TREATED WASTEWATER EFFLUENT AS A POTENTIAL SOURCE OF EMERGING BACTERIAL PATHOGENS IN SURFACE WATER

SIPHEPHILE B. T. NZIMANDE

Submitted in fulfilment of 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, University of KwaZulu-Natal (Westville Campus), Durban.

As the candidate's supervisor, I have approved this dissertation for submission.

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

PREFACE

The experimental work describe in this dissertation was carried out in the School of Life Sciences, University of KwaZulu-Natal (Westville Campus), Durban, South Africa from March 2012-December 2013, under the supervision of Professor A. O. Olaniran.

These studies represent the original work by the author and have not otherwise 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 has been duly acknowledged in the text.

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I……. 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.
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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: Siphephile B. T. Nzimande and Ademola O. Olaniran (2014). Adaptation and survival mechanisms of emerging bacterial pathogens in surface waters. *African Journal of Microbiology Research* (Submitted).

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Siphephile B. T. Nzimande

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-I never knew the strength in my wings until the day I decided to jump and immediately felt the wind carry me. The endless support, love and encouragement has been that undying wind-

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ABSTRACT

Rivers and estuaries are the major sources of the earth's fresh water, and are also the major recipient of treated wastewater effluent discharge, thus leading to their deterioration. This study was aimed at evaluating the microbiological and physico-chemical quality of final effluents of two independent wastewater treatment plants within the eThekwini Municipality as well as their effect on the receiving water bodies. Water samples were collected over a 12 month sampling period at designated points: before and after tertiary treatment, as well as upstream and downstream of the receiving rivers. Bacterial analyses of collected samples were conducted using standard membrane filtration methods and selective media, allowing for the presumptive enumeration of prevalent emerging bacterial pathogens. Analysis of the samples revealed that the effluent samples were not of acceptable standards for most of the parameters analysed throughout the sampling period. Percentage bacterial reduction varied between 19.5 – 99.9%, 23.3 – 99.9%, 8.2 – 99%, 29.1 – 99.9%, and 2.4 – 99% for *Aeromonas* spp., *Pseudomonas* spp., *Listeria* spp., *Yersinia* spp. and *Legionella* spp. respectively after chlorination. Temperature, pH, dissolved oxygen, biochemical oxygen demand, chemical oxygen demand, total dissolved solids values ranged between 12 – 27 °C, 6.41 – 7.88, 4.01 – 7.66, 1.40 – 9.61, <10 – 309.06, and 200.03 – 710.00 mg/L, respectively, while turbidity and total suspended solids ranged between 6.48 – 71.02 NTU and 0.01 – 5.93 mg/L respectively, across the sampling period. Presumptive *Aeromonas* spp. and *Listeria* spp. recovered from the water samples were further identified using biochemical tests and PCR methods, followed by the construction of antibiograms of all confirmed isolates, with some isolates showing resistance to a number of commonly used antibiotics. For the tested *Aeromonas* species, complete resistance was observed against ampicillin, penicillin, vancomycin, clindamycin and fusidic acid, followed by cephalosporin (82%), and

erythromycin (58%), with 56% of the isolates found to be resistant to nalidixic acid and trimethoprim, the antibiotic resistance index (ARI) ranging from 0.25 – 0.58. *Listeria* spp. displayed the highest resistance against penicillin, erythromycin and nalidixic acid, with all 78 (100%) tested species displaying resistance, followed by ampicillin, trimethoprim, nitrofurantoin and cephalosporin with 83.33%, 67.95%, 64.10% and 60.26%, respectively. The ARI for the *Listeria* spp. ranged between 0.13 (resistance to 3 test antibiotics) – 0.5 (resistance to 12 of the test antibiotics). Characterization of the virulence gene markers and enzymes in *Aeromonas* and *Listeria* species confirmed the level of potential pathogenicity of the isolates. Of the 78 tested *Listeria* spp., a total of 26.92% (21) were found to contain virulence genes, 14.10% (11), 5.12% (4) and 21% (17) of these species were found to harbour the *act*A, *plc*A and *iap* genes respectively, while 11.54% (9) contained more than one virulence gene. Of the 100 tested *Aeromonas* spp., 52% harboured the *aer*, while 68% tested positive for the *lip* virulence associated gene. In addition, up to 35% of the *Listeria* spp. were positive for haemolysin enzyme and negative for gelatinase and *protease*, while 57%, 81% and 100% of the *Aeromonas* spp. were positive for haemolysin, gelatinase and protease enzyme. The prevalence of these emerging pathogens in treated effluent presents a potential threat to the health of surrounding communities, considering that they are not included in the current guidelines and therefore cannot be monitored. The obtained results further highlight the need for revised standards which include emerging pathogens.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1. Introduction

Water paucity and increasing human populations in developing countries, have applied additional pressure on available water resources, consequently leading to an increased dependence on river and estuary waters. This is of increasing human and public health concerns considering that the majority of the available water sources are often polluted, harbouring copious levels of contaminants. The commonly found contaminants range broadly from pathogenic microorganisms to highly toxic chemical substances, which have a negative impact not only on humans, but also on animals and other ecological and chemical pathways in water ecosystems (Egun, 2010; Luger and Brown, 2010; UNEP 2012). Furthermore, numerous studies have shown that fresh water sources are of poor microbial and physico-chemical quality, thus posing a greater threat than previously thought (Crockett, 2007).

Freshwater pollution is frequently related to the continued discharge of improperly treated effluent from wastewater treatment plants into freshwater sources (Hoogeboezem, 2007; Odjadjare and Okoh, 2010). This has been linked with inadequately operated treatment plants, ineffective and outdated treatment processes, as well as improper pathogen monitoring techniques. Wastewater treatment processes therefore continues to be amongst the main concerns all over the world, since water is a primary need for survival, thus requiring that great measures be taken to ensure hazard-free reuse and redistribution of the treated wastewater effluent.

Inadequately treated wastewater consists of vast quantities of bacteria, some of which are harmless to man, however, some emerging bacterial pathogens have been documented to cause serious diseases, some which may result in serious infections and at worst, death if untreated (Rollins *et al*., 2003). Exposure to pathogen contaminated water also increases infection probability as well as cases of disease outbreak, especially in poorer communities with suppressed or compromised immune systems, more so in underdeveloped regions in South Africa (Egun, 2010; Luger and Brown, 2010; Momba and Kaleni, 2002; Philippaeux, 1998; UNEP 2010; Venter *et al.,* 2007; WHO, 2008; WSSCC, 2008).

Pathogens have been in existence for centuries, dating back to the biblical ages where plagues and pandemics were on the rise and spreading, resulting in the death of millions of people (Morens *et al*., 2004). Black Death, measles, small pox, and HIV/AIDS are some of the few pandemics that caused worldwide confusion for ages, since most of the causatives had not been identified or characterised then (WHO, 2008). Treatment became available before the 1950's, with these years being widely known as the breakthrough years in infections and diseases with the discovery of penicillin, drugs and vaccines (Morens *et al*., 2004). In spite of these new discoveries and developments, the sudden occurrence of emerging infections did not cease to result in massive deaths all around the world, as microbes evolved with the times (Morato *et al*., 2003). Till this day and age the fight between microbes and the human race continues as the discovery of emerging infections reveals that they are multiple steps ahead of common human understanding (Abraham, 2011; Crockett *et al*., 2007).

The prevalence of emerging bacterial pathogens in treated wastewater effluent is of concern, since presence of these organisms cannot be tracked during normal treatment processes. this is due to the fact that South Africa currently has no set guidelines for emerging bacterial

pathogens, leading to their release and dissemination into the environment unmonitored (Bartie *et al.*, 2002). Some of these microorganisms also display high microbial or antibiotic resistance trend, which can be linked to the acquisition of resistance genes due to increased usage of these agents in the medical, agricultural and veterinary applications (Goni-Urriza *et al*., 2000; Huddleston *et al*., 2006). Their adaptation to their environment and acquisition of resistance and virulence genes further aid in their ability to escape conventional treatment processes (Almuzara *et al*., 2007). Eze *et al*. (2009) suggested that even highly vigorous conventional water treatment will struggle to remove more that 99% of emerging bacterial pathogens on a routine basis. Reports of emerging bacterial pathogens which have become resistant to disinfection process of conventional water and wastewater treatment systems are common (Cabral, 2010; Fischbach *et al.*, 2009). Some of these pathogens have been shown to thrive in the presence of common disinfectant agents, such as chlorine and ozone used for the treatment of wastewater (Odjadjare *et al*., 2012). The frequent occurrence of these pathogens in the environment and the continuous battle to eradicate them in water sources reveals that these microorganisms have developed survival strategies that promote their exponential growth and reproduction (Morens et al., 2004).

1.1 Important emerging water-borne bacterial pathogens

1.1.1 *Aeromonas* spp.

Aeromonas species are Gram-negative, rod-shaped organisms, which in the recent years have gained recognition in the public health sector as opportunistic organisms (Cabral, 2010). Infections caused by this organism result in primary or secondary septicaemia, red sore disease and other water associated wound infections (skin and soft tissue infections), eye infection, meningitis and pneumonia as well as bone and joint enteritis among other

infections (Sartory *et al*., 1996). *Aeromonas* spp. is the main causative agent of childhood diarrhoea, fatal gastric variceal and clinical syndromes inevitably arise in immune compromised patients. *Aeromonas* spp. have previously been isolated from patients with diarrhoea in the presence or absence of other enteropathogens, but its involvement in gastro enteritis remains unknown (Obi *et al*., 2007; Vila *et al*., 2003).

1.1.2 *Pseudomonas* spp.

Pseudomonas species are Gram-negative, rod shaped and the most vigorous, fast-swimming bacteria responsible for causing urinary tract infections, dermatitis, bone and joint infections, gastrointestinal infections and systemic infections and are known to evade host defences. *Pseudomonas* spp. infections cause death in cystic fibrosis (CF) disease patients. They are responsible for the production of Toxin A, which is a potential cytotoxin, lethal to a variety of DNA chimas and also have the ability to cause disease by inhibiting protein synthesis, direct cytopathic effects and interferes with the immune function of the host which ultimately leads to death (Picardo and Giroux, 2004). *Pseudomonas aeruginosa* is one of the most resistant emerging pathogens and has been found to contain intrinsic resistance factors (Langsrud *et al*., 2003a; Dreeszen, 2003).

1.1.3 *Listeria* spp.

Listeria species were for a while only known to be associated with food and were therefore classified as food pathogens, until their discovery in water in recent years (Paillard *et al*., 2005). *L. monocytogenes,* the common human pathogen can cause meningitis, encephalitis, septicaemia, abortion, premature birth, stillbirth and abscesses. It may also give rise to gastroenteritis or an influenza-like disease. The worldwide case fatality rate for listeriosis is

estimated to be as high as 36%, among the risk groups, which include neonates, elderly people, pregnant women, and immuno-compromised individuals. Listeriosis has flu-like symptoms (muscle ache, fever) and gastroenteritis which may spread to the nervous system (invasive infection) and may lead to headache, confusion, loss of balance and convulsions (Arslan and özdemir, 2008). *Listeria monocytogenes* is tolerant to extreme pH, temperature and salt concentration, and causes major problems in pregnant women, neonates, the elderly, cancer and diabetic patients and immuno-compromised individuals (Paillard *et al*., 2005). Without antibiotic treatment, septicaemia, meningitis, encephalitis, abortion and ultimately death can occur. About 2400 cases of listeriosis are reported annually in the US alone (Mead *et al*., 1999). One of the biggest listeriosis outbreaks in history was reported in the 1980's in Los Angeles resulting in a 63% fatality rate in neonatal foetal infections and 37% in pregnant women with immune-compromised among the infected (Linnan *et al*, 1988).

1.1.4 *Yersinia* spp.

Yersinia species are mainly associated with food-borne illnesses but have lately been discovered in sewage and faecal polluted waters (Rollins *et al*., 2003). These are invasive pathogens with the ability to penetrate the stomach lining and gain entry into the lymphatic system and blood, resulting in Yersinosis. This results in intestinal inflammation due to the release of toxins which prevent electron transport chain function (Sinha *et al*., 2000). Symptoms include watery diarrhoea, abdominal pain, fever and arthritis. Swelling of lymph nodes results in death within a week of initial infection. *Yersinia* species survive for longer periods of time in aquatic environments and at low temperatures such as 4 °C for as long as 18 months at both alkaline and neutral pH (Perdek *et al*., 2002). Yersinosis plagues have a 40% - 60% fatality rate with untreated septicaemia and pneumonia cases resulting in 100% mortality while treated cases have a 30% - 50% mortality rate (Rollins *et al*., 2003). These

organisms have the ability to survive in different domestic animals including dogs, cats, pigs, rodents, rabbits, sheep, cattle, horses, and cats.

1.1.5 *Legionella* spp.

Legionella pneumophila is a motile, rod-shaped, Gram-negative, aerobic bacterium. *Legionella* grows in warm aquatic environments with rust, algae, and organic particles. The organism can survive in tap water at room temperature for over a year, with some *Legionella* species also found to survive for as long as 2.5 years in low and nutrient limiting environments, after being released into the environment (Palmer *et al*., 1995). Waterborne associated outbreaks of some of these organisms have been recorded in history; however the link between their occurrence in the environment and the actual cause is vague (Crittenden *et al*., 2005).

1.2 Possible sources of emerging bacterial pathogens in surface water

Rivers and estuaries are the major sources of the earth's fresh water and at the same time the recipients of wastewater effluent. These fresh water sources are highly impacted by the amount of pollution they are exposed to, including pollution from surrounding communities, floods, illegal waste discharge from hospitals, industrial companies as well as wastewater effluent discharge which is not up to the expected standards (UNEP, 2010).

1.2.1 Improper sanitation, illegal dumping, floods and overpopulation

The increasing numbers and complex dynamics in human populations is a huge contributing factor to the global water crisis and developing countries seem to be the most affected (Frederiksen, 1996). Urbanization and industrialisation are also major contributing factors to the pressure placed on all water sources, as well as the pollution thereof (UNEP, 2010). The United Nations Environmental Programme has predicted that the world population is bound to exceed 9 billion people with urban populations expected to double by the year 2050, and one fifth of the population will be living near the coast by the year 2015. Currently almost 900 million people do not have access to safe water worldwide, while 2.6 million lack adequate sanitation. On the 21st March 2012, the South African Human Rights Commission highlighted that 16 million South Africans still do not have adequate access to safe water and sanitation, compounding the problem of waterborne disease (NICD, 2012).

Urbanisation has resulted in the observable population rise in most cities and urban areas worldwide. Poorer communities living in undeveloped areas therefore flock to the cities which are already highly populated in pursuit of civilisation which promises life of improved living and higher rates of employment. Over population and limited land space has inevitably led to the building and increase of sprawling informal settlements which are overpopulated, and mostly lack any form of basic sanitation and drinking water facilities as well as waste dumping or collection systems (UNEP, 2010). This answers why most informal settlements are usually established around or in the vicinity of rivers, which are then utilised and exploited to suit the multiple needs of the surrounding communities such as dumping, sanitation and the use of the same water for basic housekeeping needs such as bathing, cooking and laundry (Odjadjare *et al*., 2010). Storm water runoff into rivers can contribute to faecal pollution not only from surrounding human populations, but also from diverse sources such as domestic animals and wildlife (Santo Domingo and Edge, 2010).

1.2.2 Untreated sewage discharge

Two million tons of sewage wastes enter the world's waterways on a daily basis in developing countries , 90% of these sewage wastes are not treated and are discharged directly into nearby rivers (UNEP, 2010). Waterborne pathogens of faecal origin continue to contribute largely to the world wide outbreaks of infectious disease in many areas around the world. While developed countries have placed importance on the monitoring and protection of surface water and also made much progress in municipal wastewater treatment and management of agricultural wastes, faecal contamination of drinking and recreational water sorces still contribute to outbreaks of infectious diseases (Santo Domingo and Edge, 2010).

1.2.3 Wastewater effluent

The discharge of inadequately treated sewage effluent into rivers and estuaries has a huge impact on the health of surrounding human populations and ecosystems (Luger and Brown, 2010). The poor quality of raw and treated wastewater has customarily been considered as a source of pathogenic microorganisms. Sewage and wastewater treatment plants have shown to be rich reservoirs of antibiotics because the antibiotics that are utilised for treatment of infections are not all processed by human bodies. Some of them are expelled as waste and end up in wastewater treatment plants (Adetunji and Isola 2011; Jury *et al*., 2010). Not only are wastewater treatment plants great sources of emerging bacterial pathogens, they are also responsible for the harbouring and later release of multidrug resistant microorganisms. The prevalence and survival of pathogens after treatment processes poses a major threat to both the receiving environment and ecosystems, most importantly to the receiving communities primarily dependant on these waters (Luger and Brown, 2010).

It was traditionally believed that discharge of 200 cfu/ 100 mL faecal coliforms in wastewater effluent had none or limited downstream microbial effects, due to the dilution effect that the water source has on the incoming effluent (Crockett, 2007). This was closely linked to the belief that infection of human beings occurred only when large microbial doses were ingested. However, the increasing resistance to standard water and wastewater treatment processes further challenges the traditional methods and beliefs. Emerging pathogens exhibit extended survival periods in these adverse environments, proving to affect sensitive subpopulations (immuno-compromised, elderly, pregnant and children), and require extremely low doses for human infection (Crockett, 2007). Traditional methods are progressively moving into the irrelevant and ineffective phase, as the emerging bacterial pathogens become more resilient, gain resistance and adapting to the most adverse environments (Cabral, 2010).

1.2.4 Hospital effluent

Hospital effluent has a major effect on microbial population dynamics and is known to contain microbiological contaminants including important microbial pathogens (Hoogeboezem, 2007). Bacteria can develop resistance to certain antibiotics, based on the frequency of exposure and therefore survive treatment with antibiotics which is an increasing problem in hospitals. They cause infections which are often difficult to treat with the common drugs, as multidrug resistance is developed. Bendt *et al*. (2002) studied the behaviour of antimicrobial resistant bacteria in sewage treatment plants and observed varying concentrations of these resistant microorganisms, with the highest numbers of multidrug resistant groups obtained from hospital sewage. These resistant organisms portrayed the ability to genetically transmit their antibiotic resistance genes via horizontal transmission,

transmission occurring more frequently between organisms or hospital effluent origin and other sources (Hoogeboezem, 2007).

1.2.5 Wastewater treatment processes and emerging bacterial pathogen removal

1.2.5.1 Wastewater treatment processes

Wastewater treatment facilities were established with the aim of protecting freshwater sources, the environment and the public (surrounding human population) against any harmful substances which are transported by a plethora of contaminants present in wastewater (Crockett 2007, Shatanawi *et al*., 2006). Conventional treatment is therefore accomplished by the removal of substances that have a high oxygen demand 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*., 2011). The main function of wastewater treatment is to remove solid, organic and microbiological components that may result in the pollution of the receiving water body. All wastewater treatment facilities have set standards and guidelines to ensure that the treated effluent is safe for release (Scott *et al.*, 2003). Additional consideration is given to chemical components such as ammonia, nitrate, and phosphorus, microorganisms, specific organic pollutants and metals depending on the size of the treatment facilities, the different treatment steps and the nature of the discharge.

Processes which are commonly applied in wastewater treatment include: screening, coarse solids reduction, grit removal, sedimentation, biological treatment as well as filtration (MWSF, 2011; Okoh, *et al.*, 2007). The majority of the processes work through the

application of a physical force and are collectively known as physical processes. A biological reaction coupled to an adsorption step allows micro-organisms to make use of components as part of their growth cycle by converting dissolved organic components to solids for further removal in downstream physical processes (Ashbolt, 2003).

1.2.5.1.1 Primary treatment

The primary treatment step which is the second step in treatment involves the separation of suspended solid matter and grease (USEPA, 2004). Influent is subjected to primary and secondary treatments steps which are commonly combined as a single basic treatment step, after it has been passed through a number of pre-treatment steps. Following the screening and grit removal process, the dissolved organic and inorganic constituents along with suspended solids are then removed. Suspended solids are the small particulate matter removed by; sedimentation, gravity settling, chemical coagulation, or filtration. The wastewater is channelled into a sedimentation tank, slowing down the water flow as the dissolved pollutants and suspended solids which could not be removed in the previous step gradually sink to the bottom. The settled particles collectively form a slurry of collective matter known as primary sludge which can then be removed from the tank by various methods (Environmental Canada, 2003).

1.2.5.1.2 Secondary treatment

Secondary treatment is a biological treatment process involving the addition of microorganisms which utilise the organic matter present as food supply and energy source, resulting in the removal of 90% dissolved organic matter from the wastewater (USEPA, 2004). Attached growth processes, suspended growth processes and lagoon systems are the three most common conventional methods used during secondary treatment (Upadhyay *et al*.,

2007). Attached growth process units include trickling filter, bio-towers and rotating biological contactors and involves microbial growth on surfaces. Wastewater passes over the media along with air to provide oxygen required for growth of the microorganisms, which are responsible for removing biodegradable organic material from the wastewater (Environmental Canada, 2003). Suspended growth processes remove biodegradable organic material and organic nitrogen-containing material by converting ammonia nitrogen to nitrate. In this growth processes, microbial growth is suspended in an aerated water mixture where air is pumped in, or the water is agitated sufficiently to allow oxygen transfer. Suspended growth process unit include variations of activated sludge, oxidation ditches and sequencing batch reactor (Mbwele *et al*., 2003). A wastewater lagoon or treatment pond is a scientifically constructed pond, three to five feet deep, that allows sunlight, algae, bacteria and oxygen to interact. Biological and physical treatment processes occur in the lagoon to improve water quality. The quality of water leaving the lagoon, when constructed and operated properly, is considered equivalent to the effluent from a conventional secondary treatment system. Lagoons remove biodegradable organic material and some of the nitrogen from wastewater (Larsdotter *et al*., 2003).

1.2.5.1.3 Tertiary treatment

Tertiary treatment is the additional treatment required to remove suspended and dissolved substances remaining after secondary treatment. This is accomplished using a variety of physical, chemical or biological treatment processes to remove the target pollutants (Environment Canada, 2003). Tertiary treatment include: Filtration, removal of ammonia and other specific contaminants and disinfection to destroy pathogens (MWSF, 2011; Okoh *et al*., 2007). After the treatment, wastewater effluent may contain pathogens, due to the variability in the treatment processes and constantly changing state of microorganisms. Processes used to kill or deactivate these harmful organisms are called disinfection. Chlorine is the most

widely used with ozone and ultraviolet radiation also frequently used for wastewater effluent disinfection (Hijnen *et al.,* 2006). Chlorine kills microorganisms by attacking the cell membrane structure and in turn affecting internal cellular materials and can be applied to wastewater as a gas, liquid or in a solid form. However, any free chlorine remaining in the water, even at low concentrations, is highly toxic to beneficial aquatic life (Hijnen *et al*., 2006). Therefore, removal of even trace amounts of free chlorine by dechlorination is often needed to protect fish and aquatic life. Ozone is another disinfectant normally used, and it is produced from oxygen exposed to a high voltage current. Ozone is very effective at destroying and killing viruses, bacteria and decomposes back to oxygen rapidly without leaving harmful by-products. Ultraviolet (UV) radiation disinfection is a physical treatment process that leaves no chemical traces. Organisms can sometimes repair and reverse the destructive effects of UV when applied at low doses. Furthermore, UV can only be applied on small scale basis (Hoyer, 2004).

1.2.5.2 Ineffectiveness of wastewater treatment plants for removal of emerging bacterial pathogens

Pathogens have evolved and adapted with their environment, resulting in decreasing treatment quality and an increase in the prevalence of infectious and viable pathogens (Abraham, 2011). Recent data suggests that even highly vigorous conventional water treatment will struggle to remove more that 99% of emerging bacterial pathogens on a routine basis (Eze *et al*., 2009). This is of great concern since the prevalence of pathogens in treated wastewater effluent discharged into surrounding water bodies is determined by the treatment efficiency. The occurrence of these pathogens in the environment and the continuous struggle to eradicate them in water sources reveals that these microorganisms have developed survival strategies that promote their growth and reproduction (Morens *et al*., 2004).

These adaptive and survival mechanisms can be expressed in a number of ways within the cell which has become resistant to disinfection (Morato *et al*., 2003). With this knowledge, one cannot ignore how these emerging bacterial pathogens have gained resistance to conventional wastewater treatment processes. Genetic changes and defence mechanisms have been acquired to make them more resistant, and in turn more prevalent in wastewater effluent and the environment in which they are discharged (Poole, 2005). Most of these microorganisms have been discovered to be highly resistant to a broad range of antibiotics, including other antimicrobial agents. This has been linked to the increased usage of these agents in the medical, agricultural and veterinary sectors, and has resulted in these microorganisms obtaining resistance genes (Goni-Urriza *et al*., 2000; Huddleston *et al*., 2006). The mechanisms of resistance against commonly used disinfectants include: Limited diffusion of antimicrobial agents through the biofilm matrix, interaction of the antimicrobial agents with the bioflm matrix (cells and polymer), enzyme mediated resistance, level of metabolic activity within the biofilm, genetic adaptation, efflux pumps and outer membrane structure. This further support and highlights the need for revision of the currently used wastewater treatment guidelines (Eze *et al*., 2009; Grobe *et al*., 2002; Langsrud *et al*., 2003a; Purkrtová *et al*., 2010; Sarme *et al*., 2010).

1.3 Prevalence of emerging bacterial pathogens in treated waters

The main concern in water and wastewater treatment processes is that many water-borne pathogens have adapted to their environment and have acquired resistance and virulence genes which are widely expressed in these stressful environments. As a result they now have the ability to escape conventional treatment processes (Almuzara *et al*., 2007). Emerging bacterial pathogens have become more resistant to disinfectants and other antimicrobial

agents when compared to coliform microorganisms which are commonly used as the indicators of water quality (Eze *et al*., 2009). This also implies that even in the absence of coliform indicators, emerging bacterial pathogens are still present in their numbers. Their presence in the absence of indicator microorganisms is common and in most cases they are even found dominating drinking water systems (Eze *et al*., 2009; Toze, 1997). The fact that these pathogens are also found to be present in drinking tap water which is meant to be safe and pathogen free, further supports that pathogens have become tougher and are difficult to eradicate. According to the safe water act, the maximum contamination levels for safe drinking water should be zero for all pathogens: "no tolerable lower limit" (Ashbolt, 2004; Bressler *et al*., 2009; WHO, 2008). This however remains as a mere standard which is not met, with the increasing occurrence and discovery of pathogens in many conducted studies (Khabo-Mmekoa, 2010).

A study conducted by Momba *et al*. (2006) showed that the quality of drinking water in South Africa is generally poor and therefore unfit for human consumption, especially in peri-urban, rural and remote areas with inadequate water supply services. Another study conducted by Khabo-Mmekoa *et al.* (2010) revealed that all drinking water samples collected from: Gamalakhe, Boboyi, Margate, Portshepstone, Bomela, Annelin, and Hibberdene in South Africa were predominantly contaminated with *Pseudomonas aeruginosa* and *Enterobacter* species. These microbial populations were also found to be dominant in the stools of residents of the above mentioned communities. Table 1.1 shows the population of some emerging bacterial pathogens in treated effluent and in some cases in drinking water sources, further reiterating the inefficiency of the treatment or disinfection processes.

Table 1.1: The concentrations of some emerging bacterial pathogens in surface water, drinking water and treated wastewater effluent.

Organism	Bacterial counts	Water sources*	References
***Listeria* spp.**	2.9 x 10^0 - 1.2 × 10^5 CFU/ml	E	Odjadjare *et al*., 2010
	<0.3 to 2.1×10^1 MPN/ml	E	Paillard *et al*., 2005
	5.5 × 10^{-1}MPN/ml	E	Combarro *et al*., 1997
	3.9 × 10^1 MPN/ml	E	Al-Ghazali *et al*., 1988
***Pseudomonas* spp.**	1.0 × 10^8 CFU/cm^2	D	Bressler *et al*., 2009
	1.20 × 10^4 (CFU/100 mL)	E	Odjadjare *et al*., 2012
	1.08 × 10^4 (CFU/100 mL)	E	
	2.66 × 10^4 (CFU/100 mL	E	
***Aeromonas* spp.**	1 × 10^4- 10^7 CFU/ml	S	Sortery *et al*., 1996
	20 CFU/100 ml	D	Pablos *et al*., 2009
	420 CFU/ml	E	Poffe *et al*., 1991
	75 CFU/ml	E	
***Legionella* spp.**	500 CFU/ml	S	Palmer, 1993

***S= surface water E= wastewater effluent D= drinking water**

Microorganisms that bypass treatment processes have been found to be more virulent and have also been found as the main causatives of infections which cannot be easily treated (Kaye *et al*., 2004). Odjadjare *et al*. (2010) tested *Listeria* spp. against an increasing concentration of chlorine, the commonly used disinfectant in treatment plants and found that they were not affected by the presence of chlorine, which should decrease the bacterial load with increasing chlorine concentrations as for most bacteria (AWWA, 1999; Tree *et al*.,

2003). It is therefore evident that the ineffectiveness of these established processes poses and presents a health threat worldwide (Obi *et al*., 2002).

1.4 Implications of emerging bacterial pathogens in human health

1.4.1 Epidemiology and disease outbreaks

Diseases caused by bacteria are some of the most common health hazards associated with untreated drinking and recreational waters. Many microbial pathogens in wastewater can cause chronic diseases (Table 1.2) with costly long-term effects, such as degenerative heart disease and stomach ulcer (Toze, 1997). In South Africa, the incidence of diarrhoeal disease doubled from 128.7 children below the age of 5 per 1000 in 2004 to 268.7 per 1000 in 2005. In addition, the 2006 South African Health Report attributed 15% of mortality in children below the age of 5 to gastroenteritis, second only to lower respiratory tract infections (NICD, 2012).

