11.11 11.77 10.53 21.05 16.67 23.53 5.89 5.89 10.53 10.00 12.50 11.77 17.65 23.81 86.67 100.00
YE 4	12.50 0.00 7.69 12.50 6.25 15.79 16.67 30.00 15.39 13.33 15.39 25.00 0.00 0.00 0.00 14.29 23.53 8.33 7.69 17.65 46.67 42.86 100.00
YE 3	15.00 21.05 11.77 9.53 22.23 17.39 18.75 12.50 17.65 29.41 11.11 17.65 6.25 6.25 17.65 10.53 13.05 5.89 11.77 25.00 35.00 38.89 26.67 100.00
YE 63	0.00 0.00 0.00 13.33 6.67 10.53 0.00 0.00 7.69 14.29 16.67 16.67 0.00 0.00 0.00 7.15 11.11 9.09 8.33 5.56 31.25 26.67 30.00 12.50 100.00
YE 64	0.00 0.00 0.00 6.67 7.15 5.27 0.00 0.00 8.33 7.15 8.33 8.33 0.00 0.00 0.00 7.69 11.77 10.00 9.09 5.89 25.00 20.00 33.33 13.33 83.33 100.00
YE 1	6.25 13.33 0.00 6.25 6.67 10.53 8.33 9.09 7.69 14.29 7.69 16.67 0.00 0.00 0.00 15.39 11.11 9.09 18.19 11.77 31.25 26.67 44.45 12.50 71.43 83.33 100.00
Control	0.00 0.00 0.00 11.77 0.00 4.55 7.15 7.69 6.67 12.50 6.67 14.29 0.00 0.00 6.67 21.43 15.79 7.69 0.00 10.53 35.30 16.67 25.00 5.27 75.00 62.50 75.00 100.00