Table 1.2: Acute and chronic health effect associated with microbial pathogens commonly found in water.

Pathogen	Acute effects	References
Vibrio cholera	Diarrhoea, cholera	CDC (1997)
Helicobacter pylori	Gastritis lcers and stomach cancer	
Cyanobacter	Diarrhea and potential fever	
Salmonella paratyphi	Paratyphoid fever, diarrhea	Ashbolt (2004); CDC (1997)
Legionella pneumonia	Pneumonia, respiratory illness (legionellosis)	
Yersinia	Diarrhea, gastroenteritis, reactive fever	Ashbolt (2004); CDC (1997); Sinha *et al*. (2000)
Aeromonas	Pneumonia, meningitis	Sartory *et al*. (1996)
Listeria monocytogenes	Meningitis, encephalitis, septicaemia, gastroenteritis	Arslan and özdemir (2008); Paillard *et al*. (2005)
Pseudomonas	Urinary tract infection, dermatitis, gastroenteritis	Pircardo and Giroux (2004)
Shigella spp.	Bacillary dysentery	Ashbolt (2004)
Enteropathogenic *E. coli*	Gastroenteritis	
Campylobacter jejuni	Gastroenteritis	
Various mycobacteria	Pulmonary illness	
Salmonella typhi	Typhoid fever, diarrhea	

A study conducted by Obi *et al*. (2007) presented the percentage of *Aeromonas* species isolated from patients with diarrhea in the Vhembe district of South Africa. Although the source of infection was unclear, the most common source of infection results from the consumption of contaminated water. Wastewater consists of vast quantities of bacteria, most of which are harmless to man. Some of these emerging bacterial pathogens have been documented to cause serious diseases, some which may result in death if ignored and untreated e.g. *Yersinia* species infections cause septicaemia and pneumonia resulting in 100% mortality if untreated (Rollins *et al*., 2003). Certain strains of *Pseudomonas* have been reported as the main causatives of gastroenteritis and other gastrointestinal disease outbreaks in immuno-compromised individuals (Metcalf and Eddy, 2003). The potential severity of listeriosis outbreak on the public health is disquieting since there is absence of information on the prevalence of this pathogen in South Africa. The fact that *Listeria* is not considered a waterborne pathogen globally in spite of reports in the literature suggesting that the pathogen is well established in the water supply chain is also of grave concern (Odjadjare *et al*., 2010).

Nutrients which may be contained in wastewater, especially nitrogen and phosphorus, stimulate the growth of toxic species of phytoplankton in both fresh and marine waters and the consumption of toxic algae or organisms that feed on these nutrients can cause serious harm to humans and other terrestrial animals (Akpor and Muchie, 2011). The toxins arising from these chemical compounds also have the ability to cause gastroenteritis, liver damage, nervous system impairment and skin irritation. Health problems associated with cyanotoxins have been reported in numerous countries, including Australia, Brazil, Canada, China, United Kingdom, United States of America and Zimbabwe (Department of Natural Science, 2006; WHO, 2006). Nitrate has not been found to be harmful, however its derivatives are potentially toxic. A quarter of ingested nitrate is converted to nitrite by microorganisms in the

saliva of animals. Once it reaches the bloodstream, the ability of blood to carry oxygen by converting haemoglobin into methemoglobin is impaired by nitrite. Ingestion of large amounts of nitrate or nitrite can result in methemoglobinemia in infants and susceptible individuals (WHO, 1997; Wigle 1992). Nitrites react with amino acids in the stomach to form nitrosamines, which have been found to be extremely harmful carcinogens in animals and humans (Fraser, 1995; El-Bahri *et al*., 1997; Runion, 2008).

Wastewater effluents have been shown to contain a variety of anthropogenic compounds, many of which have endocrine-disrupting properties. Reports have shown that exposure to wastewater treatment effluents containing estrogenic chemicals can disrupt the endocrine functioning of aquatic life, thus can cause permanent alterations in the structure and function of the reproductive system (Hoogenboezem, 2007). Individuals can be exposed to chemicals in wastewater in various ways. They may ingest small amounts of pollutants in their drinking water or absorb contaminants through their skin while bathing or swimming, or through inhalation of airborne droplets while showering (Perez Guzzi *et al*., 2000). They may also ingest food, such as fish that has been contaminated by waterborne pollutants. Although ammonia is not a hazard to human health at levels that ordinarily occur in the environment, exposure to it, especially in aquatic environments, can have several human health impacts. The most dangerous consequence of exposure to ammonia is pulmonary edema, followed by severe irritation to moist tissue surfaces (WHO, 1997; WHO, 2006). Eutrophication of water sources may also create environmental conditions that favour the growth of toxin producing cyanobacteria. Chronic exposure to such toxins produced by these organisms can cause gastroenteritis, liver damage, nervous system impairment, skin irritation and liver cancer in animals (Akpor and Muchie, 2011; EPA, 2009; WHO, 2006).

1.4.2 Prevention and treatment

Prevention of emerging waterborne bacterial diseases or outbreaks is mostly dependant on the infectious cycle of a particular organism. For example: person-to-person transmission may be minimised or inhibited by the improvement of hygiene and sanitary conditions as well as the education of the public, more importantly the rural communities in developing countries. The presence of microorganisms in natural water sources due to the discharge of inadequately treated wastewater effluent may be minimised by the reviewing of guidelines and implementation of stricter surface water protection policies (NICD, 2012). The problem encountered in under-developed or developing countries is that many poor communities are without proper sanitation facilities and in most cases do not have access to safe water sources. Instead of decreasing, these problems seem to be rising with the increases seen in population masses flocking to urban cities and towns.

The diversity of microorganisms and the on-going changes and adaptations to environments seen in microbial populations continues to pose a threat to human health, challenging many efforts aimed at prevention and treatment of diseases. In 2012, Center for Disease Control and Prevention reported on their focus and aims to ensure that disease outbreaks are prevented by improving on the following: The strengthening of public health fundamentals, including infectious disease surveillance, laboratory detection, and epidemiologic investigation, the identification and implementation of high-impact public health interventions to reduce infectious diseases and the development and advancement of policies to prevent, detect, and control infectious diseases (NICD, 2012). The above mentioned foci would include the modernization of infectious disease surveillance, and an increase in clinical laboratories for disease control and prevention, including human resource and trained staff workers. This would result in the development of high- impact equipment or tools, and vaccines both for disease detection and treatment as well as updated databases. The increase

in community and individual efforts and engagement in the prevention of disease would result in the strengthening of public awareness on a global scale.

1.4.3 Public health control

Wastewater contains a wide variety of microbial pathogens most of which have shown to be of no harm to human populations, however some of these bacteria, protozoa and viruses have shown to be a health threats to populations and communities, who rely on water highly polluted by such pathogenic contaminants. The contaminants in wastewater have the ability to cause a wide range of infections and diseases, such as typhoid, dysentery, diarrhoea, skin and tissue infections and other intestinal disorders (APHA, 2001; EPA, 1996; DWAF, 1996; Obi *et al*., 2002). Microbial agents responsible for all the above are the most common wastewater contaminants, thus their widespread study and research around the world. *E. coli, Listeria, Salmonella, Vibrio, Campylobacter* are some of the microorganisms usually found in wastewater polluted waters (CDC, 1997; Absar, 2005).

In South Africa, the Centre for Enteric Diseases was officially established in 2002 and tasked with developing new and relevant strategies for prevention and combating enteric diseaseS, as well as providing information to combat diarrhoeal diseases in South Africa. In addition, the centre monitors trends in diarrhoeal pathogen incidence and identifies areas for the introduction of additional interventions (NICD, 2012). Mortality from waterborne diseases could be prevented with the implementation of good sanitation, hygiene, vaccination, antibiotic treatment and oral rehydration therapy either through outbreaks or following disasters. In developing countries, many waterborne diseases or outbreaks cannot be prevented either due to lack of public health infrastructure or the complications arising from other factors such as malnutrition and decreased immunity (Woodall, 2008, Igbinosa *et al*.,

2012). Improvement in public health diagnostic and detection procedures is essential in the protection of the public, including infants, as well as the continuous monitoring of surface water bodies to identify potential water-borne pathogens (Igbinosa *et al*., 2012).

1.4.4 Potential implications on the growing immuno-compromised populations

Listeria infections are reported to have the highest (up to 50%) mortality rate amongst emerging bacterial pathogens. This is of grave concern to South Africa, with a high number of HIV/AIDS infected people, coupled with a high level of drug and alcohol abuse, which has led to many immune-compromised people in the country. If a new type of *Listeria* had to break out, majority of South Africans' lives would be at risk. Obi and Bessong (2002) reported the isolation of *Aeromonas* species from 13.3% HIV patients with chronic diarrhoea in rural communities in the Limpopo Province of South Africa, highlighting that immuno-compromised populations can also suffer from *Aeromonas*-associated chronic diarrhoea. This is especially worrisome in a province with a high number of immune-compromised individuals due to the extremely high HIV and TB.

1.5 Pathogenesis and virulence determinants

Investigating the resistance mechanisms and pathways that emerging bacterial pathogens choose to utilise when exposed to stressful conditions is of utmost importance. This will not only reveal the utilised defence systems, but will also shed light on how these pathogens acquire such resistance (Grobe *et al*., 2001). Resistance within microorganisms may range from great specificity to very low specificity levels, posing a great challenge in determining which mechanisms are responsible for which type of resistance (Chapman, 2003b; Morato *et al*., 2003). Bacteria may use one or multiple mechanisms against a single agent or class of agents and a single change may result in resistance to several different agents or even

multiple unrelated drug classes (Kaye *et al*., 2004; Langsrud *et al*., 2003a). It was found that heavy metals, chlorine and antimicrobial resistance are closely related since microorganisms that were resistant to one showed resistance to another to a certain degree (Cloete, 2003). This phenomenon, known as cross resistance plays a significant role in the disinfection process, particularly chlorination (Chapman, 2003b; Langsrud *et al*., 2003b). The disinfection step in wastewater treatment is aimed at inactivating any pathogenic components that might still be present in the wastewater effluent (Ford, 2006). Disinfection time and concentration varies within different facilities, resulting in different results and varying residual disinfectant and pathogen concentrations. Other factors such as temperature, pH, and turbidity can also affect this process (Koo *et al*., 2008). It has been recorded that the enhanced sensitivity of progeny of chlorine exposed cells might be caused by chlorine-induced mutations that result in the loss of some factor influential in preventing the lethal activity of chlorine, such as the ability to repair nucleic acid damage or cell envelope damage (Poole, 2005). The processes used in water treatment (coagulation, flocculation, sedimentation and filtration) are very effective in achieving high levels of particle removal when conducted appropriately, including the removal of the pathogens. Failure to remove particulate matter makes the disinfection process unreliable (Grobe *et al*., 2002).

Multidrug resistance and metal tolerance are the most studied topics in microbiology, due to their importance in the clinical or medical sectors as well as industrial application sector (Tirodimos *et al*., 2011). Antibiotic resistance is the most widely spread resistance trait of many emerging bacterial pathogens especially *Pseudomonas* spp. (Langsrud *et al*., 2003a). Studies revealed that the acquisition of antibiotic resistance is sometimes not at all affected by their presence in an environment as previously thought, but may also be reliant on other factors and chemical compositions (Eze *et al*., 2009; Sarme *et al*., 2010). Eze *et al*. (2009)

revealed that the genes coding for both heavy metal resistance and antibiotic resistance are located on the same structure, meaning that metal tolerant microorganisms were also multidrug resistant (Grobe *at al*., 2002). Substances such as heavy metals and biocides resulting from industrial waste discharge are the most influential factors for this acquired resistance in microorganisms. Recent studies conducted on microbes inhabiting metal rich environment and those in minimum metal concentrations, proved that metal exposed microorganisms portrayed greater resistance than those in metal poor conditions (Eze *et al*., 2009).

1.5.1 Virulence markers aiding in pathogenesis

Bacterial pathogens which have been found as the main causative agents in many of the water-borne diseases such as diarrhoea, septicaemia and skin infections have been studied broadly over the past years. In these pathogens, multiple virulence genes and markers aiding in pathogenesis have been discovered, explaining their resistance against the body's defence mechanisms and persistence in infections. Virulence markers such as extracellular toxins (cytotoxins and enterotoxins), adhesins, and haemolysins have been discovered (Igbinosa *et al*., 2012). No definite link has been established between virulence factors and the development of infections; however a study conducted by Sechi *et al*. (2002) suggested a possible link. In the study, *Aeromonas* species were investigated for haemolysis and protease and gelatinase production which have been attributed to increasing the rate of infection. The study found a greater prevalence of the above mentioned virulence genes among strains isolated from patients with diarrhoea, which correlated with similar studies conducted by other authors (Wang *et al*., 1996, Sechi *et al*., 2002). De Oliveira Scoaris *et al*. (2008) reported on the necessity for organisms to contain more than a single extracellular enzyme to ensure virulence. Although these enzymes undoubtedly play a role in pathogenicity, however

no specific extracellular enzyme has been proven to be solely responsible for the virulence of a particular organism. The AHCYTOEN gene which codes for the cytolytic enterotoxin gene has also been found in environmental samples obtained in aquatic environments but not from clinical isolates suggesting that aquatic environments may act as reservoirs of potentially virulent *Aeromonas* species (Sechi *et al*., 2002). The presence and expression of these virulence genes has been found to be dependent on environmental conditions such as temperature (Gonzalez-Serrano *et al*., 2002; Igbinosa *et al*., 2012). The diversity of microbes and their mechanisms and properties of virulence and pathogenicity are too great to reliably predict emergence and future human health impacts.

1.5.2 Resistance to antibiotic treatment

Antibiotics are one of the great discoveries of the 20th century; however, resistance began to spread even in the earliest years of the budding antibiotic era (Stokes and Gillings, 2011). Exposure to lowered concentrations of antimicrobials, as well as the constant evolution and adaptations of the bacterial genome contributed largely to the resistance phenomena (Zhang *et al*., 2009). Antibiotic resistance continues to be the main challenge in microbial infections and treatment studies, as infectious microbes continue to gain resistance against treatment and therefore becoming worse, despite the ever-increasing resources devoted to combat these problems (Zhang *et al*., 2009).

Microorganisms pose a major health threat to humans, due to their disease causing ability (Ashbolt, 2004; Samie *et al*., 2009). The occurrence of microorganisms and widespread occurrence of disease outbreaks has led to the extensive use of disinfectants and antibacterial agents found in common soaps, detergents, sanitizers and disinfectants. This has resulted in major changes in environmental microorganisms which have evidently become tougher and

more resistant to treatment (Stokes and Gillings, 2011). The increasing rate of antibiotics and antibacterial agents usage, has resulted in microorganisms which are highly resistant to killing or treatment, even affecting those which are beneficial to man (Silva, 2006). There are numerous routes of antimicrobial agent entry into the environment, and studies have shown that introduction by these routes has changed the antibiotic susceptibility of the microbes in those environments or changed the predominant microbial populations (Stokes and Gillings, 2011).

Medical and pharmaceutical discharge from hospitals has contributed largely to the increase in antibiotic concentration and therefore has led to the rise of highly resistant bacterial populations (Naviner *et al*., 2011). There are over 700 antibacterial household products manufactured over the past 5 years, in the form of toothpastes, paints, cement, sprays as well as common kitchen products, these products have been found to contain alcohols, bleaches such as chlorine as well as ammonium compounds and many human pathogens have been documented to be resistant to these products (Silva, 2006; Zhang *et al*., 2011). Agricultural and crop industries increased use of antimicrobials also has a huge effect on microbial populations in the environment, since these waters usually run off into nearby rivers and estuaries (Adetunji and Isola, 2011; Zhang *et al*., 2011). Antimicrobials are being used in animal feed production and subtherapeutic levels as growth promoters.

Sewage and wastewater treatment plants may be a rich reservoir of antibiotics because the antibiotics taken in are not all processed by the bodies. Sludge remaining after wastewater treatment at most treatment plants have been discovered to contain microorganisms (above 40%) which are resistant to multiple antibiotics (Jury *et al*., 2010; Silva, 2006). Rivers contaminated with urban and agricultural effluent have shown bacterial populations with

greater antibiotic resistance than areas upstream of the contamination source (Falcão *et al*., 2004).

One of the most significant factors contributing to antibiotic resistance is the common and most notable ability of bacteria to share genetic resources via horizontal or lateral gene transfer (HGT/LGT) (Munir *et al*., 2011). HGT has increased the spread of resistance between different microbial populations, while also increasing their survival and reproduction. Antibiotics are not responsible for causing resistance, instead, they allow naturally resistant variants within a population to survive and reproduce while those individuals without the resistance factor die and are eliminated (Stokes and Gillings, 2011). Once in a bacterial population, antibiotic resistance can spread rapidly among related as well as unrelated bacteria which acquire resistance from their neighbours via the HGT phenomenon (Adetunji and Isola, 2011; Stokes and Gillings, 2011). Antibiotic resistance is encoded in DNA, and bacteria have the ability to exchange DNA, especially in the form of plasmids which are circular, small and self-replicating DNA components with the ability to pass resistance very rapidly (Charpentier and Courvalin, 1999; Stokes and Gillings, 2011).

Some diseases are no longer treatable with antibiotics and some diseases that were previously susceptible to a variety of antibiotics are now untreatable. The Centre for Disease Control (CDC) approximated that 70% of infections that people get while hospitalized are now resistant to at least one antibiotic. Resistance to antibiotics is rapidly outpacing the ability to synthesize new drugs (Zhang *et al*., 2009). Considering the minority of bacteria which can potentially be of great harm to human beings, treatment for these rare infections seems to be an increasing obstacle. Wastewater treatment plants are the principal recipients of enteric

bacteria with multiple antibiotic resistances (Jury *et al*., 2010; Munir *et al*., 2011; Silva, 2006).

A number of mechanisms are responsible for antibiotic resistance; these may include enzymatic inactivation of antibiotics, the alterations of the target sites of antimicrobial agents, the development of bypass pathways around antimicrobial targets, presence of multidrug-efflux pumps, as well as reductions in bacterial cell-wall permeability (Cloete, 2003; Eze *et al*, 2009; Sarme *et al*., 2010; Zeng, 2004). Resistance mechanisms are usually specific for a particular antibiotic, with membrane-impermeability-mediated resistance usually resulting in a multidrug-resistance phenotype. Although the mechanisms of resistance to specific antibiotics are well known, the molecular mechanism of multidrug resistance is poorly understood (Zeng, 2004).

Bacteria have significant ability to develop resistance to every antibiotic they are exposed to; it can be anticipated that even bacterial species such as *Listeria*, which are still considered to be susceptible to almost all antibiotics, will evolve toward multi-resistance (Adetunji and Isola, 2011; Arslan and özdemir, 2008; Charpentier and Courvalin, 1999). *Listeria* species are opportunistic pathogens with non-specific clinical symptoms (NHS, 2009). Infection results in a disease known as listeriosis. Despite efficient antibiotic therapy, listeriosis represents a public health problem since it is fatal in multiple cases (Arslan and özdemir, 2008). In general, isolates of *L. monocytogenes*, as well as strains of other *Listeria* spp., are susceptible to a wide range of antibiotics except cephalosporins and fosfomycin (Adetunji and Isola, 2011). Studies have described the transfer of enterococcal and streptococcal plasmids and transposons carrying antibiotic resistance genes by conjugation from *Enterococcus-Streptococcus* to *Listeria* and between species of *Listeria* (Charpentier and Courvalin, 1999).

Pseudomonas aeruginosa contains the multidrug efflux pumps whose expression is linked to that of outer membrane proteins, such as the MexAB-OprM system (mainly involved in antibiotic resistance) (Sagripanti and Bonifacino, 2000; Simoês *et al*., 2009; Tirodimos *et al*., 2011). *Pseudomonas* spp. also has the ability to easily pick up resistance genes via uptake of DNA and mutations in adverse conditions (Picardo and Giroux, 2004). Adaptation and resistance acquisition may even result from a slight genetic change, altering functional target and in turn reducing the affinity of the used antimicrobial (Kaye *et al*., 2004). Inactivation of enzymes remains the predominant mechanism of resistance to several major classes of antimicrobial agents. The occurrence of phenotypic variation leading to increased agglutination and biofilm formation has been consistently linked to resistance to chlorine, acid, osmotic and oxidative stresses in a number of bacteria, indicating that changes in phenotypic characteristics associated with increased biofilm formation are important at increasing resistance to environmental stress (Drenkard, 2003).

1.5.3 Resistance to chlorine and other treatment technologies

Chlorine and chlorine containing chemical solvents are commonly used for disinfection due to their killing effect on most pathogenic microorganisms (Dreeszen, 2003). Free chlorine is most effective at a pH of 5 to 7, where HOCl is the predominant form and the effectiveness declines with increased pH. Higher chlorine concentrations may be required to ensure adequate disinfection when the pH of water is high (Koo *et al*., 2008). Five part per million is the recommended residual chlorine concentration for the killing of pathogenic bacteria for a 5-10 minute contact time at pH 7-8.5 (Dreeszen, 2003). The major disadvantages of chlorine are that it is not stable in water and the concentration decreases rapidly. Exposure to sunlight or other strong light including agitation will accelerate the reduction of chlorine (Ibusquiza,

2011). Chlorine may also react with organic matter and other oxidizable contaminants in the supply water and within the piping distribution systems (Grobe *et al*., 2001).

Resistance mechanisms vary within different microorganisms, with most of the phenotypic adaptations being genetically expressed before being manifested physically. These mechanisms include: limiting the intracellular concentration of the antimicrobial agent by decreased influx or increased efflux, neutralization of the antimicrobial agent by enzymes that reversibly or irreversibly inactivate the drug, alteration of the target so that the agent no longer interferes with it as well as the elimination of the target altogether by the creation of new metabolic pathways (Kaye *et al*., 2004). Of the resistance mechanisms, altered outer cellular layers, stress response factors and the presence of efflux pumps are the most studied (Cooper *et al*., 2010; Simoês *et al*., 2009; Tirodimos *et al*., 2011). Different biocides have specific and specialized modes of action, including the differences in the reaction sites located on the cell's outer cell structure (Chapman, 2003b). The effect of these biocides (chlorine, monochloramine etc.) on pathogens may also be reliant on several important factors such as contact time, concentration of disinfectant, temperature and pH (Russell, 2003). The difference in outer cellular membranes of different microorganisms is another factor affecting the action and degree of biocide effect.

Compared to Gram-positive bacteria, Gram negative bacteria are found to be more resistant to numerous biocides due to the presence of the outer membrane which decreases permeability (Cloete, 2003). Also the outer membrane of Gram-negative bacteria may provide an additional intrinsic barrier that prevents drugs from reaching these targets (Cloete, 2003; Kaye *et al*., 2004). Lowered permeability decreases the movement of molecules across the cell membrane, creating a permeability barrier and reducing the uptake of foreign

particles (Loughlin *et al*., 2002). This may result in decreased uptake of disinfectants which act against microorganisms which are widely used in wastewater and drinking water treatment processes. The modifications in outer membrane permeability by both alterations in porin protein channels and by the up-regulation of intrinsic multidrug efflux pumps may comprise a component of the mechanisms contributing to resistance in many Gram-negative organisms (Grobe *et al*., 2002; Kaye *et al*., 2004). *Aeromonas* and *Pseudomonas* spp. have the ability to transform toxic compounds into non-toxic compounds, rigidify the cell membrane via alteration of the cell membrane including altering the phospholipid composition, altering the cell surface to make the cells less permeable, form vesicles that remove the solvent from the cell surface and efflux of the toxic compounds via efflux pumps in an energy dependent process (Loughlin *et al.,* 2002; Lăzăroaie, 2009). The targets of most antimicrobial agents are located either in the cell wall, cytoplasmic membrane, or within the cytoplasm (Ghosh *et al*., 2011). Resistant *P. aeruginosa* down-regulated genes encoding membrane proteins, particularly genes related to permeases and transporters of carbohydrates and catabolites (Small *et al*., 2006).

Another factor which contributes to antimicrobial resistance is encapsulation, a process whereby microbial cells secrete an extracellular capsule layer (Grobe *et al*., 2001). Resistance in *Pseudomonas aeruginosa* and *Legionella* spp. results from a physical change in the cell membrane, affecting permeability. Studies where the capsular material of *Pseudomonas* was isolated to measure various components such as hexose sugar and proteins, revealed that a structural change does occur in resistant microorganisms (Kim *et al*., 2009). Free chlorine reacts with extracellular capsular materials and the extracellular material can accumulate to a great concentration. The reaction between chlorine and the extracellular material can be slow due to the increase in free chlorine demand (Shrivastava *et al*., 2004). Studies have shown

that the maintenance of residual chlorine cannot guarantee total prevention of bacterial prevalence in turn not resulting in further microbial reduction. A link between chlorine and antibiotic resistance was made, but the mechanism of chlorine-induced antibiotic resistance in bacteria still remains unknown. It is possible that chlorine can increase expression of the multidrug efflux pumps, leading to resistance to disinfection by-products as well as antibiotics (Xi *et al*., 2009).

The discovery of *Listeria* dates back to the 1920's, initially in animals, and was finally known as an animal pathogen, until the 1980's where it was discovered as a human pathogen, firstly discovered as a food borne pathogen and later as a water-borne pathogen (Ibusquiza, 2011). *Listeria* shows an increase in doubling time as well as the ability to withstand 100 times more biocide concentration; they also contain multidrug efflux pumps which have very low specificity (Kim *et al*., 2009). This further confirms that no single mechanism can account for the general observation of resistance, and that all act in concert (Sagripanti and Bonifacino, 2000).

1.6 Survival mechanisms of emerging bacterial pathogens

Investigating the resistance mechanisms and pathways that emerging bacterial pathogens choose to utilise when they are exposed to stressful conditions is of utmost importance. This does not only reveal the utilised defence systems, but could also shed some light on how these pathogens acquire such resistance (Grobe *et al*., 2001). The mechanisms of resistance against commonly used disinfectants include: limited diffusion of antimicrobial agents through the biofilm matrix, interaction of the antimicrobial agents with the biofilm matrix (cells and polymer), enzyme mediated resistance, level of metabolic activity within the

biofilm, genetic adaptation, efflux pumps and outer membrane structure (Eze *et al*., 2009; Grobe *et al*., 2002; Langsrud *et al*., 2003a; Purkrtová *et al*., 2010; Sarme *et al*., 2010).

Multidrug resistance has been mainly responsible for the emergence or re-emergence of several pathogens, especially *Pseudomonas aeruginosa* (Sharma, 2003). Resistance within microorganisms may range from great specificity to very low specificity levels; posing a great challenge in determining which mechanisms are responsible for which type of resistance (Chapman, 2003b; Morato *et al*., 2003). Literature reveals that bacteria may use one or multiple mechanisms against a single agent or class of agents, with a single change resulting in resistance to several different agents or even multiple unrelated drug classes (Kaye *et al*., 2004; Langsrud *et al*., 2003a). It was found that heavy metal, chlorine and antimicrobial resistance is closely related since microorganisms that were resistant to one showed resistance to another to a certain degree (Cloete, 2003). This phenomenon is known as cross resistance and plays a significant role in the disinfection process, particularly chlorination (Chapman, 2003a; Langsrud et al., 2003a). The disinfection step in wastewater treatment is aimed at inactivating any pathogenic components that might still be present in the wastewater effluent (Ford, 2006). Disinfection time and concentration varies within different facilities, resulting in different outcomes and varying residual chlorine and pathogen concentrations. Other factors such as temperature, pH, and turbidity can also affect this process (Koo *et al*., 2008). The particle removal processes used in water treatment (coagulation, flocculation, sedimentation and filtration) when conducted appropriately are very effective in achieving high levels of particle removal, including the removal of the pathogens. Failure to remove particulate matter makes the disinfection process unreliable (Grobe *et al*., 2002).

Multidrug resistance and metal tolerance are the most studied topics in microbiology, due to their importance in the clinical or medical sectors as well as industrial application sector, respectively (Baker-Austin *et al*., 2006). Antibiotic resistance is the most widely spread resistance trait in emerging bacterial pathogens especially in some enteropathogens, such as *Pseudomonas* species (Langsrud *et al*., 2003a). Studies revealed that the acquisition of antibiotic resistance is sometimes not at all affected by their presence in an environment as previously thought, but may also be reliant on other factors and chemical compositions (Eze *et al*., 2009; Sarme *et al*., 2010). Eze *et al*. (2009) revealed that the genes coding for both heavy metal resistance and antibiotic resistance are located on the same structure, meaning that metal tolerant microorganisms were also multidrug resistant (Grobe *et al*., 2002). Substances such as heavy metals and biocides resulting from industrial waste discharge are the most influential factors for this acquired resistance in microorganisms. Recent studies conducted on microbes inhabiting a metal rich environment and those in minimum metal concentrations, proved that metal-exposed microorganisms portrayed greater resistance than those in metal poor conditions (Eze *et al*., 2009). Tests were performed to determine the degree of resistance to antibiotics, antimicrobial agents and metals. Of the tested microbial genera, *Pseudomonas* showed the highest resistance to all the tested parameters (Shrivastava *et al*., 2004). Jydegaard-Axelson *et al.* (2005) observed an increase in the expression of genes encoding for glutamate decarboxylase, which is essential for survival in strong acid, as well as increased amounts of branched fatty acids in the membranes were observed in an acid tolerant strain of *L. monocytogenes* under elevated CO_2 and anaerobiosis. However, an acid sensitive strain responded differently to CO_2 and N_2 under the same conditions. The various survival and adaptation strategies employed by emerging bacterial pathogens include; symbiotic associations, cell membrane alterations, use of efflux pumps, changes in cell physiology and gene expression as discussed in section 1.6.1 to 1.6.5.

1.6.1 Symbiotic associations

A bacterial biofilm is a community of bacterial cells enclosed or surrounded in a self-produced polymeric matrix that is adherent to an inert or living surface (Morris and Hagr, 2005). It is estimated that only 1% of bacteria exist in the familiar, free-floating, or planktonic form, with the remaining 99% existing in protective biofilms (Morris and Hagr, 2005). Biofilm structures develop when planktonic bacteria become sessile and adhere to a surface in the presence of water. Different stages of attachment, colonization, and growth lead to the establishment of a mature biofilm colony (Sagripanti and Bonifacino, 2000). These structures are protective layers with protein and polysaccharides which surround the microorganism, in turn offering maximum protection (Purkrtová *et al*., 2010). Once the biofilm becomes established, it is then considered as a fortress from which bacteria exhibit spectacular defence mechanisms against antimicrobial agents (Kim *et al*., 2009). Bacterial cells making up a biofilm have a more complex behavioural system than that of suspended cells. Colonies show altered characteristics involving complicated intercellular communication systems, enabling them to exploit their environment (Kim *et al*., 2009). Within the biofilm population, there appears to be a division of labour which also adds to the overall resistance of the biofilm body (Morris and Hagr, 2005). Biofilm structures reduce or eliminate particle associated contact between the disinfectants and microbial cell (Poole, 2005).

Aeromonas, Pseudomonas, Listeria, Yersinia and *Legionella* species have all been associated with robust biofilm structure formation, as a stress response and protective measure. Studies on *Listeria* species have shown the existence of these organisms in biofilm structures. *Pseudomonas* cells have also been shown to exist in a biofilm structure. The biofilm structure acts as a shield for the microorganisms, preventing the diffusion of chlorine into the

microbial cells, thus requiring a longer time to be killed or eliminated compared to the planktonic free living cells which require a much shorter time (Grobe *et al*., 2002). *Legionella* species have shown the rare ability to propagate and form biofilm structures with free living amoebae such as; *Acanthamoeba, Naegleria* and *Hartmanella*. These amoebae have the ability to support *Legionella* growth within their cells, thus shielding the cells and enabling them to survive and become resistant to inappropriate environmental conditions. These sheltered cells have the ability to withstand temperatures as high as 45-53 ºC, including lower pH levels as well as free chlorine levels of 0.3-0.5 ppm (Burak and Zeybek, 2009).

1.6.2 Cell membrane alterations

Chlorine and chlorine containing chemical solvents are commonly used for disinfection due to their killing effect on most pathogenic microorganisms (Dreeszen, 2003). Free chlorine is most effective at a pH of 5 to 7, where HOCl is the predominant form, with a decline in effectiveness with increased pH. Higher chlorine concentration may be required to ensure adequate disinfection when the pH of water is high (Koo *et al*., 2008). Five ppm for a 5-10 minute contact time is the recommended residual chlorine concentration for killing off pathogenic bacteria at pH 7 - 8.5 (Dreeszen, 2003). The major disadvantage of chlorine is that it is not stable in water and the concentration decreases rapidly. Exposure to sunlight or other strong light including agitation will accelerate the reduction of chlorine (Ibusquiza, 2011). Chlorine may also be used up by reacting with organic matter and other oxidizable contaminants in the water supply and within the piping distribution systems (Grobe *et al*., 2001). Chlorine and associated compounds interact with the cell wall, forming openings and pores and therefore allowing for uncontrolled entry into the cell (Cloete, 2003).

Pseudomonas aeruginosa is one of the most resistant emerging bacterial pathogen and has been found to contain intrinsic resistance factors (Langsrud *et al*., 2003a; Dreeszen, 2003). It is the most resistant non-spore forming bacteria to most bacteriocides, due to the superior barrier properties of its outer membrane. An increase in the unsaturated fatty acid ratio was observed in disinfectant resistant *P. aeruginosa* cells and similar results were observed in the membrane lipid profiles (Cloete, 2003). Their biofilms are found to be metabolically active, leading to an increase in cell biomass, cell density and extracellular proteins and polysaccharide. *Pseudomonas* contained 29% of the total proteins and 61% of the total polysaccharide (Simoês *et al*., 2009). Biofilm surfaces are responsible for the absorption and catalytic destruction of disinfectant agent (Simoês *et al*., 2009). *Pseudomonas aeruginosa* contain a low permeable outer membrane which prevents or blocks entry of many agents to their site of action. Analysis of the outer membrane proteins of adapted cells revealed an increase in certain proteins and a sudden appearance of some proteins not found in cells which were not adapted (Langsrud *et al*., 2003a). The outer membrane protein increase from 4% to 14% can be found in the most resistant cells, depending on the strain (Loughlin *et al*., 2002). During the same study, an unknown fatty acid was detected using mass spectroscopy, indicating that there is a reasonable increase in structural components. Resistant cells showed a decrease in phosphotidylcholine concentration (20%- 6 %), with the lipid concentrations were as follows: 10% diphosphotidylglycerol, 50% phosphatidylethanolamine, 20% phosphatidylglycerol and 10% phosphatidylcholine (Loughlin *et al*., 2002). A decrease in hydrophobicity and surface charge leads to an increase in the negative charge with increasing resistance (Kim *et al*., 2009).

1.6.3 The presence of efflux pumps

Gram-negative bacteria including *Pseudomonas* spp., *Aeromonas* spp., *Legionella* spp. and *Yersinia* spp. contain multidrug transporters characterized by a common three component organization: a membrane fusion protein that is associated with the cytoplasmic membrane, a transporter protein that exports the substrate across the inner membrane, and an outer membrane protein (OMP) that facilitates the passage of the substrate across the outer membrane (Drenkard, 2003). *Pseudomonas aeruginosa* contains the multidrug efflux pumps whose expression is linked to that of outer membrane proteins, such as the MexAB-OprM system (mainly involved in antibiotic resistance) (Sagripanti and Bonifacino, 2000; Simoês *et al*., 2009; Tirodimos *et al*., 2011). The lack of specificity observed in MexAB–OprM system is very interesting and its natural physiological role indistinct (Ghosh *et al*., 2011). The OprM subunit is the outer membrane-anchored lipoprotein that is assumed to form the antibiotic discharge duct across the outer membrane. The MexA subunit anchors to an inner membrane via N-terminal fatty acid. Removal of fatty acids liberates a protein from the membrane and the protein becomes freely soluble in aqueous solutions. Therefore, MexA is assumed to link MexB and OprM, helping the assembly of the functional pump unit.

Pseudomonas spp. also has the ability to easily pick up resistance genes via uptake of DNA and mutations in adverse conditions (Picardo and Giroux, 2004). Adaptation and resistance acquisition may even result from a slight genetic change, altering functional target and in turn reducing the affinity of the used antimicrobial (Kaye *et al*., 2004). Inactivating enzymes remain the predominant mechanism of resistance to several major classes of antimicrobial agents. The occurrence of phenotypic variation leading to increased agglutination and biofilm formation has been consistently linked to resistance to chlorine, acid, osmotic and oxidative stresses in a number of bacteria, indicating that changes in phenotypic characteristics

associated with increased biofilm formation are important at increasing resistance to environmental stress (Drenkard, 2003).

1.6.4 Changes in cell physiology

Resistance mechanisms vary within different microorganisms, with most of the phenotypic adaptations being genetically expressed before manifesting. These mechanisms include: limiting the intracellular concentration of the antimicrobial agent by decreased influx or increased efflux, neutralization of the antimicrobial agent by enzymes that reversibly or irreversibly inactivate the drug, alteration of the target so that the agent no longer interferes with it as well as the elimination of the target altogether by the creation of new metabolic pathways (Kaye *et al*., 2004). The subsequent resistance mechanisms such as altered outer cellular layers, stress response factors and the presence of efflux pumps are the most studied, with many references existing in literature (Cooper and Hanlon, 2010; Simoês *et al*., 2009).

1.6.4.1 Physiology and metabolism

Different biocides have specific and specialized modes of action, including the differences in the reaction sites located on the cell's outer cell structure (Chapman, 2003b). The effect of these biocides (chlorine, monochloramine etc.) on pathogens may also be reliant on several important factors such as contact time, concentration of disinfectant, temperature and pH (Russell, 2003). The difference in outer cellular membranes of different microorganisms also affects the action and degree of biocide effect. Gram negative bacteria are found to be more resistant to numerous biocides due to the presence of the outer membrane which decreases permeability (Cloete, 2003). The outer membrane may also provide an additional intrinsic barrier that prevents drugs from reaching these targets in Gram-negative bacteria (Cloete, 2003; Kaye *et al*., 2004).

Lowered permeability decreases the movement of molecules across the cell membrane, creating a permeability barrier therefore reducing uptake of foreign particles (Loughlin *et al*., 2002). This may result in the decreased uptake of disinfectants which are widely used in wastewater and drinking water treatment processes. The modifications in outer membrane permeability by both alterations in porin protein channels and by the up-regulation of intrinsic multidrug efflux pumps may comprise a component of the mechanisms contributing to resistance in many gram-negative organisms (Grobe *et al*., 2002; Kaye *et al*., 2004). *Aeromonas* and *Pseudomonas* spp. have the ability to transform toxic compounds into non-toxic compounds, rigidify the cell membrane via alteration of the cell membrane including the phospholipid composition, alter the cell surface, make the cells less permeable, form vesicles that remove the solvent from the cell surface and efflux of the toxic compounds via efflux pumps in an energy dependent process (Lăzăroaie, 2009; Loughlin *et al*., 2002). The targets of most antimicrobial agents are located either in the cell wall, cytoplasmic membrane, or within the cytoplasm (Ghosh *et al*., 2011).

1.6.4.2 Neutralization of disinfectants

Pseudomonas spp. also produces acyl-homoserine lactone (HSL) quorum-sensing signal compounds, which react with biocides. Hexanoyl HSL, 3-oxohexanoyl HSL, 3-oxododecanoyl HSL are also involved in biofilm formation, with the 3-oxo moiety reacting with HOCl. All HSL compounds carrying the 3-oxo group have the ability to react with oxidising halogen groups by consuming it (Borchardt *et al*., 2001). *Pseudomonas* spp. defence mechanisms involve the transfer of genetic material including virulence and resistance gene between monospecies and polyspecies of closely associated microorganisms (Picardo and Giroux, 2004). The most common resistance trait is penetration failure of

bactericidal agents as well as their neutralization before reacting with the target site of specific microbial cells (Morris and Hagr, 2005).

1.6.4.3 Alginate production

Study of *Pseudomonas* spp. biofilms revealed that they have the ability to produce alginate, an extracellular polymeric substance which contains branches resembling the N-acyl homoserine lactone groups (Morris and Hagr, 2005). Planktonic *Pseudomonas aeruginosa* also secretes the alginate polysaccharide which provides the cell with protection against a variety of host factors (Leid *et al*., 2005). The role and the mechanism of action of alginate in both the sessile and planktonic microbial cell forms in their protection is still unknown (Morris and Hagr, 2005; Shrivastava *et al*., 2004). Wood *et al*. (2006), showed that *Pseudomonas* spp. with a mucoid phenotype had enhanced survival in chlorinated waters and that removal of the slime layer increased susceptibility to chlorine. Alginate production is regulated by a cassette of genes, *algTmucABC*. AlgT is a sigma factor involved in the positive regulation of the mucoid phenotype in *P. aeruginosa*. MucA and MucB inhibit the production of AlgT in a negative feedback loop (Cochran *et al*., 1999). When spontaneous null mutations in *mucA* or *mucB* occur, AlgT is up-regulated, resulting in increased production of alginate (Cochran *et al*., 1999). It has been suggested that alginate promotes biofilm formation.

Encapsulation is another factor which contributes to microbial resistance (Grobe *et al*., 2001). Resistance in *Pseudomonas aeruginosa* and *Legionella* species results from a physical change in the cell membrane, affecting permeability. In studies where the capsular material of *Pseudomonas* was isolated to measure various components such as hexose sugar and proteins, it was revealed that a structural change does occur in resistant microorganisms (Kim

et al., 2009). Free chlorine reacts with extracellular capsular materials and the extracellular material can accumulate to a great concentration. The reaction between chlorine and the extracellular material can be slow due to this, increasing the free chlorine demand (Shrivastava *et al*., 2004). Studies have shown that the maintenance of residual chlorine cannot guarantee total prevention of bacterial prevalence. A link between chlorine and antibiotic resistance was made, and three mechanism of chlorine-induced antibiotic resistance in bacteria still remains unknown. It is possible that chlorine can increase expression of the multidrug efflux pumps, leading to resistance to disinfection by-products as well as antibiotics (Xi *et al*., 2009).

The outer membrane of microorganisms may create a diffusion barrier to antimicrobial agents, since diffusion into the biofilm is affected by ionic surface charge interactions between the glycocalyx and antimicrobial agents. One of the ways by which this is accomplished, is by size exclusion or sieving affected by the viscosity of the glycocalyx (Chapman, 2003b). The polyanionic nature creates a charge interaction barrier to diffusion of cationic antimicrobial agents (Cloete, 2003). Enzyme mediated resistance is another trait of resistant microbial pathogens; these enzymes have the ability to transform bacteriocides from their toxic to a non-toxic form. The mechanism of action of these enzymes is similar to those involved in heavy metal resistance, useful in biodegradation of toxic pollutants (Eze *et al*., 2009; Sarme *et al*., 2010). The Gram negative cell envelope has evolved to regulate passage of substances into and out of the cell at increasing specificities (Morris and Hagr, 2005). All the cell envelope components play a role in barrier mechanism except that the peptidoglycan is spongy and therefore highly permeable (Cloete, 2003). The metabolic state of microorganisms growing within a biofilm has been reported to be suppressed. The physiological state of cells and the nature of the habitat can lead to considerable variation in

the susceptibility of bacteria to bactericides (Kim *et al*., 2009). Envelope and barrier composition are altered under nutrient deprivation. Sub-inhibitory concentrations of bacteriocides can confer a resistant phenotype. Cloete (2003), discovered that the resistant phenotype is mostly lost upon removal of the bacteriocidal stress. Due to the arrangement of biofilm cells, some cells within the biofilm are slow growing or in a starved state with reduced respiration. Slow or non-growing cells are less susceptible to a variety of antimicrobial agents when compared to cells grown in rich media at high specific growth rates (Kim *et al*., 2009). Wang and Gu (2003) analysed *Aeromonas* species growth and behaviour in water at a water reserve and reported that these species had the ability to produce chitinase which aids in the adsorption and multiplication on different zooplanktons and pytoplanktons. The exopolymeric matrix of *Listeria* spp. biofilms is charged, hydrated and surrounds individual cells and micro colonies, it therefore affects the access solutes (Cloete, 2003). The polymers act as ion exchange resin and therefore actively removing strongly charged molecules from the solution. Chlorine therefore poorly penetrates through anionic matrices, chemically reacting and neutralizing the reactive molecules (Cloete, 2003; Sagripanti and Bonifacino, 2000). A commonly observed disadvantage is that chlorine can be inactivated in the presence of organic material (Shrivastava *et al*., 2004).

1.6.5 Changes in gene expression

Bacterial efflux systems capable of accommodating antimicrobials generally fall into five classes. The major facilitator (MF) super family, the ATP-binding cassette (ABC) family, the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) and the multidrug and toxic compound extrusion (MATE) family. Three such efflux systems have also been described for *P. aeruginosa*, MexA-MexB-OprM, MexC-MexD-OprJ and MexE-MexF-OprN. *Pseudomonas aeruginosa* genes for the efflux systems (Cml, CmlA, CmlB)

and pump family (MF), are located mostly in plasmids while others are chromosomal (Poole, 2005). The MexAB-OprM and EmrE pumps of *P. aeruginosa* have also been reported to provide a very modest contribution to intrinsic resistance to these antimicrobial agents (Liao and Shollenberger, 2003). A recent study suggests that the MexAB-OprM efflux system of *P. aeruginosa* promotes the release of molecules ultimately important for the virulence of this organism, though the actual virulence-related factors exported were not identified (Poole, 2005; Xi *et al*., 2009). This efflux system is well known for its non-specificity and is linked to chlorine resistance (Xi *et al*., 2009). Two novel putative exopolysaccharide gene clusters, *pea* and *peb*, were identified in *P. putida* KT2440, both genes encode products that stabilize the biofilm structure. The gene clusters *alg* and *bcs*, which code for proteins mediating alginate and cellulose biosynthesis, were also discovered and these play minor roles in *P. putida* KT2440 biofilm formation and stability under the conditions tested (Nilsson *et a*l., 2011). All efforts directed towards the isolation of the chlorine resistance plasmid in *Pseudomonas* have not been successful to date, regardless of the fact that it is linked to multidrug resistance (Shrivastava *et al*., 2004). *Listeria* spp. was found to express a stress-response-related *cplC* gene, which encodes a protein (CplC ATPase) that is produced under stress conditions (Rodrigues *et al*., 2011). The *oxyR* gene system responsible for the prevention and repair purposes of radical induced damage was also discovered. Induction of these systems in response to radicals results in the synthesis of proteins such as catalases, superoxide dismutase, and alkyl hydroperoxidases. The regulation of these systems is complex, featuring overlapping expression with each other and several other stress response regulations as well. One of the features of these systems is cross-resistance to oxidants; induction by hydrogen peroxide can protect against hypochlorous acid and vice versa (Chapman, 2003a).

1.7 Wastewater guidelines and the protection of surface waters

1.7.1 Current guidelines: Review and reliability

The Durban Wastewater Managing Department (DWM), controlled and monitored by the Department of Water Affairs (DWA) has set specific guidelines to monitor, maintain and protect the state of the surface water in South Africa. The DWA is responsible for monthly sampling and testing of wastewater effluent to ensure that current water constitutions meet the appropriate legislative requirements (DWAF, 1996). The detection, isolation and identification of the many different types of microbial pathogens associated with wastewater would be difficult, time consuming and hugely expensive undertaking if attempted on a regular basis. Indicator microorganisms are therefore used to determine the relative risk of the possible presence of pathogenic microorganisms in a sample (Scott *et al*., 2003). Besides the routine monitoring for total coliform (TC) and feacal coliform (FC) bacteria that have been used for assessing water quality, several types of alternative microorganisms have been suggested as indicators of water quality, feacal pollution and public health risks (Toze, 1997). To ensure that these indicators function effectively, the following should be true for such microorganisms: the indicator should be a member of the intestinal microflora of warm-blooded animals; should be present in the presence of pathogens, and absent in pathogen-free or uncontaminated samples; it should be present in greater population numbers compared to the pathogenic counterparts; the indicator and pathogen need to be of equal resistance to environmental factors as well as to disinfection in water and wastewater treatment plants; it must not have the ability to multiply in the environment; it should be non-pathogenic and easily, rapidly and inexpensively detected (Bitton, 2005). These include enterococci, *Clostridium perfringens*, and coliphage among others. In 1986, USEPA published new criteria for bacteria based on studies of the occurrence of illness in swimmers. Feacal coliforms proved not to have a reliable relation with illness occurrence, thus the new criteria

was no longer supported, instead Enterococcus or *E. coli* were recommended because they showed good correlations with illness and therefore more protective of public health (Toze, 1997).

Different countries have shown to have differing standards, mainly based on each countries outbreak history and current possible health risks; however the differences are not too great. Table 1.3 represents guidelines from 4 different countries, indicating that South African guidelines seem to be of first world standard irrespective of its third world status.

Table 1.3: The comparison of drinking water quality guidelines

Guidelines	Australia		Canada		South Africa	
Total Coliforms:	0cfu/100ml	<400 MPN/100ml	0 cfu /100ml	<35 cfu/100mL	0-5 cfu / 100ml	0 cfu / 100ml
Feacal Coliforms:	0cfu/100ml	<200 MPN /100ml	0 cfu /100ml	200 cfu/100mL	0 cfu / 100ml	0 cfu / 100ml
Nitrates:	50 mg/ L	10 mg/L	45 mg/L	NAG	NAG	1.5 mg/L
Nitrites:	3 mg/L	1 mg/L	NAG	NAG	0-6 mg/L	1 mg/L
pH:	6.5-8.5*	5-9	6.5-8.5*	6-8.5	6.0-9.0	5.5-7.5
Sulphate:	500 mg/L	700 mg/L	<500 mg/L	NAG	0-200 mg/ml	NAG
Total suspended Solids:	NAG	35 mg/L	NAG	<25 mg/L	NAG	10 mg/L
Turbidity:	0.1 NTU	NAG	0.1 NTU	50 NTU	0-1 NTU	NAG
Residual chlorine:	5 mg/L	0.5 mg/L	NAG	<0.02 mgL	NAG	0 mg/L
Temperature:	Not necessary	40 ºC	Not necessary		25 ºC	25 ºC
COD	NAG	120 mg/L	NAG	150 mg/L	NAG	30 mg/L

Australia Environment protection, 2003; Australian drinking water guidelines, 2011; Canada gazette 2012; Department of water affairs and forestry, 1998; Government Gazette, 1984; Guidelines for Canadian drinking water quality, 2010

NAG - No Available Guidelines

1.7.2 Indicator microorganisms

1.7.2.1 Current indicator microorganisms

Indicator microorganisms are those microorganisms which can provide information about the state

(safety and health) of a water body through the organism's presence, condition, or numbers (EPA, 2011). *Escherichia coli* was used as the most suitable indicator in the early centuries, until it was discovered that its removal during wastewater treatment stages is much more easily achieved when compared to the other emerging bacterial pathogens. Pathogens and possibly emerging pathogens were found to be more resistant to the application of traditionally employed stages of wastewater or drinking water treatment (Hörman *et al*., 2004; USEPA, 2000, Slawson *et al*., 2011). Non-pathogenic bacteria known as coliforms and faecal coliform have been used to indicate the presence or absence of pathogenic wastewater bacteria (EPA, 1996; APHA, 2001).

Faecal coliform testing has been accepted as the best indicator of faecal contamination because it is easier to test for coliforms. Faecal coliform counts of 100 million per 100 millilitres may be found in raw domestic sewage. Detectable health effects have been found at levels of 2300 to 2400 total coliforms per 100 milliliters in recreational waters. Disinfection, usually chlorination, is generally used to reduce these pathogens (EPA, 1996; Absar, 2005). Testing for the presence of indicator microorganisms such as faecal coliforms, total coliforms and enterococci are imperative to ensure their reduction to safe levels with no health hazards such as disease outbreaks resulting from exposure to such waters (Ashbolt, 2004; Samie *et al*., 2009). It is assumed that the presence of these microorganisms is a sure

indicator of the presence of pathogens, especially enteropathogens (Hoogenboezem, 2007; Perdek *et al.,* 2002).

1.7.2.2 Indicator-pathogen relationship with respect to human health

Human health risks linked to surface water usually used for recreational purposes and other domestic needs, especially in much poorer communities have not been extensively studied. In the past years, a significant increase in the pathogen detection and quantification studies has been observed (Slawson, 2011). Molecular detection methods such as quantitative polymerase chain reaction (QT-PCR) and multiplex PCR have resulted in better sensitivity compared to the traditional monitoring of organisms. Several studies have resulted in the identification of pathogens of human health concern with very little to no correlation with the known and employed indicator microorganisms (AWRA, 2007; Paillard *et al*., 2005; Pablos *et al*., 2009; Poffe *et al*., 1991; Odjadjare *et al*., 2010). There are however a few studies such as that conducted by Wade *et al*. (2006), which found a strong correlation or association between microbial indicators and human health risks. This was seen where indicator microorganisms were detected in the occurrence of gastro intestinal illnesses. Wilkes *et al*. (2009), reported on the lack of a relationship found between pathogens and indicator microorganisms, with varying cases supporting the detection of pathogens in the absence of indicators (Slawson, 2011). This has led to the possible suggestions that no single indicator is responsible or suitable for the detection of pathogens, considering that many have shown varying strengths of resistance and are prone to develop adaptive traits based on their current environments and need for survival.

1.7.2.3 Alternative indicators

Although coliform bacteria are utilized worldwide as indicator microorganisms, it is of utmost importance to understand the present limitations of these organisms and their implementation in the current guidelines (Tyagi *et al*., 2006). It has been proved that the conventional indicators of faecal origin i.e. coliform bacteria (total and faecal coliforms), used to evaluate microbiological quality of waters provide erroneous information. They do not adequately reflect the occurrence of pathogens in disinfected wastewater effluent due to their relatively high susceptibility to chemical disinfection and failure to correlate with protozoan parasites and enteric viruses (Harwood *et al.*, 2005). Thus, the public health is not protected by using these common indicators, since methods for the detection of sewage borne pathogen become complex, qualitatively unreliable and do not ensure complete safety of water for the consumer. Therefore, the approach is to select some unconventional indicator microbes whose presence presumes that contamination has occurred and suggests the nature and extent of contaminants.

Clostridium perfringens has been considered greatly as an indicator of faecal pollution (José Figueras and Borrego, 2010; Harwood *et al*., 2005). It has been recorded as one of the organisms which is more resistant to conventional chemical treatment and is more suitable and successful in the monitoring of faecal contamination (José Figueras and Borrego, 2010). The World Health Organisation (2008) however expressed concerns related to this organism in that it had a longer survival rate and could therefore be detected long after the pollution. *Pseudomonas* and *Aeromonas* species have also been considered as possible indicator organisms based on their occurrence in faecal polluted waters as well as their resistance to chemical disinfection (Health Canada, 2006). The use of bacteriophages has been the most supported for bacterial analyses. These virus particles infect faecal bacterial organisms

without affecting or infecting human populations. They have been supported due to their more reliable correlation to the presence of pathogens when compared to other coliform bacteria (José Figueras and Borrego, 2010; Roop, 2007). Coliphage meet several of the ideal indicator organisms' criteria and therefore seem to be the better alternative for coliforms (Roop, 2007).

Emerging bacterial pathogens have been discovered in many treated waters, whether it be treated wastewater effluent or treated drinking water. Their prevalence is prominent resulting in a widespread increase in health problems around the world. To combat or decrease these cases, the adaptation and defence mechanisms of these bacterial pathogens to environmental stresses and bactericidal or bacteriostatic agents need to be fully understood. This, however, is a challenge and will remain so for as long as no attention is paid to the different defence mechanism and the specifics of the modes of action. Based on previous literature and findings, it is evident that these bacterial pathogens are indeed prevalent, but minimal research has been done on how they resist treatment and disinfection (Shrivastava *et al*., 2004). It is therefore a challenge to pinpoint the direct cause of decreased susceptibility in treatment processes especially in emerging pathogens that are not broadly studied including *Listeria* and *Yersinia* spp. The recorded mechanisms are known but are not fully understood and this creates a tainted picture and view on the survival of these emerging bacterial pathogens, resulting in many grey areas and assumption based findings rather than fact. However, the discovery of emerging pathogens in treated water, in the absence of indicator organisms, indicates that the treatment processes are not effective in efficient pathogen removal (Toze, 1997).

These findings have placed much doubt on the reliability of indicator organisms and their use in water treatment monitoring (Wade *et al*., 2006; Graczyk *et al*. 2010). It has been recognised that the microbial indicator parameters do not behave in the same way as emerging bacterial pathogens in the same waters (Stanfield *et al*., 2002). Blackburn *et al.* (2004) reported that more than 40% of the water samples linked to disease outbreaks contained acceptable coliform levels, further re-iterating the inadequacy of the use of these coliforms as indicators of water quality.

South Africa currently has no set guidelines for emerging bacterial pathogens and their presence in the discharged wastewater effluent cannot be monitored (Bartie *et al*., 2002). Emerging pathogens have been implicated in a wide range of disease outbreaks which may result in death if ignored or untreated e.g. *Yersinia* causes septicaemia and pneumonia resulting in 100% mortality if untreated (Rollins *et al*., 2003). The fact that these pathogens are also found to be present in drinking tap water which is meant to be safe and pathogen free, further proves that guidelines need to be revised. According to the safe water act, the maximum contamination levels for safe drinking water should be zero for all pathogens "no tolerable lower limit" (WHO, 2008). It is therefore evident that the ineffectiveness of these established processes poses a health threat worldwide (Obi *et al*., 2002).

1.8 Scope of the present study

The presence of emerging bacterial pathogens cannot be tracked or monitored during traditional treatment processes, mainly due to the absence of set guidelines for emerging bacterial pathogens in South African wastewater, leading to their release and dissemination into the environment (Bartie *et al.*, 2002; Odjadjare, 2012). Reports of inadequate removal of emerging bacterial pathogens in wastewater effluent in developing country are on the rise,

however most studies which have focused on water quality, particularly in South Africa have focused mostly on drinking or potable water supply with limited reports on treated wastewater effluent as a source of pathogens (Mackintosh and Colvin, 2003; Obi *et al*., 2007; Obi *et al*., 2008). It is therefore imperative that South African waters be monitored for the presence of these pathogens in wastewater effluents in the interest of the environment as well as public health. Some of these microorganisms have displayed high microbial or antibiotic resistance trends, which can be linked to the increased usage of antimicrobial agents in multiple industries, thus resulting in the acquisition of resistance (Goni-Urriza *et al*., 2000; Huddleston *et al*., 2006; Vandan et al., 2011). The acquisition of resistance and virulence genes as well as the adaptation to adverse environmental conditions further aids their ability to escape conventional treatment processes (Almuzara *et al*., 2007; Jury *et al*., 2010). This project therefore aimed at evaluating the prevalence of *Aeromonas* spp., *Pseudomonas* spp., *Legionella* spp., *Listeria* spp., and *Yersinia* spp. in the treated effluent from two wastewater treatment plants in Durban, to determine the possible correlation between the microbial load and the physico-chemical profiles of the treated effluent and the receiving surface water as well as characterize the antibiotic resistance profiles and virulence determinants of selected *Listeria* and *Aeromonas* species recovered from these water samples.