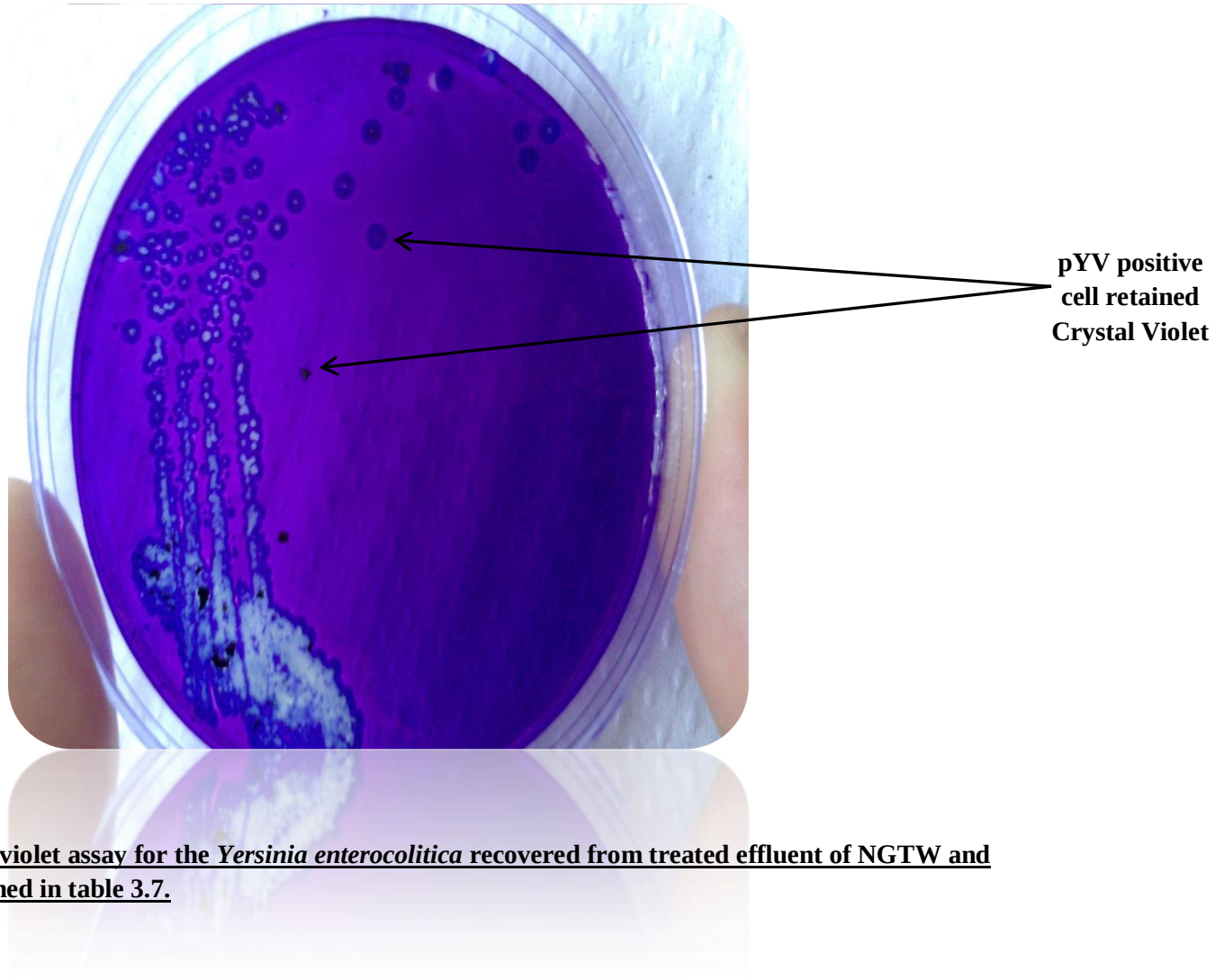


Fig 1: Crystal violet assay for the *Yersinia enterocolitica* recovered from treated effluent of NGTW and NWWTP defined in table 3.7.

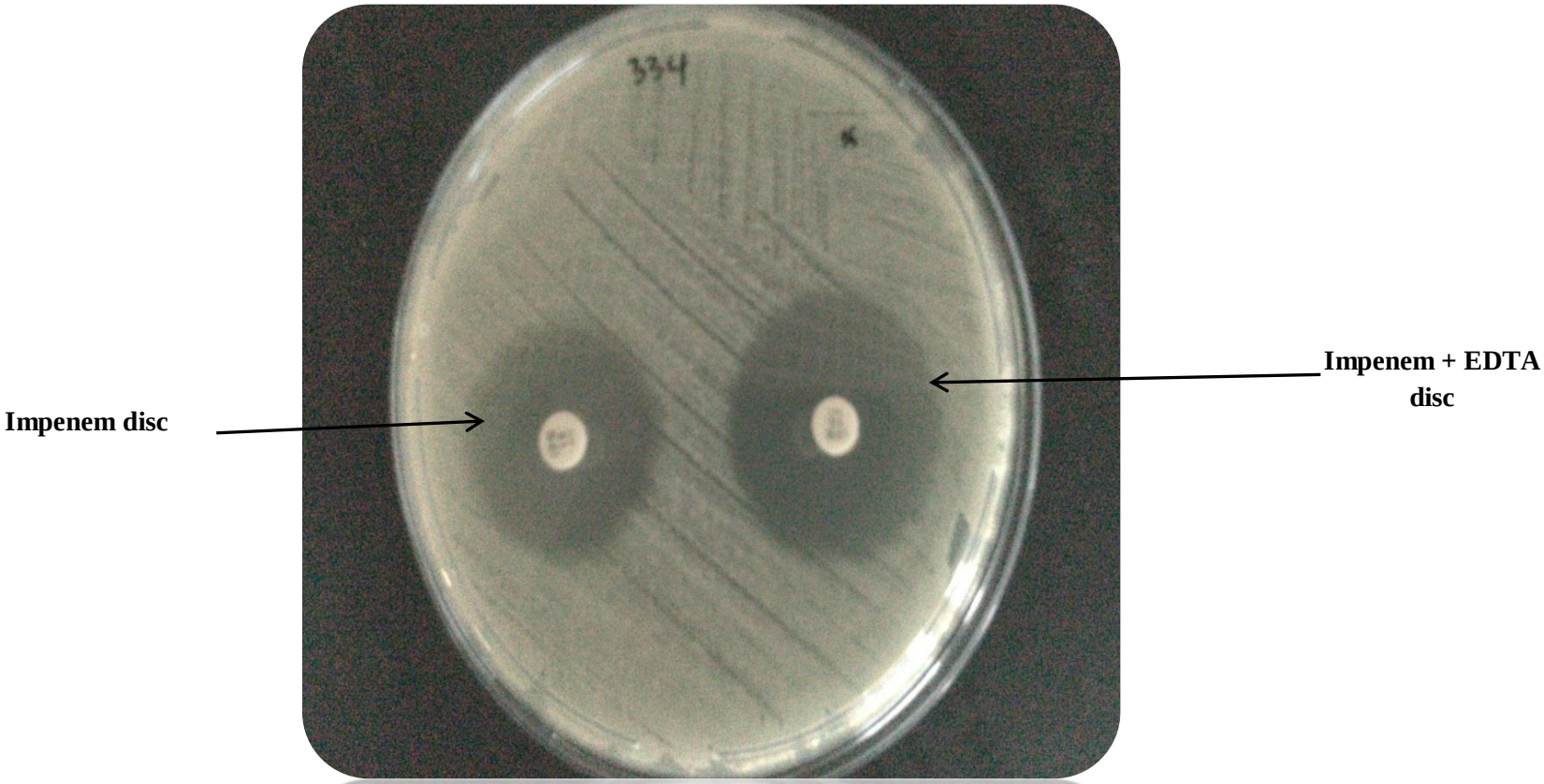


Figure 2: Metallo β- lactamase production assay of *Yersinia enterocolitica* recovered from NGTW and NWWTP defined table 3.7.