1.8.1 Hypotheses

It was hypothesized that inadequately treated wastewater effluent is a source of multidrug-resistant emerging bacterial pathogens in surface waters in Durban and that some of these emerging bacteria are resistant to commonly used disinfectants and harbour virulence genes.

1.8.2 Objectives

The following objectives were established:

1.8.2.1 To determine the effect of seasonal variability changes on the populations of emerging bacterial pathogens in the treated wastewater effluent and receiving bodies.

1.8.2.2 To correlate microbial load with physicochemical parameters of the collected water samples.

1.8.2.3 To characterize virulence markers in selected emerging bacterial pathogens.

1.8.2.4 To establish the antibiotic resistance profiles of selected emerging bacterial pathogen to the currently used antibiotics.

1.8.3 Aims

1.8.3.1 Enumeration of presumptive population of *Aeromonas* spp., *Pseudomonas* spp., *Legionella* spp., *Listeria* spp., *Yersinia* spp. in the collected water sample using the membrane filtration technique and selective media.

1.8.3.2 Determination of physical and chemical profiles of the water samples namely; pH, temperature, phosphate, nitrate, sulphate, residual chlorine, chemical oxygen demand, biochemical oxygen demand, turbidity, total dissolved solids and total suspended solids and electrical conductivity.

1.8.3.3 Confirmation of selected emerging bacterial pathogens identity isolated from the treated wastewater effluent samples and receiving surface waters using standard biochemical tests and PCR methods.

1.8.3.4 Determination of antimicrobial resistance profiles of selected emerging bacterial pathogens using the antibiotic Kirby Bauer disk diffusion method.

1.8.3.5 Detection of virulence genes in selected emerging bacterial pathogens using specific primers

CHAPTER TWO

EMERGING BACTERIAL PATHOGEN ENUMERATION AND PHYSICOCHEMICAL ANALYSIS OF TREATED WASTEWATER EFFLUENT AND THE RECEIVING RIVERS

2.1 Introduction

Water forms a part of the most important resources world-wide, having the ability to directly or indirectly affect the world's economy and health status (Obi *et al*., 2007). Furthermore the backbone of many life systems, and important for industrial and agricultural processes, including multiple domestic uses and basic water functions (Hu, 2009). South Africa being a developing country, still struggles with meeting the basic water needs of its people. Many poor and rural communities still tend to rely more on river water for many of their water needs e.g. drinking, cooking, washing and recreational purposes (Odjadjare and Okoh, 2010). Therefore the state and quality of available water sources is of importance world-wide.

This has been demonstrated by the constant government efforts to better improve the provision and distribution of water that is of acceptable quality and environmental standard. Thus, working towards the improvement of wastewater treatment which greatly impacts the surrounding water milieus in both rural and urban communities is a priority (DWAF, 2003). However most of the wastewater treatment plants in South Africa do not meet the expected standards, which is monitored and regulated via the green drop certification programme being implemented by the Department of Water Affairs.

The inadequate treatment of wastes has a negative impact on natural water sources, which are the common receiving water bodies of treated effluent, as well as on the surrounding ecosystems and human population by altering chemical and physical properties of the

affected water. Chemical and biochemical interactions in these natural water systems can be influenced by both physical and chemical parameters such as pH, temperature, salinity electrical conductivity and nutrient load of the treated effluent (Igbinosa and Okoh, 2009). Wastewater effluent has been reported to contain a plethora of pathogenic microorganisms and chemical compounds some of which have been proven to pose serious threats to the surrounding environment as well as other biological systems dependant on this water, affecting biodiversity, human health and other aquatic dependant businesses such as the fish industry. Such contaminants include emerging bacterial pathogens which have been a threat to both animal and human survival over the past decades. Bacterial pathogens have also become more resistant to disinfectants and other antimicrobial agents when compared to coliform microorganisms which are commonly used as the indicators (fecal coliforms, total coliforms and enterococci) (Eze *et al*., 2009). This also implies that even in the absence of coliform indicators, emerging bacterial pathogens are still present in their numbers. Their presence in the absence of indicators is common and in most cases are even found dominating drinking water systems (Eze *et al*., 2009; Toze, 1997). Microbial pathogens such as *Aeromonas, Pseudomonas, Listeria, Yersinia* and *Legionella* with disease causing abilities have been reported in multiple cases. Some of these microorganisms have very high mortality rates and therefore are of a major threat if ignored or untreated, as most of them have been classified as opportunistic pathogens (Metcalf and Eddy, 2003; Rollins *et al*., 2003). This is of grave concern in South Africa, a country with a high number of HIV/AIDS pandemic, coupled with a high level of drug and alcohol abuse, which has led to many people being immune-compromised.

South Africa currently has no set guidelines for emerging bacterial pathogens (Bartie *et al.*, 2002), and their presence in the discharged wastewater effluent cannot be monitored (Bartie

et al., 2002). These pathogens have been implicated in a wide range of disease outbreaks which may result in death if ignored or untreated e.g. *Yersinia* causes septicaemia and pneumonia resulting in 100% mortality if untreated (Rollin *et al*., 2003). Although a considerable number of studies have been carried out on various pathogens isolated from wastewater effluents in South Africa, there exist little or no current reports in literature on the prevalence of *Aeromonas, Pseudomonas, Listeria Yersinia* and *Legionella* species isolated from wastewater effluents and the surrounding water bodies. This chapter aims at evaluating the prevalence of *Aeromonas* spp., *Pseudomonas* spp., *Legionella* spp., *Listeria* spp., and *Yersinia* spp. in the treated effluent samples and receiving waterbodies in Durban, to determine the effect of seasonal variability changes on the populations of emerging bacterial pathogens in the treated wastewater effluent and receiving bodies, and to correlate microbial loads/populations with physicochemical parameters of the collected water samples.

2.2 Materials and methods

2.2.1 Description of study site

Wastewater and river samples were collected from two different wastewater treatment plants located in the Durban area, namely: Northern Wastewater Treatment Works (NWTW) and New Germany Wastewater Treatment Works (NGWTW) (Figure 2.1). The NWTW is situated in the centre of industrial factories and residential areas and receives sewage containing both industrial and domestic wastes. The plant is situated very close to the Umgeni River into which it discharges its effluent. The river is surrounded by informal settlement communities which are dependent on the river water for recreation purposes, washing, bathing, cooking and drinking. The New Germany Sewage Plant processes about 90% of industrial waste in their final wastewater and the remaining is predominantly domestic wastes (Olaniran *et al*., 2005). It is surrounded by industrial factories processing

dyes, detergents and oil, which are often released by these factories and received by the treatment plant. This plant releases its effluent into the Aller River. Informal settlements are located in the close vicinity of the plant, and treatment of wastewater at both the plants is carried out using 3 main treatment steps, namely: The primary, secondary and the tertiary treatment step, which is accomplished via the chlorination step prior to the releasing of the effluent into either the Umgeni or Aller Rivers.

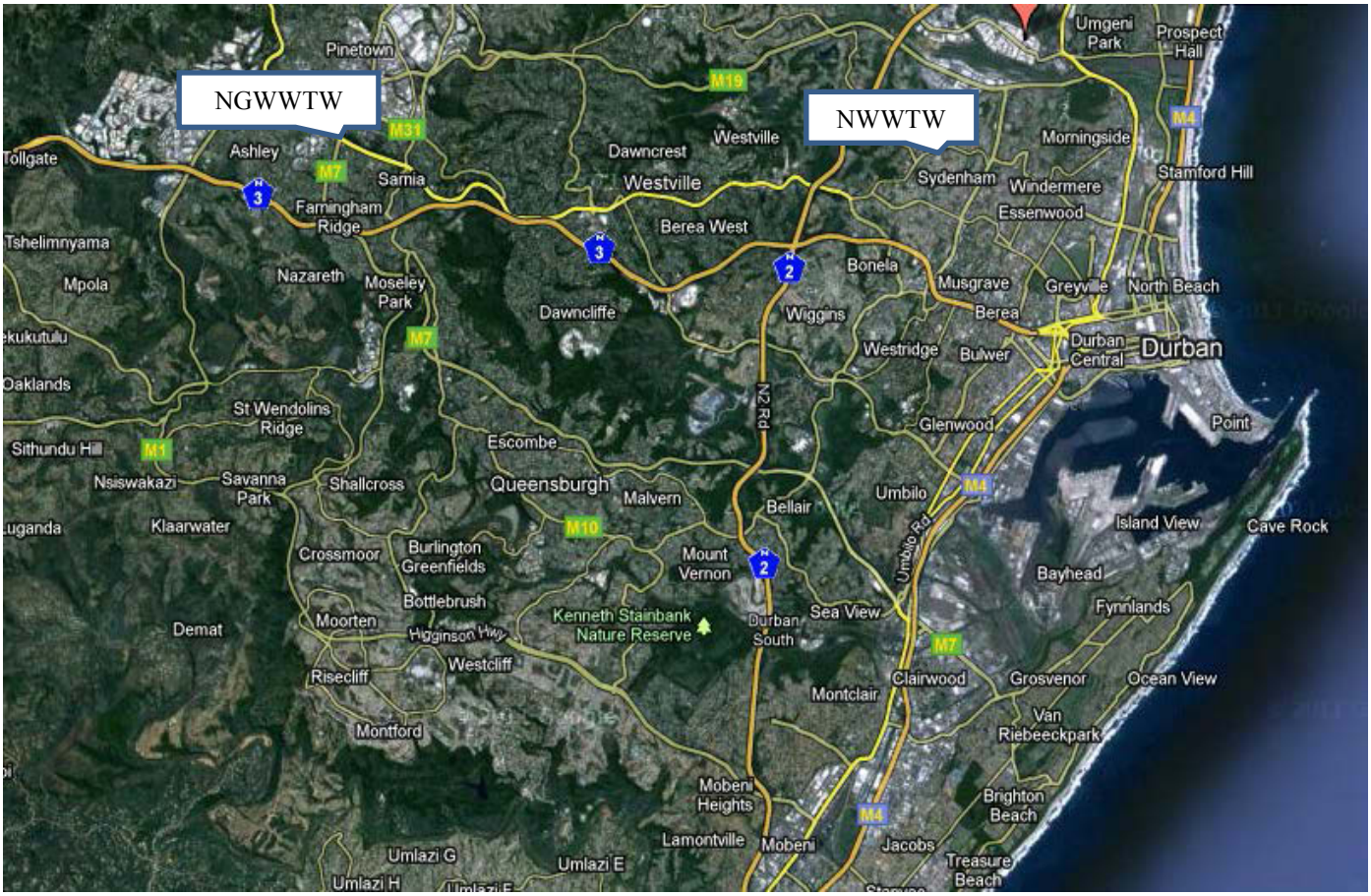

Figure 2.1: Map showing the location of the sewage treatment plants investigated in this study in the Durban area (www.googlemaps.com).

2.2.2 Collection of water samples

Water samples were collected on a monthly basis between March 2012 and February 2013 at the pre-designated points, namely, the final effluent (before chlorination) and the discharge point (after chlorination), and approximately 500 meters up and downstream of the discharge point into receiving waters. Five litre plastic bottles were sterilized with alcohol (70% v/v) and rinsed with sample water prior to collection. The samples were collected by submerging the container into the water against the water flow, leaving enough head space for the mixing of the sample (Palmer, 1993) and were transported at 4°C to the University of KwaZulu-Natal (Westville Campus), Durban.

Table 2.1: Cardinal points for wastewater and river water samples collected at the Northern and New Germany WWTPs.

	Northern WWTP		New Germany WWTP	
Sample points	**Latitude**	**Longitude**	**Latitude**	**Longitude**
BC	29° 47. 775'	0.30° 59. 754'	29° 48. 353'	0.30° 53. 829'
DP	29° 47. 988'	0.30° 59. 518'	29° 48. 353'	0.30° 53. 835'
US	29° 48. 203'	0.31° 00. 83'	29° 48. 340'	0.30° 53. 724'
DS	29° 48. 519'	0.31° 00. 083'	29° 48. 345'	0.30° 53. 847'

Legend: BC= Before chlorination DP= Discharge point US= Upstream DS= Downstream

2.2.3 Bacterial analyses

Wastewater samples were serially diluted with sterile distilled water and 50 ml of the appropriate dilutions were filtered through 0.45 µm pore sized filters, using standard membrane filtration methods (Obi *et al*., 2002). The membrane filtration apparatus, funnels and supports were sterilized by autoclaving for 15 min at 121°C. Membrane filters were then aseptically transferred onto different selective media (Table 2.1) specific for the organism to be identified (Fiorentini *et al*., 1998). Plates were incubated appropriately and then examined

for typical colonies, which were enumerated and expressed as colony forming unit per ml (CFU/ml). Presumptive isolates were inoculated separately onto fresh selective media to obtain pure culture, before sub-culturing onto nutrient agar plates for identification.

Table 2.2: Selective media and incubation temperatures for the cultivation of the emerging bacterial pathogens.

Pathogen	Media used	Colour of presumptive colonies	Incubation Temperature (°C)	Incubation period (h)
***Aeromonas* spp.**	Rimler-shotts agar	Yellow	37	20
***Listeria* spp.**	*Listeria* Chromogenic agar	Blue	35	24 – 48
***Legionella* spp.**	Buffered Charcoal-Yeast Agar (BCYE)	White	37	24
***Yersinia* spp.**	*Yersinia* selective agar base (CIN)	Colourless-pink	28	24-48
***Pseudomonas* spp.**	CHROM agar *Pseudomonas*	Cream	37	24 – 72

Working stock cultures were preserved on nutrient agar at 4°C and sub-cultured every second week and on glycerol stocks at -20°C for long term storage.

2.2.4 Physicochemical analysis

The physicochemical parameters of the collected water samples were evaluated as follows; pH was tested using Beckman 320 pH meter and temperature using the standard mercury thermometer. Phosphate, nitrate, sulphate and residual chlorine were determined at Clean Stream Scientific Services, Pretoria South Africa. Chemical oxygen demand (COD) was determined using the COD Cell Test (MERCK) by digesting the sample in a pre-heated thermoreactor HACH PRB 200 at 148°C for 120 minutes and thereafter read with the Spectroquant TR40 (MERCK). The biological oxygen demand (BOD) CD401 probe was used to determine BOD and turbidity was determined with HACH 2100P Turbidimeter. Total

dissolved solids, total suspended solids, and electrical conductivity were determined using HACH HQ40d Portable Meter, using the CDC401 PROBE.

2.3 Results

2.3.1 Bacterial analysis of wastewater effluent and receiving surface waters

During the 12 months sampling period (March 2012- February 2013), all the emerging bacterial pathogens investigated, namely: *Aeromonas, Pseudomonas, Listeria, Yersinia and Legionella* spp. were recovered from the four sampling points of the wastewater treatment plants.. The discharge and downstream points at the New Germany wastewater treatment plant had no *Listeria* species recovered for 8 of the 12 months samplingperiod (Table 2.4), with the months of March, June, July and December displaying counts for all sampling points. Different bacterial and seasonal trends were observed for both wastewater treatment plants, with each plant displaying varying results per tested organism. Of the five tested organisms, *Legionella* and *Yersinia* spp. were mostly recovered at higher concentration for both treatment plants, displaying similar patterns of microbial loads at the different sampling points. *Aeromonas* and *Pseudomonas* spp. also displayed similar recovery trends, while *Listeria* spp. were present at much lower concentrations at both plants.

At the NWWTP, *Aeromonas* spp. populations were obtained at higher populations before chlorination between the spring and summer months, with a presumptive population of 231000 x 10^3 CFU/ml recorded in December. Lower counts were observed during the autumn and winter seasons, with counts as low as 4.54 x 10^3 CFU/ml observed in January. Over the 12 months sampling period, bacterial populations (CFU/ml x 10^3) ranged between 4.42 – 23100, 0.53 –329000, 0.01 – 1470, 20.60 – 140000, and 40.13 – 95000 for *Aeromonas,*

Pseudomonas, *Listeria*, *Yersinia* and *Legionella* spp. respectively. Four out of the five tested bacteria were recovered at lowest concentrations in January and the highest concentrations observed in December for *Aeromonas* and *Yersini*a spp., September for *Pseudomona*s spp. and June for *Legionella* species. *Listeria* spp. counts at NWWTP were recovered at much lower concentrations when compared to the other microorganisms, with the highest counts recovered during the winter season.

The results obtained before and after tertiary treatment, allowed for the evaluation of the effectiveness of tertiary treatment for the removal of the emerging bacterial pathogens. The NWWTP displayed low reduction levels for the investigated bacteria for most of the sampling period, with an increase in populations observed in some months after chlorination. Percentage bacterial reduction varied between 19.5 – 99.9, 23.3 – 99.9, 8.2 – 99, 29.1 – 99.9, and 2.4 – 99 for *Aeromonas* spp., *Pseudomonas* spp., *Listeria* spp., *Yersinia* spp. and *Legionella* spp. respectively (Table 2.3).

The microbial counts obtained at the NGWWTP (Table 2.4) displayed a similar pattern to that observed at NWWTP and a relationship could be established between most of the tested organisms. The same trend was observed for most of the microbial populations, if a population increase or decrease was observed in one, the same would hold for the rest of the tested organisms. A positive correlation was observed mostly for *Aeromonas, Pseudomonas, Yersinia* and *Legionella* displaying significance levels with P-values < 0.01.

Table 2.3: Presumptive counts of selected emerging bacterial pathogens in wastewater effluents and the receiving water bodies (NWWTP).

		Bacterial counts (CFU/ml x 10^3)											
		Summer			Autumn			Winter				Spring	
***Aeromonas* sp.**	**Sampling point**	**December**	**January**	**February**	**March**	**April**	**May**	**June**	**July**	**August**	**September**	**October**	**November**
	BC	231000.00 ± 15556.35	4.54 ± 3.46	61.13 ± 1.96	900.00 ± 0.00	1170.00 ± 42.43	320.00 ± 28.28	6270.00 ± 42.43	5370.00 ± 98.99	150000.00 ± 14142.14	148000.00 ± 11313.71	4460.00 ± 56.57	8300.00 ± 84.85
	DP	186000.00 ± 5656.85	43.87 ± 3035	871.33 ± 62.17	2066.67 ± 115.47	370.00 ± 42.43	730.00 ± 14.14	4240.00 ± 56.57	6920.00 ± 113.14	5470.00 ± 98.99	264000.00 ± 8485.28	0.01 ± 0.00	6340.00 ± 56.57
	US	260.00 ± 56.57	36.20 ±3.33	51.80 ± 7.11	146.67 ± 11.55	490.00 ± 127.28	2020.00 ± 28.28	520.00 ± 56.57	120.00 ± 28.28	24.80 ± 1.13	1420.00 ± 1.13	48.30 ± 0.99	41.20 ± 1.41
	DS	3.55 ± 0.21	40.87 ±1.50	13.60 ± 5.57	780.00 ± 103.92	570.00 ± 14.14	2350.00 ± 70.71	140.00 ± 84.84	2920.00 ± 113.14	25.50 ± 0.71	1410.00 ± 0.71	1770.00 ± 98.99	40.10 ± 0.71
***Pseudomonas* sp.**	**BC**	180000.00 ± 28284.27	0.53 ± 0.06	15.47 ± 4.11	1986.67 ± 23.09	2850.00 ± 70.71	690.00 ± 14.14	5620.00 ± 28.28	3720.00 ± 169.71	117000.00 ± 9899.49	329000.00 ± 15556.35	3340.00 ± 1442.50	10050.00 ± 127.28
	DP	138000.00 ± 2828.73	55.73 ± 21.02	2120.00 ± 450.78	1293.33 ± 613.30	2150.00 ± 14.14	870.00 ± 42.43	4050.00 ± 42.43	1950.00 ± 14.14	3790.00 ± 14.14	363000.00 ± 18384.78	0.00 ± 0.00	6140.00 ± 113.14
	US	190.00 ± 14.14	19.87 ± 0.23	20.00 ± 0.00	15.93 ± 3.52	370.00 ± 42.43	2450.00 ± 70.71	540.00 ± 28.28	350.00 ± 14.14	26.80 ± 0.85	510.00 ± 98.99	21.80 ± 0.28	16.00 ± 0.57
	DS	7.10 ± 0.42	21.00 ± 1.00	13.13 ± 5.95	17.60 ± 2.12	580.00 ± 56.57	2750.00 ± 14.14	160.00 ± 28.28	800.00 ± 84.85	29.30 ± 1.27	580.00 ± 28.28	1440.00 ± 84.85	10.40 ± 1.98
***Listeria* sp.**	**BC**	0.23 ± 0.04	0.01 ± 0.00	0.01 ± 0.00	273.33 ± 110.15	620.00 ± 28.28	0.40 ± 0.00	1470.00 ± 98.99	0.91 ± 0.01	0.03 ± 0.01	0.30 ± 0.14	0.03 ± 0.01	58.30 ± 1.84
	DP	0.24 ± 0.00	1.33 ± 0.99	0.14 ± 0.23	0.13 ± 0.12	670.00 ± 98.99	1.00 ± 0.00	1350.00 ± 14.14	0.98 ± 0.01	0.12 ± 0.00	0.60 ± 0.00	0.01 ± 0.00	23.30 ± 0.4
	US	0.20 ± 0.00	0.00 ± 0.00	0.37 ± 0.00	0.56 ± 0.14	920.00 ± 28.28	1.40 ± 0.28	7.20 ± 0.57	0.01 ± 0.00	0.06 ± 0.01	0.06 ± 0.00	0.01 ± 0.00	0.09 ± 0.00
	DS	0.06 ± 0.01	0.00 ± 0.00	3.33 ± 2.89	0.22 ± 0.17	790.00 ± 14.14	0.04 ± 0.01	3.40 ± 0.28	0.35 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
***Yersinia* sp.**	**BC**	140000.00 ± 14142.14	20.60 ± 1.04	79.73 ± 0.23	3320.00 ± 1177.79	630.00 ± 42.43	1710.00 ± 42.43	117000.00 ± 4242.64	6350.00 ± 14.14	79000.00 ± 1414.21	104000.00 ± 5656.85	130000.00 ± 8485.28	10900.00 ± 84.85
	DP	85000.00 ± 7071.07	25.89 ± 0.45	951.33 ± 131.25	2266.67 ± 461.88	630.00 ± 42.43	770.00 ± 42.43	83000.00 ± 1414.21	6900.00 ± 141.42	19000.00 ± 1414.21	335000.00 ± 1414.21	0.02 ± 0.00	6920.00 ± 113.14
	US	470.00 ± 42.43	23.33 ± 3.06	65.13 ± 4.46	74.27 ± 22.33	1170.00 ± 42.43	2820.00 ± 169.71	970.00 ± 42.43	790.00 ± 70.71	33.10 ± 0.42	860.00 ± 28.28	350.00 ± 14.14	70.00 ± 42.43
	DS	3.00 ± 1.13	45.33 ± 5.03	11.53 ± 7.34	60.07 ± 0.12	880.00 ± 113.14	2520.00 ± 28.28	390.00 ± 14.14	4990.00 ± 14.14	32.90 ± 0.14	750.00 ± 14.14	3680.00 ± 113.14	230.00 ± 14.14
***Legionella* sp.**	**BC**	638000.00 ± 2828.43	40.13 ± 17.21	368.00 ± 13.86	602000.00 ± 0.00	6450.00 ± 42.43	6770.00 ± 42.43	954000.00 ± 8485.28	9540.00 ± 84.85	809000.00 ± 15556.35	511000.00 ± 7071.07	557000.00 ± 7071.07	13680.00 ± 169.71
	DP	623000.00 ± 1414.21	75.67 ± 13.59	7333.33 ± 5485.74	41266.67 ± 230.94	490.00 ± 127.28	7980.00 ± 28.28	659000.00 ± 1414.21	11070.00 ± 98.99	113000.00 ± 1414.21	550000.00 ± 14142.14	0.05 ± 14142.14	9450.00 ± 268.70
	US	2350.00 ± 14.14	7333.33 ± 577.35	102.67 ± 36.07	61266.67 ± 230.94	16010.00 ± 42.43	14420.00 ± 197.99	13450.00 ± 155.56	6850.00 ± 70.71	51.90 ± 0.71	4670.00 ± 127.28	2960.00 ± 127.28	1290.00 ± 70.71
	DS	67.50 ± 0.71	7026.67 ± 6039.22	97.33 ± 4.62	6466.67 ± 115.47	10750.00 ± 155.56	12990.00 ± 268.70	4370.00 ± 14.14	6850.00 ± 70.71	44.50 ± 0.42	4240.00 ± 56.57	7290.00 ± 56.57	1820.00 ±113.14

Legend: BC= Before chlorination DP= Discharge point US= Upstream DS= Downstream

Spring= September-November Autumn= March-May
Summer= December-February Winter= June-August

Over the sampling period, bacterial populations (CFU/ml x 10^3) in treated effluent samples before the chlorination step ranged between 17 – 9613.3, 19.2 –8820, 0.01 – 331, 70 – 11480, and 258 – 46600 for *Aeromonas, Pseudomonas*, *Listeria*, *Yersinia* and *Legionella* spp. respectively. The lowest concentrations recovered for *Aeromonas, Pseudomonas*, *Yersinia* and *Legionella* were recorded in October while the highest concentrations were observed in June. Similar to NWWTP, *Listeria* spp. counts at NGWWTP were at much lower concentrations compared to the other microorganisms, with the highest *Listeria* counts obtained during the winter season. Emerging bacterial pathogens were also recovered at the receiving water bodies throughout the sampling period. Counts obtained upstream of the receiving river were notably higher than any of the counts obtained at other points of sampling. Of the four samples, those collected at the discharge point and downstream of the receiving river had the lowest bacterial counts, in most cases during the sampling period.

Bacterial reduction at the NGWWTP after chlorination was considerably high, reaching reduction levels as high as 100% for *Listeria* and 99.9% for the other four emerging bacterial pathogens tested (Table 2.4). The month of May displayed very poor reduction of all tested microorganisms, with a few other cases displaying the failure to reduce or preferably eliminate the tested organisms after chlorination.

Table 2.4: Presumptive counts of selected emerging bacterial pathogens in wastewater effluents and the receiving water bodies (NGWWTP).

		Bacterial counts (CFU/ml x 10^3)											
	Sampling point	Summer			Autumn			Winter			Spring		
		December	January	February	March	April	May	June	July	August	September	October	November
***Aeromonas* sp.**	**BC**	6380.00 ± 28.28	9613.33 ± 400.67	2746.67 ± 1092.03	1000.00 ± 56.57	310.00 ± 14.14	270.00 ± 42.43	8130.00 ± 14.14	480.00 ± 113.14	1450.00 ± 70.71	6200.00 ± 141.42	17.00 ± 1.41	2970.00 ± 240.42
	DP	0.20 ± 0.01	0.04 ± 0.01	0.19 ± 0.18	960.00 ± 0.00	0.00 ± 0.00	450.00 ± 42.43	140.00 ± 28.28	330.00 ± 42.43	0.16 ± 0.01	0.10 ± 0.00	2.20 ± 0.28	0.00 ± 0.00
	US	7240.00 ± 56.57	39.20 ± 01.39	1646.67 ± 313.90	150.00 ± 14.14	20.00 ± 0.00	3790.00 ± 70.71	11430.00 ± 127.28	8460.00 ± 141.42	2080.00 ± 113.14	50.00 ± 14.14	1020.00 ± 28.28	2120.00 ± 56.57
	DS	0.12 ± 0.01	0.40 ± .40	26.67 ± 11.55	3380.00 ± 28.28	20.00 ± 0.00	570.00 ± 42.43	90.00 ± 14.14	7950.00 ± 14.14	0.04 ± 0.00	420.00 ± 84.85	990.00 ± 14.14	0.01 ± 0.00
***Pseudomonas* sp.**	**BC**	5560.00 ± 56.57	2266.67 ± 230.94	1666.67 ± 577.35	820.00 ± 28.28	210.00 ± 14.14	910.00 ± 42.43	8820.00 ± 141.42	310.00 ± 42.43	1050.00 ± 98.99	3740.00 ± 56.57	19.20 ± 1.13	3220.00 ± 84.85
	DP	13.70 ± 0.42	0.81 ± 1.03	0.17 ± 0.06	7700.00 ± 141.42	0.00 ± 0.00	960.00 ± 28.28	210.00 ± 14.14	210.00 ± 14.14	0.13 ± 0.01	0.10 ± 0.00	3.80 ± 0.28	0.00 ± 0.00
	US	5990.00 ± 14.14	2293.33 ± 257.16	1466.67 ± 461.88	3.90 ± 0.14	1.80 ± 0.28	5000.00 ± 282.84	12800.00 ± 791.96	9170.00 ± 42.43	3980.00 ± 28.28	1440.00 ± 56.57	3500.00 ± 141.42	3330.00 ± 98.99
	DS	0.09 ± 0.02	18.93 ± 1.01	133.33 ± 57.74	600.00 ± 0.00	0.50 ± 0.14	570.00 ± 14.14	30.00 ± 14.14	9650.00 ± 155.56	0.02 ± 0.00	190.00 ± 70.71	1190.00 ± 14.14	0.00 ± 0.00
***Listeria* sp.**	**BC**	0.01 ± 0.00	36.00 ± 6.93	2.00 ± 2.00	0.40 ± 0.00	230.00 ± 14.14	0.41 ± 0.04	331.00 ± 12.73	0.40 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.00 ± 0.00	0.11 ± 0.04
	DP	0.34 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	2.15 ± 0.07	0.00 ± 0.00	0.00 ± 0.00	10.00 ± 2.83	0.47 ± 0.10	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	US	0.04 ± 0.03	8.83 ± 1.02	0.00 ± 0.00	0.48 ± 0.06	50.00 ± 14.14	0.09 ± 0.01	420.00 ± 2.83	0.08 ± 0.00	0.33 ± 0.00	0.04 ± 0.00	0.00 ± 0.00	0.02 ± 0.00
	DS	0.22 ± 0.03	24.67 ± 4.09	0.00 ± 0.00	3.30 ± 0.14	20.00 ± 0.00	0.00 ± 0.00	1.00 ± 1.41	0.75 ± 0.01	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
***Yersinia* sp.**	**BC**	8290.00 ± 155.56	1660.00 ± 588.90	7540.00 ± 2132.32	1860.00 ± 28.28	70.00 ± 14.14	4180.00 ± 28.28	11480.00 ± 113.14	2890.00 ± 127.28	2040.00 ± 84.85	1890.00 ± 127.28	298.00 ± 2.83	1660.00 ± 28.28
	DP	0.22 ± 0.01	2.11 ± 1.83	0.17 ± 0.06	6000.00 ± 0.00	0.00 ± 0.00	1580.00 ± 28.28	380.00 ± 28.28	2590.00 ± 42.43	1.07 ± 0.04	6000.00 ± 0.00	12.10 ± 0.42	0.00 ± 0.00
	US	6450.00 ± 2955.71	31.33 ± 7.53	1780.00 ± 192.87	1450.00 ± 70.71	1.95 ± 0.07	12590.00 ± 70.71	12590.00 ± 70.71	23600.00 ± 282.84	1270.00 ± 98.99	1450.00 ± 42.43	3720.00 ± 113.14	1380.00 ± 28.28
	DS	0.15 ± 0.01	2.33 ± 2.04	226.67 ± 11.55	1150.00 ± 14.14	2.83 ± 0.04	1070.00 ± 14.14	50.00 ± 14.14	20200.00 ± 282.84	0.13 ± 0.00	1150.00 ± 56.57	4780.00 ± 28.28	0.00 ± 0.00
***Legionella* sp.**	**BC**	8900.00 ± 84.85	8006.67 ± 11.55	46600.00 ± 5716.64	4990.00 ± 989.95	8030.00 ± 14.14	4800.00 ± 0.00	16230.00 ± 14.14	8850.00 ± 127.28	3600.00 ± 141.42	4990.00 ± 11314	258.00 ± 2.83	7260.00 ± 113.14
	DP	0.32 ± 0.02	8.05 ± 1.70	161.33 ± 84.88	6400.00 ± 1131.37	0.31 ± 0.01	6400.00 ± 28.28	6400.00 ± 14.14	8650.00 ± 127.28	1.89 ± 0.07	0.08 ± 0.00	30.20 ± 2.55	0.02 ± 0.01
	US	9420.00 ± 254.56	2000.00 ± 40.00	23333.33 ± 5773.50	30800.00 ± 282.84	2000.00 ± 56.57	119000.00 ± 1414.21	610000.00 ± 14142.14	36900.00 ± 1271.79	1270.00 ± 98.99	6380.00 ± 28.28	8780.00 ± 254.56	258000.00 ± 2828.43
	DS	0.22 ± 0.01	2.87 ± 2.48	5946.67 ± 1654.25	61200.00 ±	270.00 ± 42.43	6000.00 ± 1979.90	4280.00 ± 84.85	34800.00 ± 1131.37	0.18 ± 0.00	4250.00 ± 70.71	8600.00 ± 56.57	0.12 ± 0.02

Legend: BC= Before chlorination DP= Discharge point US= Upstream DS= Downstream Spring= September-November Autumn= March-May Summer= December-February Winter= June-August

2.3.2 Physico-chemical parameters of the collected water samples

The physico-chemical parameters observed at the Northern wastewater treatment plant over the 12 month sampling period are shown in Table 2.5. The temperature and pH readings ranged between 12 – 27 °C and 6.41 – 7.88 respectively. For these parameters, the lowest readings were observed in September and the highest in February. There was no significant change in temperature readings across the sampling points for each month. Dissolved oxygen (DO), biochemical oxygen demand (BOD), chemical oxygen demand (COD) and total dissolved solids (TDS) ranged between 4.01 – 7.66, 1.40 – 9.61, <10 – 309.06, 200.03 – 710.00 mg/L respectively. Turbidity and total suspended solids readings ranged between 6.48 – 71.02 NTU and 0.01 – 5.93 respectively. Higher DO readings were noted more commonly at the discharge point than before chlorination point in March, May, October and November. A similar trend was noted for BOD, except for November when a higher reading was obtained before chlorination. Residual chlorine reading ranged broadly between 20.40 – 1090.00 mg/l, with higher concentrations observed in autumn and winter and lower in spring and summer. No notable trend was observed for the rest of the parameters determined.

Temperature and pH readings at the NGWWTP (Table 2.6) ranged between 12 – 26 °C and 6.34 – 8.08 respectively. The lower readings were observed during winter and spring; with values obtained upstream and downstream of the receiving Aller River samples commonly lower than those in samples collected before and after chlorination. DO, BOD, COD and TDS readings ranged between 7.07 – 8.69, 2.20 – 11.04, 24.33 – 313.61, 153.07 – 567.33 mg/L respectively. DO and BOD readings were higher at the discharge point (after chlorination) than before chlorination as well as higher upstream than in the downstream samples. The residual chlorine readings ranged between 22.31 – 677.37 mg/L throughout the sampling period, with the month of June displaying higher readings than usual. No notable trend was observed for the remainder of the physico-chemical parameters.

The correlations among the physicochemical and/or bacterial parameters were studied and results are presented in Tables 2.7 - 2.14. The correlation matrices obtained for NWWTP, before chlorination (Table 2.7) revealed a strong positive correlation between COD and the presumptive populations of *Aeromonas, Yersinia* and *Legionella* ($p<0.01$), followed by *Pseudomonas* and *Listeria* ($p<0.05$). Turbidity values also displayed a positive correlation with *Yersinia* and *Legionella* spp. populations. A positive correlation was observed between COD and all the tested organisms, however a stronger correlation was formed between *Aeromonas* and *Yersina* spp. ($p<0.01$) compared to the other organisms ($p<0.05$) obtained at the discharge point (Table 2.8). Results of samples obtained from this point also displayed a significant positive correlation between turbidity, TSS and *Legionella* spp. populations ($p<0.05$). Total suspended solids also showed significant positive correlation with the BOD readings. Tables 2.9 and 2.10 display the correlation results obtained for the upstream and downstream samples. A significant negative correlation ($p<0.05$) was observed between temperature readings and all the tested microorganisms, at both the sampling points. Most of the physico-chemical correlations were insignificant, while those which showed some level of correlation, such as BOD and COD displayed a negative one.

Table 2.5: Physico-chemical profiles of treated wastewater effluents from NWWTP and the receiving water bodies over the sampling period.

			Temperature	DO	BOD	COD	Conductivity	Resistivity	Salinity	TDS	TSS	pH	Turbidity	Nitrate	Phosphate	Residual Cl_2
			°C	mg/L	mg/L	mg/L	(µS/cm)	Ω.cm	%	mg/L	mg/L		Ntu	mg/L	mg/L	mg/L
AUTUMN	March	BC	26.00	5.58	2.23	90.44	839.67	1191.00	0.41	411.33	0.01	7.11	7.91	-	-	-
		DP	25.00	4.01	5.13	<10	888.00	1126.00	0.44	436.00	0.03	7.36	23.40	-	-	-
		US	26.00	5.19	5.62	161.33	757.67	1320.33	0.37	370.00	1.24	7.25	16.67	-	-	-
		DS	25.50	5.10	5.62	191.94	714.67	1400.00	0.35	348.67	1.13	7.24	15.27	-	-	-
	April	BC	22.00	4.69	3.30	127.89	783.33	1276.67	0.38	383.00	0.10	7.38	53.98	<0.057	2.11	116.35
		DP	22.00	4.80	3.44	152.89	903.67	1106.67	0.44	444.00	0.07	7.22	71.02	<0.057	2.10	219.64
		US	21.00	4.32	8.49	309.22	997.67	1004.00	0.49	491.00	0.02	7.29	18.35	0.86	0.23	260.56
		DS	21.00	4.80	6.33	303.00	676.33	1478.67	0.33	329.00	0.01	7.40	14.60	0.32	0.38	108.40
	May	BC	21.93	5.38	1.40	71.56	853.00	1173.00	0.42	418.00	0.04	7.02	20.65	0.16	2.72	198.93
		DP	21.00	4.81	3.04	< 10	966.00	1036.00	0.48	475.00	0.02	7.06	14.22	0.20	2.34	186.80
		US	21.00	5.71	4.52	51.00	2115.67	473.00	1.08	1067.00	0.01	6.87	13.58	1.03	1.08	1090.00
		DS	22.00	5.04	5.06	301.33	621.00	1612.33	0.30	302.00	5.93	6.85	13.58	1.72	0.82	214.37
WINTER	June	BC	13.50	4.35	4.01	113.33	703.00	1423.33	0.34	342.67	0.02	7.26	7.08	5.98	12.38	150.16
		DP	12.67	5.13	4.31	207.17	757.33	1320.33	0.37	368.33	0.02	7.27	6.48	3.92	9.02	163.67
		US	13.00	4.48	9.61	113.83	1082.33	924.00	0.54	534.33	0.01	7.57	6.95	2.40	2.17	918.93
		DS	12.00	5.22	7.34	89.39	633.33	1580.00	0.31	308.00	0.01	7.76	10.89	1.78	1.44	164.90
	July	BC	14.80	4.10	2.13	116.56	724.67	1370.67	0.34	348.33	0.03	7.20	20.30	1.00	1.83	555.13
		DP	15.30	4.49	3.10	291.22	849.67	1177.00	0.42	416.33	0.03	7.00	25.67	0.89	0.75	202.80
		US	14.93	4.49	7.80	311.72	1428.67	700.00	0.72	710.00	0.01	6.96	12.82	0.79	-0.03	210.33
		DS	15.83	4.53	6.75	311.72	606.00	1650.33	0.29	294.67	0.02	6.93	23.18	1.35	0.09	191.87
	August	BC	21.00	4.10	1.54	310.11	754.66	1319.33	0.37	370.33	0.04	6.85	56.37	-	-	93.36
		DP	19.00	4.49	2.23	182.78	840.00	1190.33	0.41	411.67	0.05	7.09	68.53	-	-	43.08
		US	20.00	4.49	7.80	105.89	899.00	1112.33	0.44	441.33	0.02	7.12	28.73	-	-	46.14
		DS	19.00	4.53	5.74	309.56	676.00	1479.00	0.33	329.67	0.02	7.26	20.76	-	-	45.01

DO= Dissolved oxygen **BOD= Biochemical Oxygen Demand** **COD= Chemical Oxygen Demand** **TDS= Total Dissolved Solids** **TSS= Total Suspended Solids**
US= upstream **DS= downstream** **BC= before chlorination** **DP= discharge point**

…Continuation of Table 2.5

			Temperature	DO	BOD	COD	Conductivity	Resistivity	Salinity	TDS	TSS	pH	Turbidity	Nitrate	Phosphate	Residual Cl_2
			°C	mg/L	mg/L	mg/L	(µS/cm)	Ω.cm	%	mg/L	mg/L		Ntu	mg/L	mg/L	mg/L
SPRING	September	BC	25.67	6.41	1.91	308.67	864.00	1156.67	0.42	423.67	0.03	6.76	20.73	0.15	1.70	31.49
		DP	26.00	6.61	2.52	309.00	789.67	1266.33	0.39	386.33	0.02	6.82	19.27	0.08	1.90	39.10
		US	26.00	8.12	7.90	55.56	509.33	1963.00	0.25	246.67	0.01	6.41	10.67	0.17	0.10	29.58
		DS	25.50	7.56	7.22	139.67	790.33	1265.33	0.39	386.33	0.01	6.52	11.50	1.47	0.16	40.10
	October	BC	22.00	7.31	3.18	306.89	831.67	1202.33	0.41	407.33	0.03	6.60	30.53	0.88	1.88	39.46
		DP	23.00	7.96	3.93	109.89	832.33	1200.67	0.41	408.00	0.02	6.75	28.50	0.82	1.57	34.37
		US	24.00	7.70	7.08	195.33	499.33	2003.00	0.24	241.33	0.36	7.02	17.07	1.02	0.37	32.51
		DS	24.00	7.64	6.93	148.00	689.67	1449.33	0.34	336.00	0.01	6.91	29.03	1.30	0.42	24.96
	November	BC	23.00	6.87	3.49	123.78	907.67	1102.00	0.44	445.33	0.05	6.79	39.97	0.28	3.53	20.40
		DP	22.00	6.86	3.35	287.22	970.00	1031.00	0.48	477.33	0.03	6.68	48.53	0.31	3.69	34.17
		US	21.00	7.62	8.38	241.78	429.00	2330.00	0.20	206.87	0.01	6.86	21.33	0.97	0.64	33.62
		DS	22.50	7.42	7.42	246.11	718.00	1392.33	0.35	350.00	0.01	6.72	14.10	3.38	0.45	28.17
SUMMER	December	BC	25.00	6.92	3.27	170.67	872.33	1760.33	0.43	428.00	0.05	6.78	36.13	0.24	1.82	24.17
		DP	21.00	7.60	3.54	153.56	961.67	1806.00	0.47	473.00	0.02	6.69	31.77	0.77	1.37	28.64
		US	22.00	7.50	7.04	274.33	415.33	2082.00	0.20	200.03	0.01	6.85	12.20	0.39	0.03	24.66
		DS	22.00	7.59	7.52	205.33	735.67	2010.33	0.36	359.33	0.01	6.64	10.33	1.95	0.35	29.12
	January	BC	24.00	7.60	3.72	306.89	804.00	1244.00	0.39	393.67	0.02	6.84	12.67	< 0.017	4.70	47.07
		DP	23.00	7.66	3.77	109.89	841.33	1188.33	0.41	412.33	0.04	6.87	32.67	< 0.017	4.69	56.64
		US	24.00	7.61	7.45	195.33	323.67	3093.33	0.15	155.10	0.03	7.04	11.40	0.58	0.23	25.04
		DS	24.00	7.66	6.67	148.00	672.00	1488.33	0.33	327.33	0.01	6.92	8.72	1.22	0.12	36.18
	February	BC	25.00	5.92	1.74	306.89	925.33	1080.67	0.45	454.33	0.07	7.80	40.37	< 0.017	3.51	40.02
		DP	25.00	6.87	2.81	109.89	931.00	1188.33	0.46	457.67	0.03	7.88	44.07	< 0.017	2.71	65.13
		US	25.00	7.48	7.56	195.33	319.33	3093.33	0.15	153.07	0.02	7.41	6.37	0.22	0.03	52.33
		DS	27.00	7.51	8.19	148.00	641.33	1488.33	0.31	312.33	0.01	7.77	5.94	0.16	0.53	56.61

DO= Dissolved oxygen **BOD= Biochemical Oxygen Demand** **COD= Chemical Oxygen Demand** **TDS= Total Dissolved Solids** **TSS= Total Suspended Solids**
US= upstream **DS= downstream** **BC= before chlorination** **DP= discharge point**

Table 2.6: Physico-chemical profiles of treated wastewater effluents from NGWWTP and the receiving water bodies over the sampling period.

			Temperature	DO	BOD	COD	Conductivity	Resistivity	Salinity	TDS	TSS	pH	Turbidity	Nitrate	Phosphate	Residual Cl_2
			°C	mg/L	mg/L	mg/L	(µS/cm)	Ω.cm	%	mg/L	mg/L		Ntu	mg/L	mg/L	mg/L
AUTUMN	March	BC	25.67	8.16	2.20	154.33	878.33	1125.67	0.44	436.00	0.01	7.12	6.65	-	-	
		DP	26.00	8.16	3.12	239.06	970.33	1023.67	0.48	477.33	0.02	7.18	5.71	-	-	
		US	26.00	8.58	7.79	141.67	431.33	2320.00	0.21	207.97	0.00	7.52	5.16	-	-	
		DS	25.50	8.62	4.97	313.61	615.00	1622.67	0.30	299.00	0.01	7.51	7.32	-	-	
	April	BC	20.17	8.40	3.53	199.28	790.33	1265.33	0.39	386.67	0.05	6.82	1.34	2.81	1.09	191.79
		DP	20.17	8.51	3.58	180.33	884.00	1131.33	0.43	433.67	0.03	6.70	1.44	2.35	0.86	188.63
		US	17.67	8.69	8.87	102.28	328.67	304.33	0.16	157.67	0.01	6.92	8.75	1.29	0.17	55.86
		DS	19.00	8.69	7.84	115.39	627.33	1594.33	0.30	305.33	0.06	7.01	17.10	0.82	0.28	132.50
	May	BC	19.00	8.29	3.29	312.78	1148.33	871.00	0.57	567.33	0.04	6.93	28.70	<0.057	0.05	343.10
		DP	18.50	8.19	4.10	245.33	1253.67	797.67	0.62	621.33	0.03	6.93	30.30	<0.057	<0.025	308.53
		US	13.67	8.78	11.04	305.61	363.67	2.75	0.17	174.67	0.00	6.63	3.18	0.84	<0.025	108.67
		DS	16.07	8.59	9.27	302.67	939.67	1064.33	0.46	462.00	0.02	6.83	17.80	0.51	<0.025	249.60
WINTER	June	BC	18.00	7.89	4.18	307.17	1084.33	925.33	0.53	534.33	0.01	7.60	7.68	3.76	1.05	381.50
		DP	17.50	8.10	4.36	124.17	1166.33	857.33	0.58	577.33	0.01	7.61	7.47	1.99	0.88	677.37
		US	16.00	8.22	9.85	26.33	383.67	2610.00	0.18	184.53	0.00	7.93	6.87	2.38	0.66	114.57
		DS	13.87	8.06	6.95	72.28	826.67	1209.00	0.40	404.67	0.01	7.81	9.13	2.15	0.33	298.43
	July	BC	16.50	7.19	2.38	193.67	974.67	1026.67	0.47	467.30	0.02	6.73	20.78	0.47	-0.03	273.47
		DP	17.00	8.71	4.45	308.67	1069.00	935.67	0.53	523.33	0.02	6.60	24.58	0.57	0.09	261.23
		US	13.50	8.58	8.14	309.39	321.67	3.11	0.15	154.30	0.00	6.34	7.39	1.03	-0.03	97.67
		DS	14.50	8.46	8.12	298.06	706.67	1415.00	0.34	345.00	0.02	6.47	19.77	0.71	0.07	196.00
	August	BC	16.80	7.19	4.10	139.56	849.33	1187.00	0.42	416.67	0.02	6.85	19.73	1.42	0.93	38.32
		DP	16.50	8.71	4.49	309.00	958.00	1044.00	0.47	471.00	0.01	7.09	16.80	1.96	1.47	31.77
		US	14.50	8.58	10.61	207.56	706.67	1419.00	0.34	344.67	0.03	7.12	40.40	0.80	1.89	33.79
		DS	12.00	8.46	8.84	311.78	757.33	1320.67	0.37	369.67	0.02	7.26	14.10	2.29	0.59	32.90

DO= Dissolved oxygen **BOD= Biochemical Oxygen Demand** **COD= Chemical Oxygen Demand** **TDS= Total Dissolved Solids** **TSS= Total Suspended Solids**
US= upstream **DS= downstream** **BC= before chlorination** **DP= discharge point**

…Continuation of Table 2.6

			Temperature	DO	BOD	COD	Conductivity	Resistivity	Salinity	TDS	TSS	pH	Turbidity	Nitrate	Phosphate	Residual CL_2
			°C	mg/L	mg/L	mg/L	(µS/cm)	Ω.cm	%	mg/L	mg/L		Ntu	mg/L	mg/L	mg/L
SPRING	September	BC	22.00	7.97	4.76	98.22	672.00	1488.33	0.33	327.33	0.01	6.75	5.84	8.22	38.90	31.27
		DP	20.00	8.26	4.57	310.44	778.67	1284.00	0.38	380.67	0.00	6.37	16.63	0.23	17.77	22.31
		US	20.00	7.74	8.22	310.33	528.67	1890.67	0.25	256.00	0.01	6.48	15.83	2.65	14.90	35.76
		DS	20.00	7.70	7.46	189.89	542.00	1841.67	0.26	261.67	0.02	6.59	6.98	0.72	18.62	28.35
	October	BC	20.00	8.11	4.17	311.89	733.67	1363.00	0.36	358.67	0.00	6.91	20.00	1.23	45.00	36.44
		DP	20.00	8.36	4.25	239.33	697.67	1433.33	0.34	340.33	0.00	6.85	16.33	1.64	43.30	38.37
		US	17.00	8.44	7.98	35.67	458.33	2180.67	0.22	221.33	0.00	6.97	3.68	2.32	25.50	24.19
		DS	19.00	8.50	8.92	54.11	545.67	1831.67	0.26	264.67	0.01	6.98	5.10	2.47	35.90	30.45
	November	BC	20.00	7.61	4.05	69.11	892.33	1116.33	0.43	431.67	-0.15	6.82	5.51	0.50	61.20	25.57
		DP	20.00	7.64	4.22	108.56	1087.67	914.67	0.54	534.67	0.01	7.14	6.48	0.33	56.40	33.42
		US	17.00	7.84	8.27	306.78	472.00	2120.33	0.23	228.00	0.01	7.12	8.11	2.11	29.10	56.91
		DS	18.00	7.60	7.96	257.56	715.00	1397.33	0.35	349.00	0.03	7.16	16.53	1.27	55.90	28.07
SUMMER	December	BC	22.00	7.07	2.88	93.33	552.67	1760.33	0.27	267.67	0.05	6.55	4.41	0.11	17.80	28.40
		DP	22.00	7.43	3.38	300.44	568.00	1806.00	0.27	275.33	0.04	6.45	29.43	< 0.017	21.50	30.42
		US	20.00	7.88	7.20	24.33	480.33	2082.00	0.23	232.00	0.05	6.47	32.10	< 0.017	1.58	40.31
		DS	20.00	7.70	6.96	35.33	497.00	2010.33	0.24	241.00	0.05	6.51	28.10	0.51	8.40	40.40
	January	BC	23.00	7.24	3.52	311.89	848.00	1176.67	0.42	417.67	1.12	6.59	9.43	< 0.017	31.80	50.82
		DP	23.00	7.71	3.80	239.33	849.33	1177.33	0.42	416.33	0.00	6.61	9.29	< 0.017	39.70	68.97
		US	22.00	7.71	7.11	35.67	410.33	2440.00	0.20	197.63	-0.11	6.71	10.80	1.21	26.00	25.75
		DS	22.00	8.03	8.23	54.11	522.00	1915.00	0.25	253.00	0.01	6.73	10.60	0.84	29.50	24.37
	February	BC	24.00	7.54	4.30	311.89	925.33	1080.67	0.45	454.33	0.01	7.72	3.93	< 0.017	54.90	31.71
		DP	24.00	7.75	4.24	239.33	931.00	1188.33	0.46	457.67	0.00	7.87	4.02	< 0.017	55.30	42.00
		US	21.00	7.22	7.64	35.67	319.33	3093.33	0.15	153.07	0.00	7.50	8.83	0.44	28.10	35.99
		DS	23.00	7.94	8.71	54.11	641.33	1488.33	0.31	312.33	0.01	8.08	5.80	0.39	41.50	25.18

DO= Dissolved oxygen **BOD= Biochemical Oxygen Demand** **COD= Chemical Oxygen Demand** **TDS= Total Dissolved Solids** **TSS= Total Suspended Solids**
US= upstream **DS= downstream** **BC= before chlorination** **DP= discharge point**

Table 2.7: Pearson's correlation coefficient of selected physicochemical and bacterial variables of water samples before chlorination at the NWWTP.

Variables	Turbidity	COD	*Aeromonas*	*Pseudomonas*	*Listeria*	*Yersinia*	*Legionella*
Turbidity	1	.486**	.296	.213	.213	.605**	.551**
COD		1	.501**	.398*	.398*	.563**	.520**
Aeromonas			1	.978**	.978**	.694**	.613**
Pseudomonas				1	1.000**	.613**	.571**
Listeria					1	.613**	.571**
Yersinia						1	.793**
Legionella							1

**. Correlation is significant at the 0.01 level *. Correlation is significant at the 0.05 level (2-tailed).

Table 2.8: Pearson's correlation coefficient of selected physicochemical and bacterial variables of water samples after chlorination at the NWWTP

Variables	Turbidity	COD	BOD	TSS	*Aeromonas*	*Pseudomonas*	*Listeria*	*Yersinia*	*Legionella*
Turbidity	1	.011	-.288	.625**	.203	.271	.009	.276	.537**
COD		1	-.222	.058	.341*	.437**	.357*	.331*	.189
BOD			1	-.370*	-.066	-.026	-.015	-.142	-.280
TSS				1	.109	.149	.116	.145	.200
Aeromonas					1	.966**	.266	.976**	.545**
Pseudomonas						1	.265	.946**	.626**
Listeria							1	.369*	.240
Yersinia								1	.615**
Legionella									1

**. Correlation is significant at the 0.01 level *. Correlation is significant at the 0.05 level (2-tailed).

Table 2.9: Pearson's correlation coefficient of selected physicochemical and bacterial variables of water samples upstream of the Umgeni River

Variables	pH	Temperature	COD	BOD	*Aeromonas*	*Pseudomonas*	*Listeria*	*Yersinia*	*Legionella*
pH	1	-.099	.076	.274	-.222	-.321	.479**	-.317	-.228
Temperature		1	.060	-.153	-.560**	-.488**	-.793**	-.542**	-.331*
COD			1	.376*	.009	-.008	-.298	.038	-.469**
BOD				1	-.146	-.150	.092	-.116	-.539**
Aeromonas					1	.892**	.369*	.855**	.567**
Pseudomonas						1	.361*	.908**	.410*
Listeria							1	.411*	.330*
Yersinia								1	.490**
Legionella									1

**. Correlation is significant at the 0.01 level *. Correlation is significant at the 0.05 level (2-tailed).

Table 2.10: Pearson's correlation coefficient of selected physicochemical and bacterial variables of water samples downstream at the Umgeni River

Variables	pH	Temperature	Turbidity	COD	BOD	*Aeromonas*	*Pseudomonas*	*Listeria*	*Yersinia*	*Legionella*
pH	1	-.004	-.456**	-.092	-.128	-.651**	-.594**	.548**	-.657**	-.405*
Temperature		1	-.007	.043	-.398*	-.409*	-.519**	-.679**	-.359*	-.619**
Turbidity			1	.007	-.022	.013	.118	-.357*	.029	-.012
COD				1	-.109	-.256	-.340*	-.474**	-.387*	-.229
BOD					1	.190	.365*	.285	.274	.305
Aeromonas						1	.860**	.084	.900**	.769**
Pseudomonas							1	.202	.901**	.748**
Listeria								1	.134	.329*
Yersinia									1	.709**
Legionella										1

**. Correlation is significant at the 0.01 level *. Correlation is significant at the 0.05 level (2-tailed).

Table 2.11: Pearson's correlation coefficient of selected physicochemical and bacterial variables of water samples before chlorination at the NGWWTP.

	pH	Temperature	Turbidity	TDS	COD	*Aeromonas*	*Pseudomonas*	*Listeria*	*Yersinia*	*Legionella*
pH	1	.183	-.236	.439**	.414*	.266	.202	.297	-.130	.355*
Temperature		1	-.582**	-.307	-.334*	.374*	.023	-.291	-.008	-.121
Turbidity			1	.474**	.300	-.192	.122	.159	.110	-.050
TDS				1	.786**	.096	.362*	.465**	-.084	.377*
COD					1	-.212	-.088	.537**	-.258	.287
Aeromonas						1	.727**	.323	.448**	.367*
Pseudomonas							1	.280	.709**	.701**
Listeria								1	-.023	.608**
Yersinia									1	.525**
Legionella										1

**. Correlation is significant at the 0.01 level *. Correlation is significant at the 0.05 level (2-tailed).

Table 2.12: Pearson's correlation coefficient of selected physicochemical and bacterial variables of water samples after chlorination at the NGWWTP.

Variables	pH	Temperature	Turbidity	TDS	COD	*Aeromonas*	*Pseudomonas*	*Listeria*	*Yersinia*	*Legionella*
pH	1	.068	-.434**	.208	.141	-.315	-.134	.089	-.316	-.353*
Temperature		1	-.385*	-.507**	.221	-.493**	-.278	-.456**	-.260	-.501**
Turbidity			1	.086	.121	.656**	.582**	.309	.461**	.548**
TDS				1	-.350*	.145	.085	.218	.150	.198
COD					1	.406*	.570**	.296	.184	.314
Aeromonas						1	.951**	.558**	.820**	.912**
Pseudomonas							1	.546**	.794**	.838**
Listeria								1	.383*	.538**
Yersinia									1	.635**
Legionella										1

**.Correlation is significant at the 0.01 level *. Correlation is significant at the 0.05 level (2-tailed).

Table 2.13: Pearson's correlation coefficient of selected physicochemical and bacterial variables of water samples downstream at the Aller River.

Variables	pH	TDS	BOD	*Aeromonas*	*Pseudomonas*	*Listeria*	*Yersinia*	*Legionella*
pH	1	-.135	-.068	-.444**	-.172	.509**	-.424**	-.496**
TDS		1	-.305	.318	.742**	.461**	.601**	.308
BOD			1	-.248	-.293	-.041	-.093	-.068
Aeromonas				1	.776**	-.122	.663**	.603**
Pseudomonas					1	.381*	.785**	.565**
Listeria						1	.047	.167
Yersinia							1	.495**
Legionella								1

**. Correlation is significant at the 0.01 level *. Correlation is significant at the 0.05 level (2-tailed).

Table 2.14: Pearson's correlation coefficient of selected physicochemical and bacterial variables of water samples downstream at the Aller River.

Variables	pH	Temperature	Turbidity	TDS	COD	BOD	*Aeromonas*	*Pseudomonas*	*Listeria*	*Yersinia*	*Legionella*
pH	1	.017	-.379*	.461**	.236	-.025	-.334*	-.398*	.503**	-.398*	-.359*
Temperature		1	-.391*	-.707**	-.420*	-.336*	-.538**	-.268	.006	-.376*	-.596**
Turbidity			1	.179	-.238	.056	.580**	.515**	-.172	.563**	.644**
TDS				1	.501**	.152	.381*	.179	.429**	.209	.412*
COD					1	.215	.253	.200	-.175	.223	.195
BOD						1	.084	-.021	-.251	.036	.105
Aeromonas							1	.927**	-.027	.966**	.971**
Pseudomonas								1	-.025	.975**	.906**
Listeria									1	-.106	-.039
Yersinia										1	.937**
Legionella											1

**. Correlation is significant at the 0.01 level *. Correlation is significant at the 0.05 level (2-tailed).

A positive correlation was observed between pH and TDS, COD and *Legionella*, while temperature was positively correlated with turbidity, COD, and *Aeromonas* (Table 2.11). TDS displayed a significant positive correlation with COD, *Pseudomonas*, *Listeria* and *Legionella*. Listeria was the only microorganism which was significantly, positively correlated to COD values. At the discharge point, temperature and pH show a negative correlation to the tested microorganisms (Table 2.12), while TDS and COD were positively correlated ($P < 0.05$). Tables 2.13 and 2.14 display the correlation results obtained for the upstream and downstream samples. Similar to NWWTP, a significant negative correlation ($p<0.05$) was observed between temperature readings and all the tested microorganisms, at both the sampling points. Most of the physico-chemical parameters were negatively correlated, while most displayed non-significant results. Throughout the sampling points at both the plants, the microbial populations mostly displayed a strong correlation to each other, with the exception of *Listeria* spp. which were not significantly correlated to neither of the organisms.

2.4 Discussion

Bacterial analyses of treated effluents from both plants displayed an unexpected trend for some of the sampling months, with an increase in the bacterial loads obtained at the discharge points (after chlorination) compared to counts obtained before chlorination, showing the survival of these organisms after chlorine treatment. This corroborates the findings of Odjadjare *et al*. (2010), who reported higher *Listeria* spp. populations in treated effluent after chlorination compared to the population recovered before the chlorination step. In another study by Odjadjare *et al*. (2012), *Pseudomonas* spp. populations were also obtained at higher concentrations after chlorination. This study however only focused on assessing final effluents of different plants.

Two factors could be responsible for the observed increase in the bacterial populations after the chlorination point at the NWWTP. Firstly the plant was undergoing an upgrade which could have resulted in treatment inefficiency during the sampling period. Secondly, after tertiary treatment, the water is channelled out of the plant to the DP which is approximately 4 km from the plant. The effluent pipe could have provided a conducive growth environment for the microorganisms, resulting in growth and microbial proliferation. At low tide the river water flow was inevitably directed towards the sea, allowing the water to flow from the downstream to upstream points where higher counts would be obtained. At high tide the river water flowed from upstream to downstream, where higher counts were obtained at the downstream point. This had an influence on the microbial populations obtained at both these points. Bacterial populations at NGWWTP were found to be generally lower than that of NWWTP. This could be attributed to the fact that NGWWTP is a smaller plant and treated lesser amounts of water having reduced the treatment capacity from the initial 7 to 0.5 megalitres per day. A greater reduction in the bacterial popultions was observed at this plant after chlorination step. This plant is also situated very close to the river, eliminating the use of a discharge pipe.

Bacterial populations were generally much higher in treated effluent than the receiving water bodies with the exception of NGWWTP's upstream point which is highly influence by other human activities. This could be as a result of higher nutrient levels in the wastewater effluents compared to the receiving water bodies, as previously reported (Conter *et al*., 2009; Czeszejko *et al*., 2003; Odjadjare *et al*., 2010; Pailard *et al*., 2005). The observed lower bacterial populations downstream of the Aller River at the NGWWTP could be due to the dilution effect of the treated effluent discharge high in residual chlorine concentrations to the

downstream flowing water. The narrow Aller River also has vegetation growing in it which might also serve as a filtration system to the downstream waters.

The Durban Wastewater Managing Department (DWM), controlled and monitored by the Department of Water Affairs (DWA) has set specific guidelines to monitor, maintain and protect the state of the surface water in South Africa. The DWA is responsible for monthly sampling and testing of wastewater effluent to ensure that current water policy meet the appropriate legislative requirements (DWAF, 1996). In this particular study, the five tested emerging bacterial pathogens were found to be present after tertiary treatment at both plants. This is of great concern, taking into consideration that in some cases, these pathogens were found to be present in extremely high numbers in the absence or low occurrence of commonly used indicator microorganisms (fecal coliforms, total coliforms and enterococci), which is assumed to be a sure indicator of the presence of pathogens, especially enteropathogens (Hoogeboezem, 2007; Perdek *et al.,* 2002). However, the discovery of emerging pathogens in treated water, in the absence of indicator organisms as observed in the study, indicates that the treatment processes are not effective in efficient pathogen removal (Toze, 1997). The alarmingly high counts obtained at the discharge point are worrisome, indicating the ineffectiveness of the tertiary treatment in removing these emerging bacterial pathogens and the potential health effects of these effluent discharge on the environment and many communities dependants of this water (Obi, 2002; Philippaeux, 1998, UNEP, 2010). Previous studies have shown that people who swim in waters with an increased number of pathogens showed signs of respiratory and gastrointestinal infections, fevers, chills, ear discharges, vomiting and coughing (Perez Guzzi *et al*., 2000). WHO (2008) reported that approximately 1.8 million children under five years of age die every year from water related diseases, with the under developed and developing countries such as South Africa being most

affected. The high rate of disease outbreaks resulting from contact or consumption of water from rivers into which wastewater effluent is discharged reflects on the poor treatment processes and the lack of compliance to set guidelines.

Receiving rivers of improperly treated wastewater effluent have been found to have minimized aquatic ecosystems and species diversity. It also affects the marine resource trade and allows for an increased possibility of ingesting food with toxic compounds that were absorbed by algal blooms during the process of eutrophication (Luger and Brown, 2010). Most of the rivers in the Durban Metropolitan Area suffer from eutrophication, due to contamination from untreated sewage resulting from poor treatment processes or blockage of sewage carrier pipes (DMW, 1999). The non-compliance of treated wastewater effluent has resulted in a number of disease outbreaks all around South Africa. In 2008, media reports in KwaZulu Natal claimed that large amounts of sewage effluents were being discharged into the Durban harbour killing a large population of fish and destabilizing aquatic ecosystems (Mema, 2009).

The presence of *Legionella* in alarmingly high numbers in the treated effluent tested in this study was not surprising since they are known to be more resistant to chlorine than coliform bacteria and may also be protected by amoebae and be sheltered in biofilms (Palmer *et al*., 1995). It has also been previously reported that *Y. enterocolitica* and other *Yersinia* spp. from raw sewage and sewage sludge, can survive up to 1.5 years in the environment, ultimately resulting in the contamination of drinking water. *Legionella* species were also found to survive for periods as long as 2.5 years in low and nutrient limiting environments, after being released into the environment. In addition, organisms such as *Pseudomonas* and *Aeromonas* spp., produce bacteriocins that result in significant inhibition of legionellae in treated water if

found in high numbers (Palmer *et al*., 1995). Thus, chlorine levels that eliminate microorganisms that inhibit *Legionella* spp. could result in increases in the population of indigenous *Legionella* spp. This can be true for this study considering that the *Aeromonas* and *Pseudomonas* spp. always had lower counts when compared to those of *Legionella* spp.

The *Listeria* counts obtained after tertiary treatment were found to be similar or within the same as those reported by Odjadjare *et al*. (2010). *Listeria* infections are reported to have the highest (up to 50%) mortality rate amongst foodborne pathogens. This is of grave concern in South Africa, with a high number of HIV/AIDS infected people, coupled with a high level of drug and alcohol abuse, which has led to many people being immunocompromised in the country. The potential severity of listeriosis outbreak on the public health is disquieting since there is absence of information on the prevalence of this pathogen in South Africa. The fact that *Listeria* is not considered a waterborne pathogen globally in spite of reports in the literature suggesting that the pathogen is well established in the water supply chain is also of grave concern (Odjadjare *et al*., 2010).

Aeromonas and P*seudomonas* spp. were also found to be present at both plants after tertiary treatment throughout the sampling periods. These results are similar to those obtained in similar studies (Bressler *et al*., 2009; Paillard *et al*., 2005; Pablos *et al*., 2009). These organisms have the ability to multiply under appropriate temperature and nutritional conditions and can also survive in drinking water despite treatment (Goni-Urriza *et al*., 2000). They have also been implicated in a wide range of disease outbreaks around the world including South Africa. One of the main reasons that these organism have been found to escape treatment, is the fact that they have the advantageous ability to form biofilm structures, which are highly involved in their survival and protection from disinfection (Vila *et al*., 2003). The mere fact that they are also highly present in drinking water which has

higher treatment standards is an indicator that they house numerous defence systems that provide protection during treatment. *P. aeruginosa* is especially of concern to human health today because it is a formidable nosocomial pathogen. *P. aeruginosa* is an appropriate commonly associated with a variety of health ailments including: urinary tract infections, respiratory infections, dermatitis, bone and joint infections, gastrointestinal infections, and systemic infections (Picardo and Giroux, 2004).

The physicochemical quality of the treated effluents from the two treatment plants were generally compliant to recommended limits of pH, temperature, TSS, and DO (Hitchins, 2001) set for effluents to be discharged into the receiving environment such as rivers (DWAF, 1992; CLSI, 2005). However, the remainder of the physico-chemical parameters displayed poor quality across the sampled sites and generally fell short of set guidelines/limits for turbidity, COD, phosphates and nitrates (Conter *et al*., 2009; DWAF, 1992; Hitchins, 2001, Naravaneni and Jamil, 2005). Temperature readings were commonly below the recommended limit of 25 °C for no risk stipulated in the South African water quality guidelines for domestic use (DWAF, 1996). According to Jaji *et al*. (2007), the temperature readings obtained in this study do not pose any threat to the homeostatic balance of the receiving water bodies. High turbidity readings obtained in the influent cannot be graded against guidelines, since no standard can be found for this parameter. The turbidity readings were however always higher that the 0 – 1 NTU standard set for water intended for domestic use (DWAF, 1998). Highly turbid waters may also give rise to other problems, during the treatment processes. Salinity, like turbidity has no set guidelines for treated effluent, but the conductivity readings exceeded the South African acceptable limit of 70 µ/Scm for conductivity in domestic water supply (DWAF, 1996), by extreme ranges, raising concern and disqualifying the water for domestic use. In previous studies conducted by

Igbinosa *et al*. (2009) and Fatoki *et al*. (2003), the conductivity readings obtained were also considerably higher than the set guideline. There are no guidelines set to monitor salinity readings, which in this study are below 1% for all the sampled points at both plants. However, certain companies and organisations such as SANCOR have established and put in place some criteria for aquatic ecosystems (SANCOR, 1984). Increased salinity values have a negative impact on crop yield, increase corrosion and highly salinized water often require high pre-treatment levels prior to use (DEAT, 2000). Dissolved oxygen reading seemed to fluctuate with the microbial population, probably due to the fact that in the presence of degradable organic matter, microorganisms require more oxygen to carry out the degradation and this process is oxygen demanding. DO readings obtained in this study were higher than those obtained in similar studies (Fatoki *et al*., 2003; Igbinosa *et al*., 2009; Jaji *et al.,* 2007). This again could possibly have a negative impact on the receiving water systems, considering the importance of a well-balanced biological system and that the oxygen balance of the system is essential for maintaining life within a biological system. COD values obtained at most of the sampling points were higher than the 30 mg/L South African guideline set for wastewater effluents to be discharged into the receiving water bodies (Government Gazette, 1984). COD is often linked to both the organic and inorganic substances which might be present in an environment, which can include organic contaminant carried by the wastewater influent into the plant, as well as from other sources. High COD values obtained at the NGWWTP could be attributed to the fact that the plant is surrounded by a number of industrial companies and treats mainly industrial wastes containing high concentrations of dyes, detergents and other toxic chemicals. High COD levels can be harmful to aquatic life and ecosystems (Fatoki et al., 2003). The high turbidity, COD and TDS obtained in this study suggesting the presence of high organic matter content which have a negative effect on the surrounding ecosystems as well as human population.

The residual chlorine values generally fell short of the recommended limit (0.3 to 0.6 mg/L) of no risk at point of use (SA Government Gazette, 1984) and this implies that the effluents may not be appropriate for domestic uses. A mixture of chlorine particles with high organic matter forms a toxic compound known as trihalomethane which is a carcinogenic compound formed as a by-product of chlorine and organic matter reaction in water systems and has been reported to have serious health implications for aquatic life and humans exposed to it (Murrell *et al*., 1999; Venkateswaran *et al*., 1989). Nitrate and phosphate levels for both plants were mostly higher that the expected or set guidelines and phosphate levels were alarmingly higher than the 1.5 mg/L guideline. Nitrate can be reduced to nitrite, which at high levels gives rise to methaemoglobinemia (Fatoki, 2003). It also results in eutrophication of receiving water bodies when values obtained exceeded the recommended limits for no risk of 0 to 0.5 mg/L (DWAF, 1996). Nitrates and phosphorus are essential nutrients important to plant growth when moderated. Excess quantities of these nutrients have the ability to stimulate excessive plant growth giving rise to algal blooms. Eutrophication could possibly affect the use of rivers and dams for recreation purposes, blocking access to waterways and giving rise to scum, which in turn could lead to the growth of blue-green algae and release toxic substances (cyanotoxins) into the water systems.

The prevalence and survival of pathogens in treated effluent after treatment processes poses a major threat both to the receiving environment and ecosystems and most importantly presents a potential threat to the health of the receiving communities primarily dependant on these waters (Luger and Brown, 2010). Microorganisms that bypass treatment processes have been found to be more virulent and have also been found as the main causatives of infections which cannot be easily treated (Kaye *et al*., 2004). South Africa currently has no set

guidelines for emerging bacterial pathogens (Bartie *et al.*, 2002), and therefore requires new and revised standards that will include emerging pathogens since treatment appears to be ineffective (Odjadjare *et al*., 2010) and the presence of these pathogens in the discharged wastewater effluent cannot be monitored (Bartie *et al*., 2002). The fact that these pathogens are also found to be present in drinking tap water which is meant to be safe and pathogen free, further proves that guidelines need to be revised. According to the safe water act, the maximum contamination levels for safe drinking water should be zero for all pathogens "no tolerable lower limit" (WHO, 2008). Some tested physico-chemical parameters also have no set guidelines making the monitoring of these factors difficult. Most of the guidelines being used are numerous decades old and are evidently outdated. There exists a need for the revision of wastewater effluent guidelines to ensure a system with no grey/uncertainty areas, especially since South Africa seems to be inclined towards the recycling of wastewater into drinking water.

CHAPTER THREE

ANTIMICROBIAL RESISTANCE AND VIRULENCE SIGNATURES OF *LISTERIA* SPECIES RECOVERED FROM TREATED EFFLUENT AND RECEIVING SURFACE WATER

3.1 Introduction

Wastewater effluent and surrounding fresh water bodies such as rivers and estuaries have been found to contain high levels of contaminants, including disease-causing bacteria such as *Listeria* species (Odjadjare and Okoh, 2010). The ability of these organisms to survive conventional wastewater treatment processes could lead to major environmental and human health problems, resulting from the highly contaminated surface waters (Odjadjare and Okoh, 2010). Previously, *Listeria* has only been associated with food related infections and disease, but has now been discovered and reported in water (Paillard *et al*., 2005). *Listeria* species are rod shaped Gram positive bacteria which are non-spore and capsule forming, with the ability to grow at a wide range of pH (4.4- 10), salinity (up to 20% concentration) and temperature (-0.4°C - 37°C) (Brugere-Picoux, 2008). Of the seven recognised *Listeria* species (*L. monocytogenes, L. ivanovii. L. innocua, L. seeligeri, L. welshimeri, L. grayii* and *L. murrayi*), only *L. monocytogenes* and *L. ivanovii* are currently deemed as pathogenic and infectious, causing diseases in animals and human beings, also being the only species that are known and have been previously reported to display β-haemolytic activity (Brugere-Picoux, 2008; Roberts and Wiednann 2003; Rodriguez-Lazaro *et al*., 2004; Schuchat *et al*., 1991). *L. ivanovii, L. seeligeri* and *L. innocua* however have also been reported to cause disease in both animals and humans (Brugere-Picoux, 2008; Cocolin *et al*., 2002; Cummins *et al*., 1994; Walker *et al*., 1994).

Listeria species are known to be oxidase negative, catalase, methyl red and Voges-Proskauer positive. *Listeria* species, mainly *Listeria monocytogenes* cause listeriosis which develops mostly in neonates, the elderly, pregnant women, and immuno-compromised individuals. *Listeria monocytogenes* can be classified as invasive or non-invasive, the former which spread to the nervous system causing meningoencephalitis, encephalitis, sepsis, abscesses and abortions resulting in a 20-30% mortality rate and the latter affecting not only the immuno-compromised but even the healthy individuals, resulting in abdominal pains, fever, diarrhoea, muscular pain, headache, nausea and vomiting (Arslan and Özdemir, 2008; Paillard *et al*., 2005).

Limited information has been documented regarding listeriosis outbreaks and incidence rates; however the disease still remains of great public health concern due to the high fatality rate, which for the past 2 decades has been estimated to be between 20 to 30 % in developed countries (Tirumalai, 2013). The prevalence of *Listeria* species in treated wastewater effluent has been reported both in developing and developed countries (Czeszejko *et al*., 2003, Paillard *et al*., 2005, Odjadjare and Okoh, 2010). Higher incidence rates have been reported to occur annually in other first world countries like the US and Europe (Mead *et al*., 1999; WHO/FAO, 2004) when compared to the low incidence or prevalence reported in Africa, Asia and South America and mostly in the 3rd world or developing countries (Rocourt, 1996; Tirumalai, 2013). This is possibly due to differing food consumption patterns between the developed and developing countries, as well as ineffective and inefficient pathogen detection methods in these countries (WHO/FAO, 2004). *Listeria* spp. are commonly reported as foodborne pathogens, however wastewater has for a long period been reported to be a potential reservoir and transporter of pathogenic *Listeria* which harbour virulence

determinants and display a notable trend of resistance to commonly used antimicrobials (Aslan and Ozdemir, 2008, Czeszejko *et al*., 2003, Paillard *et al*., 2005; Watkins and Sleath, 1981).

Wastewater treatment plants across South Africa have displayed poor bacterial pathogen removal over the years. Also, the current treatment regulations and guidelines mainly using common indicator organisms as a standard for monitoring drinking water and wastewater treatment processes have proven to be unreliable (Eze *et al*., 2009; Toze, 1997). This is of great public concern considering that an estimated 80% of South Africans depend on surface water for most of their domestic water needs (Venter, 2007, Mackintosh and Colvin, 2003). Worse cases of waterborne infections have been seen and reported mostly in poverty stricken populations which lack adequate sanitation and infrastructure as surrounding populations have easy and uncontrolled access to free-flowing highly contaminated waters to meet their water needs (Odjadjare and Okoh, 2010). Recent reports on waterborne disease outbreaks have shown to be unreliable and in most cases, the supposedly causative organisms found to represent a small percentage with the remainder causative organisms remaining unidentified, also highlighting the lack of reliable pathogen detection methods in South Africa (Odjadjare and Okoh, 2010).

This study assessed the prevalence of *Listeria* species in treated effluent of two wastewater treatment plants in Durban and the impact of the effluent discharge on the receiving water bodies (the Aller and Umgeni Rivers). The presumptive *Listeria* isolates recovered from the treated effluent and the receiving Rivers were confirmed by both the conventional biochemical tests and PCR methods. The confirmed isolates were further characterized for their virulence determinants and antimicrobial resistance profiles.

3.2 Materials and methods

3.2.1 Biochemical analyses

Identification of the presumptive *Listeria* spp. isolated from the treated effluent and the receiving rivers was performed using a range of biochemical tests including the oxidase and catalase test, carbohydrate fermentation, acid formation (TSI), indole production test and Methyl red and Voges-Proskauer test.

3.2.2 Molecular analyses

Polymerase chain reaction (PCR) was used to amplify and detect the presence of specific conserved sequences (*iap* gene) in each of the presumptive *Listeria* species isolates, using universal *Listeria* primers for further identification.

3.2.2.1 DNA isolation

Genomic DNA was isolated from biochemically identified *Listeria* species using the boiling method (Lin *et al*., 1996; Yàñez *et al*., 2003). One colony from fresh 24 hour culture was suspended in a microcentrifuge tube containing 70 µl sterile ddH_2O. The culture and water mixtures were vortexed at high speed for 1 min and heated to 100 °C for 10 min in a water bath to lyse the cells and thereafter cooled to room temperature. The tubes were vortexed again prior to centrifugation at 13 000 rpm for 5 min and the supernatant was transferred to a sterile tube and used as template in the PCR assay.

3.2.2.2 PCR identification of *Listeria* species isolates

The PCR amplification of the *iap* gene was performed in a thermal cycler (GeneAMP PCR System 2400, Bio Rad) using the primers indicated in Table 3.1. PCR conditions were as follows: Pre-denaturation at 95 °C for 5 min, denaturation at 95 °C for 1 min, annealing at 36 °C for 1 min, extension at 72 °C for 3 min and final extension 72 °C for 7 min for 30 cycles (Cocolin *et al*., 2002). PCR was performed in a final volume of 50 µl containing 50 mM KCl, 10mM Tris/HCl (pH 8.3), 3.5 mM $MgCl_2$, 0.2 µM of each dNTP, 1.25 U *Taq* DNA polymerase, 0.2 µM of each primer and 1 µl of the extracted DNA sample (Cocolin *et al*., 2002). *Listeria monocytogenes* ATCC 19115 strain was used as a positive control in all PCR assays**.**

Table 3.1: Characteristics of primers used for PCR amplification of *iap* gene.

Organism	Primer	Sequence(5′-3′)	Product size (bp)	References
***Listeria* spp.**	List-Universal1 List-Universal2	ATGTCATGGAATAA GCTTTTCCAAGGTGTTTTT	457-610	Cocolin *et al*. (2002)

Following the PCR, the amplification products were subjected to gel electrophoresis using a 1% (w/v) agarose gel and detected by staining in ethidum bromide solution (0.5 µg/ml) for 30 – 40 min (Cocolin *et al*., 2002). The gels were rinsed in water or TAE buffer to remove excess dye on the gel, and were visualized under the SYNGENE Gel documentation/ Bio-imaging system (Syngene UK).

3.2.2.3 Virulence gene characterization

A multiplex PCR assay was used for the detection of four virulence-associated genes of *Listeria monocytogenes* namely, *plcA, hlyA, actA* and *iap*, coding for the phospholipase, haemolysin, intracellular motility and p60 invasion proteins respectively (Rawool *et al*., 2007) using the primer sets indicated in Table 3.2.

Table 3.2: Primers used for PCR amplification of *Listeria* virulence genes.

Organism	Primer	Sequence(5′-3′)	Product size(bp)	References
Listeria monocytogenes	Plc A	CTG CTT GAG CGT TCA TGT CTC ATC CCC C ATG GGT TTC ACT CTC CTT CTA C	1484	Rawool *et al*., 2007
	Act A	CGC CGC GGA AAT TAA AAA AAG A ACG AAG GAA CCG GGC TGC TAG	839	
	Iap	ACA AGC TGC ACC TGT TGC AG TGA CAG CGT GTG TAG TAG CA	131	

The multiplex PCR for *Listeria* spp. was performed in a single reaction tube containing all the four primer sets for these genes, with a final reaction mixture of 50 µl containing 1x PCR buffer (100 mM Tris-HCl buffer, pH 8.3 containing 500 mM KCl, 15 mM $MgCl_2$ and 0.01% gelatin), 1 mM dNTP mix, 6 mM $MgCl_2$ and 10 µM of each primer sets (Table 3.2), 4U of Taq DNA polymerase, 5 µl of DNA and sterilized water to make up the reaction volume. PCR was carried out in a Thermocycler (GeneAMP PCR System 2400, Bio Rad) with the following conditions: Pre- denaturation at 95 °C for 2 min, denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, extension at 72 °C for 1min and final extension at 72 °C for 10 min for 35 cycles (Rawool *et al*., 2007).

3.2.3 Antimicrobial resistance profile analyses

The Kirby Bauer Disk diffusion method (CLSI, 2005) was used to determine the antibiotic resistance profile of *Listeri*a spp. isolates. The isolates were screened against a predetermined panel of 24 antibiotics belonging to 14 different classes (Table 3.3). Cultures were grown for 24 h in Luria–Bertani broth and thereafter standardized to 0.5 McFarland standard (OD_{625nm} = 0.1- 0.8) using a spectrophotometer (Biochrom, Libras), before spreading on Mueller-Hinton agar plates using sterile swabs. The plates were then dried at room temperature for 45 min before placing the discs on the plates at equidistance. The plates were incubated for 24 h at 37 °C (CLSI, 2005). Zones of clearance surrounding each disk were used to determine the level of susceptibility or resistance, and were scored based on the CLSI (CLSI, 2005) standards. The results were interpreted according to CLSI standards for *Escherichia Coli* ATCC 25922 due to lack of specific standards for *Listeria* species (Zuraini *et al*., 2001).

Table 3.3: Antimicrobials and the concentration levels used.

Antibiotic class	Antibiotics	Level (µg)	Symbol
β-Lactams	Penicillin	10	P
	Cephalothin	30	KF
Aminoglycosides	Gentamicin	10	CN
	Kanamycin	5	K
	Amikacin	30	AK
Carbapenems	Ertapenem	10	ETP
	Meropenem	10	MEM
Cephalosporin	Cefotaxime	30	CTX
	Ceftriaxone	30	CRO
Glycopeptides	Vancomycin	30	VA
Lincosamides	Clindamycin	10	DA
Macrolides	Erythromycin	15	E
Nitrofurans	Nitrofurantoin	50	F
Penicillins	Ampicillin	10	AMP
Polypeptides	Colistin	10	CT
Quinolones	Nalidixic acid	30	NA
	Mixofloxacin	5	MXF
	Ciprofloxacin	5	CIP
Sulfonamides	Trimethoprim	5	W
Tetracyclines	Tetracycline	10	TE
Other	Streptomycin	25	S
	Chloramphenicol	30	C
	Fosfomycin	50	FOS
	Fusidic Acid	10	FD

3.2.4 Protease, gelatinase and haemolysin assay

Protease activity was assayed by spreading *Listeria* strains on nutrient agar containing 1.5% skim milk. After incubation at 30°C for 72 h, the production of protease was shown by the formation of a clear zone caused by casein degradation. Gelatinase production was determined using LB agar containing gelatine (30 g/L), the plates were incubated at 30°C for 24 h and cooled for 5 h at 4°C. The appearance of turbid halos around the colonies was considered positive for gelatinase production (Sechi *et al*., 2002). Haemolysin production was assayed by culturing each strain on human blood agar at 30°C for 24 h. The production of haemolysin was observed by the formation of a clear zone caused by β-haemolysis activity of the enzyme on the blood (modified from Sechi *et al*., 2002).

3.3 Results

3.3.1 Identification of the presumptive *Listeria* spp. isolates

Based on the biochemical tests listed under section 3.2.1., isolates were confirmed as either negative or positive. Oxidase negative, catalase positive and Methyl red and Voges-Proskauer positive tested organisms were further confirmed using PCR. The resulting PCR amplification products with the expected sizes (457-610 bp, commonly 457 bp) of the universal conserved *iap* gene regions in the *Listeria* spp. isolates are shown in Figure 3.1. A total of 78 *Listeria* spp. isolates obtained from the FE (23.08%), DP (35.90%), US (20.81%) and DS (19.23%) sampling points were confirmed and identified as *Listeria* species.

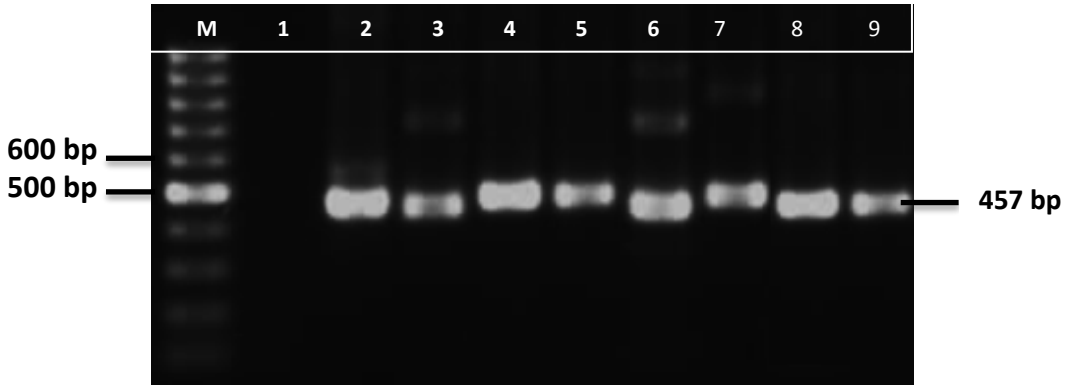

Figure 3.1: Representative agarose gel showing PCR amplicons of the *iap* gene of *Listeria* spp. isolates (lanes 2- 9), M: molecular marker and lane 1: negative control.

3.3.2 Virulence gene signatures of the *Listeria* species isolates

The agarose gel showing the multiplex PCR amplicons of three virulence associated gene products for *Listeria monocytogenes* ATCC 19115 used as the control in this study is represented in figure 3.2. The primer sets allowed for the amplification of 1484 bp (*plc*A), 839 bp (*act*A), and 131 bp (*iap*) PCR products, each represented by a single band in the expected base pair region. These genes are specific for *Listeria monocytogens*, with a minor chance of *L. ivanovii* and *L. seeligeri* detection. Of the 78 tested *Listeria* spp., a total of 26.92% (21) were found to contain virulence genes, 14.10% (11), 5.12% (4) and 21% (17) of these species were found to harbour the *act*A, *plc*A and *iap* genes respectively. Of the total number of isolates, 11.54% (9) contained more than one virulence gene (*act*A and *iap* genes). None of the virulence genes were detected in the remaining *Listeria* species.

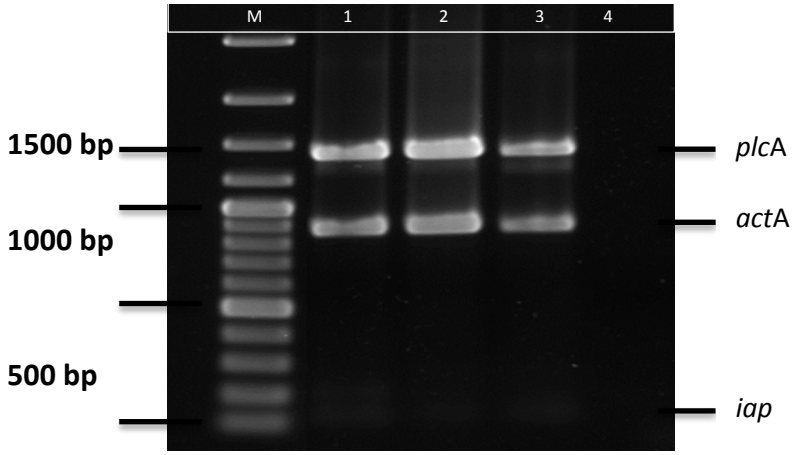

Figure 3.2: Representative agarose gel showing PCR amplicons of three virulence associated genes for *Listeria monocytogenes* (ATCC 19115). M: DNA marker (100 to 3000 bp), Lane 1-3: amplified products of three genes (*plcA*, *actA*, and *iap*), Lane 4: negative control.

3.3.3 Antimicrobial resistance profiles of *Listeria* species

The resistance and susceptibility profiles of *Listeria* spp. isolates against a broad range of antimicrobials commonly used for Enterobacteria are shown in Table 3.4. The highest resistance was observed for penicillin, erythromycin and naladixic acid, with all 78 (100%) tested species displaying resistance, followed by ampicillin, trimethoprim, nitrofurantoin and cephalosporin with 83.33%, 67.95%, 64.10% and 60.26% respectively. Of the 78 tested species, 100% sensitivity was observed for 5 of the antibiotics: streptomycin, chloramphenicol, fosfomycin, fusidic acid and meropenem, followed by ciprofloxacin, meropenem, gentamicin, clindamycin and colistin, with 96.15%, 89.74%, 72.31%, 88.46% and 79.48% of the isolates displaying susceptibility respectively. Most of the tested isolates were found to be susceptible to most of the tested antibiotics. All the tested isolates showed resistance to at least 5 of the 24 antibiotics, with 4 (5.13%) of the test isolates displaying resistance to at most 12 of the 24 antibiotics.

The antibiotic resistance index (ARI) for the *Listeria* spp. ranged between 0.13 (resistance to 3 test antibiotics) – 0.5 (resistance to 12 of the test antibiotics). The multidrug resistance patterns of the *Listeria* spp. as shown in Table 3.5, revealed that at least 1 (1.28%) of the isolates was found to be resistant to 3-4 antibiotic classes, with most of the multidrug resistant organisms being resistant to more than 5 and at most 11 antimicrobial classes. The highest multidrug resistance patterns was observed in 3 (3.85%) of the total 78 isolates, being resistant to 11 of the 24 tested antibiotics, followed by 5 (6.41%) which were resistant to 10 antibiotic classes. The highest percentage resistance was observed for 19 (24.36%) of the tested *Listeria* spp. which were resistant to 9 antimicrobial classes, followed by 16 (20.51%) and 12 (15.38%) isolates found to be resistant to 6 and 7 antimicrobial classes, respectively. None of the *Listeria* species was found to be resistant to more than 11 antimicrobial classes.

Table 3.4: Antimicrobial resistance/susceptibility profile of *Listeria* species isolates.

Antibiotic class	Antibiotics	Code	Level (µg)	Bacterial isolates		
				Listeria spp. (n=78)		
				n (Resistant)	n (Susceptible)	n (Intermediate)
β-Lactams	Penicillin	P	10	78 (100)	0	0
	Cephalothin	KF	30	47 (60.26)	24 (30.77)	7 (8.97)
Aminoglycosides	Gentamicin	CN	10	0	72 (72.31)	6 (7.69)
	Kanamycin	K	5	41 (52.56)	18 (23.08)	19 (24.36)
	Amikacin	AK	30	0	78(100)	0
Carbapenems	Ertapenem	ETP	10	22 (28.20)	48 (61.90)	8 (10.26)
	Meropenem	MEM	10	0	70 (89.74)	8 (10.26)
Cephalosporin	Cefotaxime	CTX	30	24 (30.79)	39 (50)	15 (19.23)
	Ceftriaxone	CRO	30	19 (24.36)	45(59.69)	14 (17.95)
Glycopeptides	Vancomycin	VA	30	24 (3.76)	54 (69.23)	0
Lincosamides	Clindamycin	DA	10	9 (11.54)	69 (88.46)	0
Macrolides	Erythromycin	E	15	78 (100)	0	0
Nitrofurans	Nitrofurantoin	F	50	50 (64.10)	6 (7.69)	22 (28.20)
Penicillins	Ampicillin	AMP	10	65 (83.33)	8 (10.26)	5 (6.41)
Polypeptides	Colistin	CT	10	16 (20.51)	62 (79.48)	0
Quinolones	Nalidixic acid	NA	30	78 (100)	0	0
	Mixofloxacin	MXF	5	3 (3.85)	72 (92.31)	3 (3.89)
	Ciprofloxacin	CIP	5	0	75 (96.15)	3 (3.89)
Sulfonamides	Trimethoprim	W	5	53 (67.95)	20 (25.64)	5 (6.41)
Tetracyclines	Tetracycline	TE	10	37 (47.44)	31 (39.74)	10 (12.82)
Other	Streptomycin	S	25	0	78 (100)	0
	Chloramphenicol	C	30	0	78 (100)	0
	Fosfomycin	FOS	50	0	78 (100)	0
	Fusidic Acid	FD	10	0	78 (100)	0

n= number of isolates; value in bracket represent % resistance, susceptibility or intermediates

Table 3.5: Multidrug resistance pattern in the *Listeria* spp.

Number of antibiotic classes	3	4	5	6	7	8	9	10	11
No. of isolates resistant	1	1	11	16	12	10	19	5	3
Percentage resistant	1.28	1.28	14.10	20.51	15.38	12.82	24.36	6.41	3.85

3.3.4 Protease, gelatinase and haemolysin production

Blood agar plates were hydrolysed by 25 (35%) of the tested *Listeria* species formed clear zones around the colonies indicating a positive result for the production of the haemolysin enzyme. All the tested *Listeria* isolates tested negative for gelatinase and protease enzyme production.

3.4 Discussions

It is mandatory through the South African Water Act (Act 54 of 1956) that wastewater effluent be treated to acceptable standards before release into surface water resources. However, potentially pathogenic microorganisms have been reported to prevail in treated waters in several studies conducted in South Africa indicating that wastewater effluent in most cases fails to meet the "acceptable standard" status (Kaye *et al*., 2004; Khabo-Mmekoa *et al*. 2010; Momba *et al*., 2006; Obi *et al*., 2008). Some of these studies have reported an increase in highly pathogenic and virulent strains containing virulence and antibiotic resistance genes posing a potential health threat to the public.

In the current study, the presumptively identified *Listeria* species were subjected to a range of biochemical tests and PCR methods for further confirmation, with results indicating that potentially pathogenic *Listeria* species have the ability to bypass conventional wastewater treatment processes. A similar study conducted by Odjadjare and Okoh (2010) reported on the prevalence of *Listeria* species in wastewater effluent after tertiary treatment, leading to the contamination of surrounding rivers in the Eastern Cape. There are currently no standards in place to determine the acceptable limit of *Listeria* spp. in treated effluent in South Africa. However, the "no risk limit" of 0 cfu/100 ml for faecal coliform recommended for domestic water uses by the South African government (DWAF, 1996), was exceeded in this study. This is of great concern considering that these emerging bacterial pathogens are in most cases found present in the absence of common indicator microorganisms (fecal coliforms, total coliforms and enterococci). The discovery of emerging pathogens in treated water, in the absence of indicator organisms as observed in the study, indicates that the treatment processes are not effective in efficient pathogen removal (Toze, 1997). The limited information on *Listeri*a related studies and their prevalence in wastewater, particularly in South Africa could be due to the fact that *Listeria* species are still largely associated with food associated infections (foodborne pathogen) as opposed to being fully recognised waterborne pathogens (Paillard *et al*., 2005). Few studies which have been conducted have reported similar results displaying a notable prevalence of *Listeria* species in treated effluent (Al-Ghazali and Al-Azawi, 1988; 1988; Paillard *et al*., 2005; Odjadjare and Okoh, 2010; Watkins and Sleath, 1981).

In this study, 14.10% (11), 5.12% (4) and (21%) 17 of the tested *Listeria* species were found to harbour the *act*A, *plc*A and *iap* genes respectively, while no virulence genes were detected in the remainder of the tested isolates. Based on previous findings reported by Rawool *et al*.

(2007), all the isolates containing the above listed virulence genes, could possibly be *L. monocytogenes, L. ivanovii* and *L. seeligeri*. Results obtained in this study indicate a possible presence of virulent *Listeria* strains, which could infect the users of these contaminated waters. Although the use of primers specific for *plcA*, *actA*, *hlyA* and *iap* genes are reported to be unique to pathogenic *Listeria monocytogens* (Furrer *et al*., 1991), the identity of the *Listeria* species containing virulence genes cannot be accurately identified as common pathogenic *Liseria monocytogens*, in agreement to a study conducted by Rawool *et al*., (2007) which indicated their detection in two other *Listeria* species. The remainder of the Listeria species in this study can therefore not be completely disregarded as possible *Listeria monocytogene*s strains considering that there is a possibility that some strains may lack one or more virulence determinants due to genetic mutations (Cooray *et al*., 1994). The use of PCR based methods is said to be insufficient at revealing the true pathogenic potential considering that different *Listeria monocytogene* strains display different profiles (Rawool *et al.,* 2007; Shakuntala *et al.,* 2006).

There is also limited information on the antibiotic resistance/susceptibility profiles of *Listeria* species isolated from treated wastewater effluent, with most studies on the antimicrobial susceptibility profiles of *Listeria* species focusing almost exclusively on clinical and/or food isolates. The susceptibility patterns of *Listeria* spp. obtained in this study is similar to that reported by Odjadjare and Okoh (2010), who tested 23 *Listeria* isolates against 20 antibiotics. All the 23 *Listeria* species tested in that study were sensitive to 3 of the 20 test antibiotics including amikacin (aminoglycosides), meropenem, and ertapenem (carbapenems) suggesting that these antibiotics may be the best therapy in the event of listeriosis outbreak in South Africa. Similar to the observation in this study, Hansen *et al*. (2005) reported complete sensitivity of 106 *Listeria* species isolated from humans to meropenem, and moderate

sensitivity to gentamycin, chloramphenicol, and tetracycline, while Safdar and Armstrong (2003) observed 100% sensitivity to amikacin and ciprofloxacin. Odjadjare and Okoh (2010) also reported complete sensitivity to the three antibiotics by all *Listeria* species isolated from chlorinated wastewater effluent. None of the isolates were resistant to erythromycin and penicillin. These results are similar to the results obtained in other studies looking at *Listeria* species (Abuin *et al*., 1994; Conter, *et al*., 2009; Safdar and Armstrong, 2003; Zhang *et al*., 2007). Contrary to the results obtained in this study, Odjadjare and Okoh (2010) observed resistance to the above mentioned antibiotics among the tested species. In general, *L. monocytogenes*, as well as other strains of *Listeria* spp., are susceptible to a wide range of antibiotics (Adetunji and Isola, 2011), however a notable increased resistance has been observed over the past couple of years (Marian *et al*., 2012). Studies have also described the transfer, by conjugation of enterococcal and streptococcal plasmids and transposons carrying antibiotic resistance genes from *Enterococcus-Streptococcus* to *Listeria* and between species of *Listeria* (Charpentier and Courvalin, 1999). *Listeria monocytogenes* may acquire or transfer antibiotic resistance gene from mobile genetic elements such as self-transferable and mobilizable plasmids and conjugative transposons or mutational events in chromosomal genes (Steve *et al*., 2009).

High sensitivity levels towards the β-Lactams (penicillins, cephalothins) and penicillins have been reported in literature, and are therefore considered as the main treatment drug for listeriosis (Abuin *et al*., 1994; Conter *et al*., 2009; Hansen *et al*., 2005; Zuraini *et al*., 2011; Zhang *et al*., 2007). In Contrast, results obtained in this study revealed high resistance levels towards the β-Lactams: 100%, 60.26% and 83.33% resistance to penicillin, cephalothin, and ampicillin, respectively. Similarly, Srinivasan *et al*. (2005) reported 92% and 40% resistance against ampicillin and penicillin G respectively, for *L. monocytogenes* strains isolated from

dairy farms. Arslan and Ozdemir (2008) also reported resistance against ampicillin (2.1 %) and penicillin (12.8 %) in strains of *Listeria* species isolated from white cheese. A study by Odjadjare and Okoh (2010) reported a high level of resistance to ampicillin (71%) and penicillin G (64%) by the *Listeria* isolates. The sudden occurrence of high level of resistance in *Listeria* spp. towards ampicillin and penicillin G may be attributed to their frequent use for most manifestations of listeriosis, which remain the first choice antibiotics (Steve *et al*., 2009). A dual combination of trimetroprim and mostly sulfamethoxazole is considered to be a second-choice therapy for listeriosis infection especially in patients allergic to penicillin (Mauro *et al*., 2008).

The physical and chemical properties of the wastewater effluent could largely influence the level of resistance displayed by the *Listeria* species isolated in this study. It has been widely reported that conventional wastewater treatment plants are unable to effectively remove antimicrobials such as antibiotics as well as a number of other chemicals from wastewater, thereby increasing the chances of bacterial pathogens resident in such wastewater effluent to acquire resistance to commonly used antibiotics due to selective pressures (Giger *et al*., 2003; Kummerer, 2003; Volkmann *et al*., 2004). Medical and pharmaceutical discharge from hospitals has largely contributed largely to the increase in antibiotic concentration and therefore has led to the rise of highly resistant bacterial populations (Naviner *et al*., 2011). Wastewater treatment plants have therefore been considered as a rich reservoir of antibiotic and multidrug resistant organisms since the antibiotics ingested by humans are not completely processed by the body. Some of these antibiotics are expelled as waste and wind up at wastewater treatment plants (Jury *et al*., 2010; Adetunji and Isola, 2011). Rivers contaminated with urban and agricultural effluent have shown to have greater antibiotic resistant bacterial populations than areas upstream of the contamination source (Falcão *et al*.,

2004). Antibiotic resistance in streams is also indirectly selected for by an increase in industrial wastes containing heavy metals. This could explain the findings of this study since this supports the observations of this study, both wastewater treatment plants investigated are surrounded by industrial areas, receiving wastes containing carcinogenic heavy metals and toxic chemicals. Multiple drug resistance in *Listeria* species have also been attributed to antimicrobial selective pressure and gene transfer between and amongst *Listeria* species and closely related bacteria such as *Enterococcus*, *Streptococcus* and *Staphylococcus* species (Safdar and Armstrong, 2003). Recent findings suggest an increase in the percentage of multi-antibiotic resistance over the last few years (Ebrahim *et al*., 2010; Zuraini *et al*., 2001; Mauro *et al*., 2008; Pesavento *et al*., 2010). It is therefore not surprising that a high prevalence of multi-antibiotic resistance was observed among the *Listeria* spp. in this study.

The presence of *Listeria* spp. in the discharged wastewater effluent therefore cannot be monitored (Bartie *et al*., 2002) leading to their untracked release into the environment, contaminating fresh water sources, which are easily accessible to surrounding rural communities. This would cause a health hazard for consumers who use the contaminated water for other domestic purposes. *Listeria* species in this study were found to be resistant to a range of antibiotics, some of which are traditionally used in treatment or control of infections, such as the ampicillins. This would be highly problematic if an outbreak had to occur since some of the *Listeria* species were also multidrug resistant. This is especially worrisome in a province with a high number of immunocompromised individuals due to the extremely high HIV and TB, increasing their infection potential. The presence of virulence genes also indicates that these isolates could potentially be pathogenic *Listeria monocytogens* strains which in history have been reported in a number of worldwide infection studies. This could also raise alarms and questions regarding the safety of South African fruit and

vegetable, since most farmers still depend on treated wastewater effluent, as irrigation water for their crops. The lack of information and previous literature regarding the prevalence and survival of *Listeria* species in South African wastewaters and fresh water sources coupled by the limited reports on occurrence and outbreaks in the country indicates a vast research area which needs to be investigated.

CHAPTER FOUR

ANTIMICROBIAL RESISTANCE AND VIRULENCE SIGNATURES OF *AEROMONAS* SPECIES RECOVERED FROM TREATED WASTEWATER EFFLUENT AND RECEIVING SURFACE WATER

4.1 Introduction

In recent history, *Aeromonas* species have been implicated or associated with numerous animal and human diseases (Igbinosa *et al*., 2012; Obi et al., 2008). They have grown in importance, both as food and waterborne pathogens, receiving much research attention, since the discovery of Aeromonads and their implication in gastrointestinal diseases (Ansari *et a*l., 2011; Ghenghesh *et al*, 2008; Holmberg, 1986). Seven *Aeromonas* spp., (A. *hydrophila, A. caviae, A. veronii biovar sobria, A. veronii biovar veronii, A. jandaei, A. trota,* and *A. schubertii*) are currently recognized as human pathogens (Obi *et al*., 2007).

Aeromonads are rod shaped, Gram negative, non-spore forming psychrophilic or mesophilic bacteria which are found and widely distributed in soil and mostly aquatic (fresh, marine, estuarian) environments. They are easily differentiated by the oxidase test from other related genus groups, such as *Vibrio* and *Enterobacteriacea* which were previously thought to belong to the same genus classification. They are mostly opportunistic microorganisms, mainly affecting individuals with reduced or compromised immunity, children and the elderly. *Aeromonas* spp. have been widely reported in diarrheal diseases based on their common discovery in faecal samples of patients suffering from diarrhoea and other gastrointestinal diseases (Igbinosa *et al*., 2012; Vila *et al*., 2003). *Aeromonas* infections cause gastroenteritis in healthy individuals, and may results in primary or secondary septicaemia in immuno-compromised individuals (Di Pinto *et al*., 2012). Red sore disease, water associated wound

infections (skin and soft tissue infections), eye infection, meningitis and pneumonia as well as bone and joint enteritis are some of the *Aeromonas* associated infections, amongst many other infections (Sartory *et al*., 1996). Clinical syndromes inevitably arise in immuno-compromised patients. *Aeromonas* spp. have previously been isolated from patients with diarrhoea in the absence of other enteropathogens, but its association with gastroenteritis remains unknown (Vila *et al*., 2003). Septicaemia is also often connected to other underlying diseases such as leukaemia, cirrhosis, meningitis, peritonitis, and endocarditis urinary tract and surgical wound infections, and a strong link has been established with *Aeromonas* infections (Bravo *et al*., 2003). Motile *Aeromonas* species which are usually responsible for human infections grow at optimum temperatures between 35 - 37 °C, while non-motile species that usually infect fish grow optimally at lower temperatures between 22 - 28 °C. These two groups can easily be differentiated by the different growth temperatures, as well as the indole production test (Joseph and Carnahan, 2000). All *Aeromonas* species have the ability to grow between 4.5 - 9 pH levels (Isonhood and Drake, 2002).

Inadequately treated wastewater effluent transports these emerging pathogenic species to open water sources, posing a threat to man and animals which come into contact with the contaminated waters (Igbinosa and Okoh, 2012). They are also found in high numbers both before and after sewage treatment, and have for a while been proposed as potential indicators of faecal contamination, because of their constant detection even in the absence of traditional indicator organisms (EPA, 2009; Toze, 1997; Vila *et al*., 2003). These organisms also have the ability to produce a wide range of extracellular enzymes, which are often linked to their pathogenic characteristics. These include enterotoxins, proteases, haemolysins etc. which are the main components playing a major role in the establishment of disease, as well as in initiating host infection processes (Igbinosa and Okoh, 2013; Gavin *et al*., 2003; Trower *et*

al., 2000). Regardless of continuous research, much is yet to be understood regarding the association or the contribution of these virulence determinants in infection. For instance, aerolysin (*aer*) has both haemolytic and cytotoxic activity which aid in pathogenicity, yet their mechanisms of action are not fully understood.

The wide presence of potentially pathogenic *Aeromonas* species in freshwater bodies is of major public health concern. Some studies have reported high antimicrobial resistance patterns in *Aeromonas* species, which increases the public threat, especially in cases of immunocompromised individuals with severe infections. Although many cases of *Aeromonas* infections have been reported in South Africa, it is believed that the reported cases are underestimated in developing countries, where exposure to these waterborne pathogens may possibly occur more frequently due to the country's history of outdated and ineffective wastewater treatment processes (Obi *et al*., 2008). This chapter therefore aimed at identifying and characterizing the presumptive *Aeromonas* species recovered from treated effluent of two wastewater treatment plants in Durban and the receiving water bodies (the Aller and Umgeni Rivers) for antimicrobial resistance and virulence gene signatures.

4.2 Materials and methods

4.2.1 Biochemical analyses

Identification of the presumptive *Aeromonas* spp. recovered from treated effluent and the receiving rivers was carried out using a range of biochemical tests, including; oxidase, catalase, urease, carbohydrate fermentation, acid formation (TSI), indole production tests and Methyl red and Voges-Proskauer test.

4.2.2 Molecular analyses

Polymerase chain reaction (PCR) was used to amplify and detect the presence of specific conserved sequences (*gyrB* gene) which code for the β-subunit of DNA gyrase in each of the presumptive *Aeromonas* species isolates, using specific *Aeromonas* gyrase primers for further identification.

4.2.2.1 DNA isolation

Genomic DNA was isolated from biochemically identified *Aeromonas* species using the boiling method (Lin *et al*., 1996; Yàñez *et al*., 2003). One colony from a 24 h culture was suspended in a micro-centrifuge tube containing 70 µl sterile distilled water. The culture and water mixtures were vortexed at high speed for 1 min and heated to 100 °C for 10 min in a water bath, to lyse the cells and thereafter cooled to room temperature. The tubes were vortexed again prior to centrifugation at 13 000 rpm for 5 min and the supernatant was transferred to a sterile tube and used as template in the PCR assay.

4.2.2.2 PCR amplification of *gyrB* of *Aeromonas* species

The PCR amplification of the *gyrB* gene was performed in a thermal cycler (GeneAMP PCR System 2400, Bio Rad) using forwards and reverse primers (gyrB3F: TCCGGCGGTCTGCACGGCGT, gyrB14R: TTGTCCGGGTTGTACTCGTC) with an expected product size of 1100 base pairs (Huddleston *et al*. 2006; Yàñez *et al*. 2003). The following PCR conditions were applied: Pre-denaturation at 94 °C for 2 min, denaturation at 93 °C for 30 sec, annealing at 62 °C for 30 sec and extension at 72 °C for 1 min for 30 cycles (Huddleston *et al*., 2006). PCR was performed in a final volume of 50 µl containing 50 mM KCl, 10mM Tris/HCl (pH 9), 1.5 mM $MgCl_2$, 0.2 mM dNTPs , 1U *Taq* DNA polymerase , 20 pmol of each primer and 1 µl of the extracted DNA sample (Yàñez *et al*., 2003). *Aeromonas caviae*

ATCC 15468 and *Aeromonas hydrophila* ATCC 7965 strain were used as positive controls in all PCR assays**.** Following PCR, the amplification products were subjected to gel electrophoresis using a 1% (w/v) agarose gel and detected by staining in ethidum bromide solution (0.5 µg/ml) for 30 – 40 min (Cocolin *et al*., 2002). The gels were rinsed in water or TAE buffer to remove excess dye on the gel and visualized under the SYNGENE Gel documentation/ Bio-imaging system (Syngene UK).

4.2.2.3 Virulence gene characterization

A monoplex PCR was used for the detection of two virulence-associated genes of *Aeromonas* species namely, *aer* and *lip*, coding for the aerolysin and lipase enzymes, respectively, using the primer sets indicated in Table 4.1

Table 4.1 Primers used for PCR amplification of *Aeromonas* virulence genes.

Organism	Primer	Sequence(5′-3′)	Product size(bp)	References
Aeromonas spp.	aer-F	CCTATGGCCTGAGCGAGAAG	431	Igbinosa *et al*., 2013
	aer-R	CCAGTTCCAGTCCCACCACT		
	lip-F	CA(C/T)CTGGT(T/G)CCGCTCAAG	247	
	lip-R	GT(A/G)CCGAACCAGTCGGAGAA		

The monoplex reactions were carried out in a total volume of 25 µl, containing 12.5 µl of the PROMEGA G2 Go *taq* green master mix (ANATECH), 5 µl of isolated genomic DNA and sterile double distilled water to make up the reaction volume. PCR was carried out in a Thermocycler (GeneAMP PCR System 2400, Bio Rad) with the following conditions, Aerolysin (*aer*): Pre-denaturation at 94 °C for 2 min, denaturation at 94 °C for 30 s, annealing at 64 °C for 50 s and extension at 72 °C for 10 min for 35 cycles. The Lipase (*lip*)

gene: Pre-denaturation at 94 °C for 2 min, denaturation at 94 °C for 30 s, annealing at 59 °C for 50 s and extension at 72 °C for 10 min for 35 cycles (Igbinosa *et al*., 2013).

4.2.3 Antimicrobial resistance profile analyses

The Kirby Bauer Disk diffusion method as explained in the previous chapter was used to determine the antibiotic resistance profile of *Aeromonas* spp. isolates. The isolates were screened against a predetermined panel of 24 antibiotics belonging to 14 different classes (Listed in Table 3.3 of the previous chapter). The results were interpreted according to CLSI standards for *Escherichia coli* ATCC 25922.

4.2.4 Protease, gelatinase and haemolysin assay

Protease activity was assayed by spreading cultures of *Aeromonas* strains on nutrient agar containing 1.5% skim milk. After incubation at 30°C for 72 h, the production of protease was shown by the formation of a clear zone caused by casein degradation. Gelatinase production was determined using LB agar containing gelatine (30 g/L), the plates were incubated at 30°C for 24 h and cooled for 5 h at 4°C. The appearance of turbid halos around the colonies was considered positive for gelatinase production (Sechi *et al*., 2002). Haemolysin production was assayed by culturing each strain on human blood agar at 30°C for 24 h. The production of haemolysin was observed by the formation of a clear zone caused by β-haemolysis activity of the enzyme on the blood using a modified method of Sechi *et al*. (2002).

4.3 Results

4.3.1 Identification of the presumptive *Aeromonas* spp. isolates

Aeromonas spp. isolates were confirmed as either negative or positive for the biochemical tests listed under section 4.2.1. Oxidase and catalase positive, urease negative, Methyl red positive and Voges-Proskauer negative organisms were further tesed and confirmed as *Aeromonas* spp. using PCR. The amplicons with the expected gyrB gene region sizes (1100bp) in the positively confirmed isolates are shown in Figure 4.1. A total of 100 *Aeromonas* spp. isolates obtained from the BC-before chlorination (19), DP-discharge point (52), US- upstream (9) and DS- downstream (20) were confirmed and identified as *Aeromonas* species.

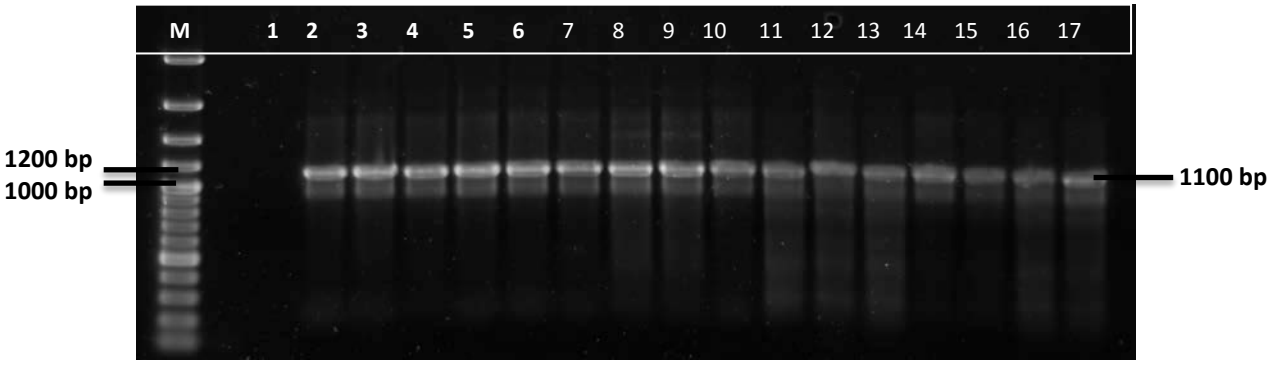

Figure 4.1: Representative agarose gel showing PCR amplicons of the *gyr*B gene of *Aeromonas* spp. isolates (lanes 2- 17), M: 100 pb molecular marker and lane 1: negative control.

4.3.2 Virulence gene signatures of the *Aeromonas* species isolates

The agarose gel in Figure 4.2 represents the PCR amplicons of the expected sizes of the aerolysin (*aer)* gene in *Aeromonas* spp. The primer sets allowed for the amplification of 431 bp product, represented by a single band in the expected base pair region. The *aer* gene is not species specific, but is most commonly found in *A. hydrophila*, *A. caviae* and *A. veroni*. Of the 100 tested *Aeromonas* spp., 52% harboured the *aer* virulence associated gene.

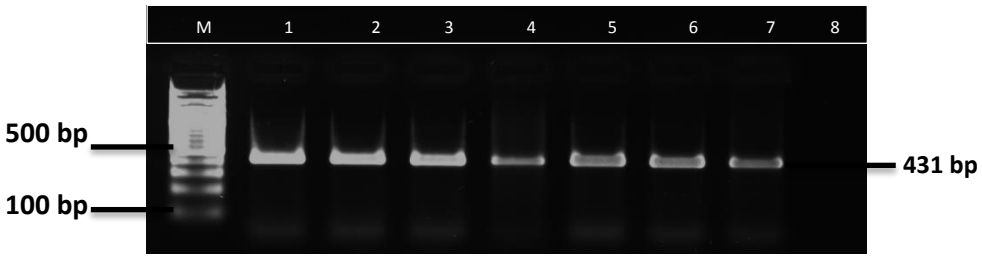

Figure 4.2: Representative agarose gel showing PCR amplicons of the Aerolysin (*aer*) virulence associated gene of *Aeromonas* spp.*,* M: DNA marker (100 bp), lane 1-7: amplified PCR products, Lane 8: negative control.

The lipase (*lip*) virulence associated gene was also detected in 68% of the tested *Aeromonas* spp. Figure 4.3 represents the PCR amplicons (247 bp) of the *lip* gene detected in tested *Aeromonas* spp. isolates. Of the 100 isolates, 29 contained both the *aer* and *lip* genes, while 12 of the tested isolates did not contain any virulence associated genes.

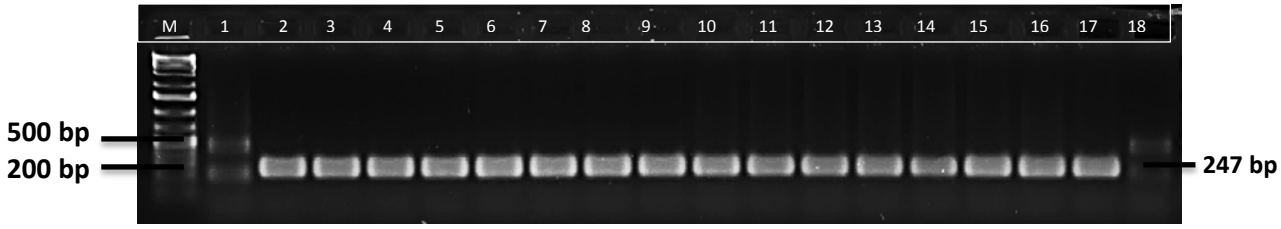

Figure 4.3: Representative agarose gel showing PCR amplicons of the Lipase (*lip*) virulence associated gene of *Aeromonas* spp.*,* M: DNA marker (100 bp), lane 1-17: amplified PCR products.

4.3.3 Antimicrobial resistance profiles of *Aeromonas* species

The resistance and susceptibility profiles of the 100 *Aeromonas* spp. isolates tested against a broad range of antimicrobials commonly used for the treatment of Enterobacteria-associated infections are shown in Table 4.2. The highest resistance (100%) was observed for ampicillin, penicillin, vancomycin, clindamycin and fusidic acid, followed by cephalosporin (82%), and erythromycin (58%), with 56% of the isolates found to be resistant to naladixic acid and trimethoprim. All the isolates were found to be sensitive to gentamycin, 95% of the isolates were sensitive to amikacin and chloramphenicol, while 94%, 88%, 86%, 83% and 82% of isolates were susceptible to ciprofloxacin, fosfomycin, colistin, mixofloxacin and cefotaxime, respectively. Slightly higher antimicrobial susceptibility numbers were observed, compared to those of resistant *Aeromonas* species, indicating that most of the *Aeromonas* species had low antimicrobial resistance levels. All the tested isolates showed resistance to at least 6 of the 24 antibiotics, with 5 of the test isolates displaying resistance to at most 14 of the 24 antibiotics.

The multidrug resistance patterns of *Aeromonas* spp. displayed in Table 4.3, show that most of the multidrug resistant isolates were resistant to at least 6 and at most 14 classes of antimicrobials, with the antibiotic resistance index (ARI) ranging from 0.25 – 0.58. The highest multidrug resistance patterns was observed in 5 of the 100 test isolates, these isolates being resistant to 14 of the 24 tested antibiotics, followed by 6 which were resistant to 13 antibiotic classes. Furthermore, 29 of the tested *Aeromonas* spp. were resistant to 8 antimicrobial classes, followed by 20 and 19 isolates found to be resistant to 7 and 9 antimicrobial classes, respectively. None of the *Aeromonas* species were resistant to more than 14 antimicrobial classes.

Table 4.2: Antimicrobial resistance/susceptibility profile of *Aeromonas* species isolates recovered from treated effluent and receiving rivers.

Antibiotic class	Antibiotics	Code	Level (µg)	Bacterial isolates		
				Aeromonas spp. (n=100)		
				n(Resistant)	n(Susceptible)	n(Intermediate)
β-Lactams	Penicillin	P	10	100	0	0
	Cephalothin	KF	30	82	15	3
Aminoglycosides	Gentamicin	CN	10	0	100	0
	Kanamycin	K	5	14	59	27
	Amikacin	AK	30	0	95	5
Carbapenems	Ertapenem	ETP	10	23	72	5
	Meropenem	MEM	10	11	79	10
Cephalosporin	Cefotaxime	CTX	30	6	82	12
	Ceftriaxone	CRO	30	22	78	0
Glycopeptides	Vancomycin	VA	30	100	0	0
Lincosamides	Clindamycin	DA	10	100	0	0
Macrolides	Erythromycin	E	15	58	11	31
Nitrofurans	Nitrofurantoin	F	50	4	72	24
Penicillins	Ampicillin	AMP	10	100	0	0
Polypeptides	Colistin	CT	10	14	86	0
Quinolones	Naladixic acid	NA	30	56	44	0
	Mixofloxacin	MXF	5	3	83	14
	Ciprofloxacin	CIP	5	0	94	6
Sulfonamides	Trimethoprim	W	5	56	38	6
Tetracyclines	Tetracycline	TE	10	19	44	37
Other	Streptomycin	S	25	16	70	14
	Chloramphenicol	C	30	4	95	1
	Fosfomycin	FOS	50	3	88	9
	Fusidic Acid	FD	10	100	0	0

n= number of isolates

Table 4.3: Multidrug resistance patterns of *Aeromonas* spp.

Number of antibiotic classes	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
No. of isolates resistant	4	20	29	19	8	6	3	6	5
Percentage resistant	4	20	29	19	8	6	3	6	5

4.3.4 Protease, gelatinase and haemolysin production

All the 100 tested *Aeromomonas* spp isolates tested positive for protease enzyme production, while 67% and 88% of the isolates tested positive for the production of haemolysin (evidenced by distint clearing zones around the colonies due to the hydrolysis of blood agar) and gelatinase, respectively.

4.4 Discussion

The increasing number of studies reporting on *Aeromonas* spp. isolated from treated waters, even drinking waters is a cause for concern, considering the severity of *Aeromonas* infections on immune-compromised individual. Results obtained in this study indicate the prevalence of *Aeromonas* species in treated wastewater effluent, reiterating the fact that they are able to withstand and survive conventional wastewater treatment processes as previously reported (Lafdal et al., 2012; Martone-Rocha, 2012; Obi *et al*., 2007). Organisms with the ability to withstand wastewater treatment and thereafter disseminated into the environment from treatment plants which are reservoirs of antimicrobial substances, easily transferring their resistance properties to other environmental bacterial organisms (Jury *et al*., 2010).

In this study, the aerolysin (*aer*) and lipase (*lip*) genes were detected in most of the tested *Aeromonas* species isolates. The aerolysin gene which was detected in 52% of the isolates is

responsible for most of the haemolytic, cytotoxic and enterotoxic activities, which play a vital role in the initial stages of the host infection process (Chopra *et al*., 1993; Igbinosa *et al*. 2013). Igbinosa and Okoh (2013) reported a high presence (43%) of the *aer* gene in *Aeromonas* species isolated from water samples. Another study by Soler *et al*. (2002), reported on the gene's presence in 26% of the tested environmental *Aeromonas* isolates. These findings support the observed rise in potentially virulent environmental *Aeromonas* isolates. This is contrary to common reports stating that the *aer* gene is commonly a trait of clinical samples, as opposed to environmental samples. The presence of this gene in 52% of the *Aeromonas* spp obtained from treated wastewater effluent and receiving river water indicates that the isolates investigated in this study are potentially pathogenic and virulent strains of either *A. hydrophila, A. caviae and A. veroni*, where this gene is commonly found (Yousr et al., 2007). High numbers of virulence gene containing *Aeromonas* species have been observed in multiple studies, displaying in environmental samples, with reports from Brazil, India, Italy and Spain alike (Balsalobre *et al*., 2009; Ottaviani *et al*., 2011; Sihna *et al*., 2000). Environmental samples are increasingly becoming more pathogenic, and this will surely have bad implications in South Africa, especially due to the large population of immuno-compromised individuals.

The haemolytic activity demonstrated by these *Aeromonas* species on human red blood cells is indicative of the production of the haemolysin virulence factor by the isolates. Several authors have suggested that this virulence determinant is usually associated with strains of *A. hydrophila* and *A. sobria* (Albert e*t al*., 2000; Kudinha *et al*., 2000; Monfort and Baleux, 1991), which are potential human pathogens. The link between haemolytic activity and enterotoxigenicity observed in this study has been well researched (Kudinha *et al*., 2000; Monfort and Baleux, 1991).

The *lip* gene which primarily plays an integrated and coherent role in pathogenicity of *Aeromonas* species was detected in 68% of the isolates. This gene is responsible for altering the host's plasma membranes, thus increasing the severity of the infection (Chuang *et al*., 1997; Nawaz *et al*., 2010; Pemberton *et al*., 1997; Lee and Kellis, 1990). The infection or pathogenicity process of *Aeromonas* is very complex and is said to involve different virulent and pathogenicity factors which either act together or separately at different stages of infection. Of the 100 *Aeromonas* species characterized, 47 contained both virulence genes, and the majority of these were isolated from the FE, BC and DS, partly providing a link between the treatment plant and its role in surface water contamination. Only a few were from the US sample, again supporting the hypothesis that wastewater treatment plant is a potential reservoir of virulent bacteria, with the possibility of spreading the virulence factor via horizontal gene transfer. Microorganisms that bypass treatment processes have been found to be more virulent and have also been found as the main causatives of infections which cannot be easily treated (Kaye *et al*., 2004).

The highest resistance (100%) was observed against ampicillin, penicillin, vancomycin, clindamycin and fusidic acid, as all the 100 tested isolates were resistant to these antibiotics. These findings were in line with those of similar studies (Igbinosa and Okoh, 2012; Lafdal *et al*., 2012; Obi *et al*, 2007; Vandan *et al*., 2011), where ampicillin and vancomycin were amongst the antibiotics which had no antimicrobial activity towards tested *Aeromonas* spp. isolates. Some studies, including that of Goni-Urriza *et al*. (2000) and Perez-Valdespino *et a l*. (2009), did not observe complete resistance to these antibiotics by the *Aeromonas* spp.tested, but a similar trend of high resistance levels was observed. The observed high resistance level of *Aeromonas* isolates tested in the current study to *β*-lactam reflective of the changing antibiotic resistance pattern of these organisms, as previous studies reported on low

resistance levels of these organisms towards this group of antibiotics (Goni-Urriza *et al*., 2000). Tetracycline resistance results obtained in this study were however different to the results of obtained by Igbinosa and Okoh (2012) where close to 80% of isolates displayed resistance, compared to only 19% of resistant isolates observed in this study, with most isolates having an intermediate result. However, lower percentage of resistance to tetracycline was also observed by Goni-Urriza *et al*. (2000) among isolates obtained from river that received wastewater samples. The high resistance pattens observed in *Aeromonas* strains, mainly towards ampicillin and vancomycin corroborates many reports and could be attributed to the frequent utilization of ampicillin as a selective agent in the most common *Aeromonas* culture media, such as the one used in this study (Vila *et al*., 2003). The high sensitivity patterns observed against gentamycin, fosfomycin, cefotaxime, amikacin, and meropenem in this study were comparable to previous findings (Igbinosa and Okoh 2012; Obi *et al*, 2007).

The high prevalence of virulence and antimicrobial resistant *Aeromonas* species obtained in this study is indicative of the severity of the threat these pathogens might pose to the health of the environment and other organisms exposed to the contaminated waters. This is of great concern as most of the surface water samples (US and DS) were collected from locations which were easily accessible to animals and human populations residing in informal settlements along the river. Some of the residents in these areas used this water to meet some of their domestic needs, such as washing and laundry as well as for fishing. Results obtained in this study suggest a high prevalence of *Aeromonas* species in the treated effluent, and receiving water bodies. These organisms harbour virulence determinants which are involved in human and animal infection, increasing the organisms' pathogenicity. Wastewater treatment plants are therefore reservoirs of pathogenic *Aeromonas* species, with the potential

to pose a risk to the health and well-being of human and animals exposed to these waters. Once in a bacterial population, antibiotic resistance can spread rapidly (Adetunji and Isola, 2011; Stokes and Gillings, 2011).The study shows that some of the tested environmental isolates possessed virulence genes which are mostly associated with clinical samples indicates an evolution of these genes in environmental isolates. Antimicrobial resistance profile also indicates that more *Aeromonas* species are becoming resistant to multiple antibiotics. Although the number of multidrug resistant *Aeromonas* species might be low, it doesn't indicate safe levels, as resistance to antibacterial agents may be strain dependent. Results from this study further highlight the need for improved treatment processes for effective removal of these emerging bacterial pathogens.

CHAPTER FIVE

GENERAL DISCUSSION AND CONCLUSION

5.1 Research in perspective

South African fresh surface water bodies are increasingly being exposed to a broad range of microbial and chemical pollutants. These diverse and mostly noxious contaminants have been traced to numerous contributing factors of varying anthropogenic and natural derived sources (UNEP, 2010). Of these sources, inadequately treated wastewater effluent has been reported as a major contributor to fresh water pollution, transporting a plethora of microorganisms, some of which are highly infectious disease-causing bacterial pathogens with alarmingly high mortality rates and chemical complexes. Contaminated fresh water sources of poor microbial and physico-chemical quality are increasingly posing a greater health and environmental threat than previously projected (Crockett, 2007). This raises serious concern in developing countries, such as South Africa, especially in rural areas where surface water and groundwater sources are still being depended upon for basic water needs (Bolonga *et al*., 2009; Odjadjare and Okoh, 2010). In 2012, sixteen million South Africans were estimated to lack adequate access to safe water and sanitation facilities. This in conjunction with contaminated water bodies undoubtedly escalates the problems of water-related infection, treatment and the spread of waterborne diseases (NICD, 2012).

Additionally, the increased usage of antimicrobial substances and their residual presence in wastewater could be potentially linked to increased antimicrobial resistance detected in microorganisms isolated from wastewater effluents, which are then later disseminated into fresh water bodies. These organisms then spread antimicrobial resistance among environmental isolates, further posing potential harm to human (Abraham, 2011; Zhang *et al*.,

2009). Waterborne pathogens of fecal origin in developing countries have been largely implicated in numerous infectious disease outbreaks, thus necessitating the need to safeguard and effectively protect surface water by the relevant regulatory authorities (Crockett, 2007; Scott *et al*., 2003; Santo Domingo and Edge, 2010; Shatanawi *et al*., 2006). There is lack of adequate knowledge pertaining to waterborne emerging bacterial pathogens, despite their frequent detection in surface water even in the absence of traditional indicator bacterial pathogens. This study therefore evaluated the prevalence of five emerging bacterial pathogens; namely *Aeromonas, Pseudomonas*, *Listeria*, *Yersinia* and *Legionella* species, in treated effluent of two wastewater treatment plants and the receiving rivers in Durban. Physico-chemical profiles of these waters were also assessed and correlated with the bacterial loads over the study period followed by antimicrobial resistance and virulence gene characterization of the positively confirmed *Aeromonas* and *Listeria* species isolates.

Bacterial analyses of treated effluents from both plants as presented in Chapter two, displayed varying trends over the sampling period, with some samples from the Northern wastewater treatment plant (NWWTP) displaying unexpectedly higher bacterial populations at the discharge points (after tertiary treatment) compared to counts obtained before chlorination. This could be attributed to a number of factors, such as an incapacitated plant, treatment inefficiency and the regrowth and proliferation of microorganisms in the effluent pipe systems. Microbial counts upstream and downstream of the receiving Umgeni River varied greatly, with microbial population recovery trends closely linked to the observed directional flow of the tidal current as well as the quality of the released effluent. Bacterial populations at the NWWTP (in CFU/ml x 10^3) ranged between 4.42 – 23100, 0.53 –329000, 0.01 – 1470, 20.60 – 140000, and 40.13 – 95000 for *Aeromonas, Pseudomonas*, *Listeria*, *Yersinia* and *Legionella* spp., respectively, over the sampling period. Bacterial counts displayed great

variability with no observable monthly or seasonal trends. The NGWWTP which was the smaller plant of the two displayed a more normalised trend of lower bacterial count at the discharge point compared to the values obtained in samples before chlorination. Counts (in CFU/ml x 10^3) varied between 17 – 9613.3, 19.2 –8820, 0.01 – 331, 70 – 11480, and 258 – 46600 for *Aeromonas, Pseudomonas*, *Listeria*, *Yersinia* and *Legionella* spp. respectively. The observed very low bacterial counts in downstream samples compared to samples upstream and at the discharge point could be due to the dilution effect of the treated effluent discharge with high residual chlorine. In NGWWTP, up to 100% reduction in *Listeria* spp population and up to 99% for the other four emerging bacterial pathogens investigated was observed after chlorination. However, similar to NWWTP, emerging bacterial pathogens were also recovered at the receiving water bodies throughout the sampling period. Results of this study highlight the ineffectiveness of wastewater treatment processes in pathogen removal (Toze, 1997). Some of the tested organisms (*Legionella* and *Yersinia*) are known to be more resistant to chlorine and survived longer than coliform bacteria (Palmer *et al*., 1995). Some of the results obtained in this study corroborate reports of previous studies (Bressler *et al*., 2009; Odjadjare and Okoh, 2010; Paillard *et al*., 2005; Pablos *et al*., 2009). The high microbial population bypassing treatment and being transferred into fresh water bodies is a cause for concern as this poses multiple threats of waterborne disease outbreaks.

The physico-chemical profile of the treated final effluent samples generally fell short of set guidelines, with respect to certain parameters, notably turbidity, COD, phosphates and nitrates, while pH, temperature, TSS, and DO displayed overall compliance to the set guidelines (Conter *et al*., 2009; DWAF, 1992; Hitchins, 2001, Naravaneni and Jamil, 2005). Extremely high turbidity, DO, COD and conductivity readings completely rule out the river waters for any domestic use. The presence of organic matter affects the biochemical

composition of a natural water system, thus affecting and altering normal biological function. A positive correlation ($P < 0.05$) was observed between some physicochemical parameters like; pH, TDS and COD. The strong correlation between microbial counts and DO readings can be attributed to the high organic matter of the water. The high turbidity, COD and TDS obtained in this study suggested a high presence of organic matter which may have a negative effect on the surrounding ecosystems as well as human population.

Virulence and antibiotic resistance profiling of confirmed *Listeria* and *Aeromonas* spp. isolates (reported in chapters 3 and 4) further provided relevant information of the potential negative impact of the effluent discharge from the two treatment plants studied. The virulence genes; *plc*A, *act*A, and *iap* associated with pathogenic *Listeria* species were detected in some of the confirmed isolates. A similar trend has been observed in environmental samples from numerous *Listeria* studies conducted in different parts of the world (Balsalobre *et al*., 2009; Ottaviani *et al*., 2011; Sihna *et al*., 2000). The presence of such virulence genes commonly found in pathogenic clinical isolates indicates that these genes have now been transferred to environmental isolates. The *aer* and *lip* genes associated with the initial stages of the infection process were detected in most of the *Aeromonas* species isolates.

As reported in chapter 3, *Listeria* species tested in this study displayed high resistance towards penicillin (100%), erythromycin (100%), nalidixic acid (100%), ampicillin (88.33%), trimethoprim (67.95%), nitrofurantoin (64.10%) and cephalosporin (60.26%). Complete sensitivity was observed towards streptomycin, chloramphenicol, fosfomycin, fusidic acid and meropenem, followed by ciprofloxacin (96.15%), meropenem (89.74%), clindamycin (88.46%), colistin (79.48%) and gentamicin (72.31%). The high *Listeria* sensitivity levels towards β-Lactams and penicillins observed in this study is similar to previous reports in

related studies (Abuin *et al*., 1994; Conter *et al*., 2009; Hansen *et al*., 2005; Zuraini *et al*., 2001; Zhang *et al*., 2007). There has been an increase in reports of multidrug resistant *Listeria* species over the past years (Marian *et al*., 2012), and this can be attributed to their gene acquisition ability from other bacterial species such as *Streptococcus* (Charpentier and Courvalin, 1999; Steve *et al*., 2009). It has been widely reported that conventional wastewater treatment processes are incapable of effectively removing antimicrobials and other chemical compounds from wastewater, thereby increasing the chances of bacterial pathogens established in such wastewater effluent to acquire resistance to commonly used antibiotics due to selective pressures (Giger *et al*., 2003; Kummerer, 2003; Volkmann *et al*., 2004). Medical and pharmaceutical wastes have contributed largely to the increase in antibiotic concentration and therefore have led to the rise of highly resistant bacterial populations in the environment (Naviner *et al*., 2011). As presented in chapter 4, all *Aeromonas* spp. tested in this study were resistant to ampicillin, penicillin, vancomycin, clindamycin and fusidic acid, followed by cephalosporin (82%), erythromycin (58%), naladixic acid (56%) and trimethoprim (56%). The observed resistance patterns are comparable to similar studies conducted by other authors (Igbinosa and Okoh, 2012; Lafdal *et al*., 2012; Obi *et al*, 2007; Vandan *et al*., 2011). Although some studies did not observe complete resistance towards ampicillin and vancomycin, a similar trend of high resistance levels has been reported (Goni-Urriza *et al*. 2000; Perez- Valdespino *et al*. 2009).

Overall, findings from this study highlight the need for the Department of Water Affairs to revise the current guidelines and standards to include the emerging bacterial pathogens. There also needs for constant evaluation of South African wastewater treatment plants to ensure efficiency and compliance to set guidelines and regulation to protect public and environmental health. This study also demonstrated that some of the treatment plants in the

Durban are not effective in the removal of emerging bacterial pathogens, considering that one of the tested plants is one of the biggest within the Durban area. Multidrug resistant organisms found in this study were also resistant to some of the commonly used antibiotics and this is particularly worrisome in a province with a high number of immunocompromised individuals due to the extremely high HIV and TB pandemic.

5.2 Potential for future development of the study

The presence of emerging bacterial pathogens in treated effluent has been reported in numerous studies; however there is limited research on the tested organisms recovered from the wastewater effluent, thus restricting ones understanding on their impact and epidemiological effects on exposed communities. Future studies should therefore focus on further characterization of the recovered emerging bacterial pathogens to ascertain their survival mechanisms, such as that of biofilm formation and interaction, the presence of efflux pumps which is associated closely to antibiotic resistance levels. Antibiotic testing can be partnered with antibiotic resistance genes screening and consequently the investigation of antibiotic resistance mechanisms utilized, ultimately giving an holistic understanding on the associated resistance mechanisms and how these differ between different organisms. More toxicity or virulence determinant assays could be performed to better understand the level of pathogenicity of the tested isolates. Finally, genetic fingerprinting of the isolates should be carried out using pulse field gel electrophoresis (PFGE) or amplified fragment length polymorphism (AFLP) to establish the level of relatedness of the isolates recovered from the different sources for contaminant source tracking. Future studies should also attempt to characterize other emerging bacterial pathogens (*Yersinia*, *Legionella* and *Pseudomonas* species) recovered from the treated effluent, as they were also found in extremely high number and could pose environmental and human health threat.

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7.0 Appendices

APPENDIX I

1. media and reagents

1.1 MRVP reagents

Methyle red solution

0.1g of methyl red

300 ml of ethanol (95%)

200 ml of deionized water added to make 500 ml of a 0.05% (wt/vol) solution in 60% (vol/vol) ethanol.

Voges-Proskauer reagents

Barritt's reagent A: 5% (wt/vol) a-naphthol in absolute ethanol

Barritt's reagent B: 40% (wt/vol) KOH in deionized water

Reagents were prepared fresh.

All the components were added to 1000ml of distilled water.

1.3 TSI slants	**g/500ml**
Beef extract	1.5
Yeast extract	1.5
Peptone	10
Glucose	0.5
Lactose	5
Sucrose	5
Ferrous sulphate	0.1
NaCl	2.5
Sodium Thiosulfate	0.15
Phenol red	0.012
Nutrient agar	6.5

1.4 Urease broth	**g/500ml**
Yeast extract	0.5
Potassium phosphate monobasic	4.5g
Potassium phosphate dibasic	4.5
Urea	10
Phenol red	0.0005

1.5 Casein	**g/500ml**

Skim milk	37.5
Nutrient agar	10

1.6 MRVP broth	**g/500ml**
Peptone	3.5
Potassium phosphate	2.5
Dextrose	2.5

All broth and media components the components were added to 500ml of distilled water, mixed, boiled and then sterilized via autoclaving at 121°C for 15 minutes.

Table: Physico-chemical profiles of treated wastewater effluents from NWWTP and NGWWTP and the receiving water bodies over the sampling period (March-February)

			Temperature	DO	BOD	COD	Conductivity	Resistivity	Salinity	TDS	TSS	pH	Turbidity	Nitrate	Phosphate	Residual Cl_2
			°C	mg/L	mg/L	mg/L	(µS/cm)	Ώ.cm	%	mg/L	mg/L		Ntu	mg/L	mg/L	mg/L
AUTUMN	March	BC	26.00 ±0	5.58 ±0.14	2.23 ±0.36	90.44 ±4.48	839.67 ±0.57	1191.00 ±1	0.41 ±0	411.33 ±0.57	0.01 ±0	7.11±0. 02	7.91 ±0.32	-	-	-
		DP	25.00 ±0	4.01 ±0.25	5.13 ±0.18	<10 ±0	888.00 ±0	1126.00 ±1	0.44 ±0	436.00 ±1.00	0.03 ±0.03	7.36 ±0.06	23.40 ±12.12	-	-	-
		US	26.00 ±0	5.19 ±0.6	5.62 ±1.01	161.33 ±1.32	757.67 ±2.51	1320.33 ±4.51	0.37 ±0	370.00 ±1.00	1.24 ±4.20	7.25 ±0.08	16.67 ±0.37	-	-	-
		DS	25.50 ±0	5.10 ±0.54	5.62 ±0.24	191.94 ±2.37	714.67 ±2.08	1400.00 ±4.25	0.35 ±1.8	348.67 ±1.15	1.13 ±1.03	7.24 ±0.05	15.27 ±0.11	-	-	-
	April	BC	22.00 ±0	4.69 ±0.25	3.30 ±0.97	127.89 ±0.57	783.33 ±2.08	1276.67 ±3.21	0.38 ±0	383.00 ±1.00	0.10 ±0.01	7.38 ±0.05	53.98 ±0.02	<0.057 ±0	2.11 ±0	116.35 ±0
		DP	22.00 ±0	4.80 ±0.58	3.44 ±0.67	152.89 ±0.57	903.67 ±2.08	1106.67 ±2.30	0.44 ±0	444.00 ±1.00	0.07 ±0.02	7.22 ±0.1	71.02 ±0.28	<0.057 ±0	2.10 ±0	219.64 ±0
		US	21.00 ±0	4.32 ±0.17	8.49 ±0.47	309.22 ±1.52	997.67 ±2.88	1004.00 ±0	0.49 ±0	491.00 ±1.73	0.02 ±0	7.29 ±0.12	18.35 ±0	0.86 ±0	0.23 ±0	260.56 ±0
		DS	21.00 ±0	4.80 ±0.30	6.33 ±0.21	303.00 ±0	676.33 ±4.35	1478.67 ±2.31	0.33 ±0	329.00 ±2.08	0.01 ±0	7.40 ±0.05	14.60 ±0	0.32 ±0	0.38 ±0	108.40 ±0
	May	BC	22.00 ±0	5.38 ±0.1	1.40 ±0.19	71.56 ±0.47	853.00 ±0	1173.00 ±0	0.42 ±0	418.00 ±0	0.04 ±0	7.02 ±0.03	20.65 ±0	0.16 ±0	2.72 ±0	198.93 ±0
		DP	21.00 ±0	4.81 ±0.01	3.04 ±0.17	< 10 ±0	966.00 ±0	1036.00 ±1	0.48 ±0	475.00 ±0	0.02 ±0.01	7.06 ±0.02	14.22 ±0.17	0.20 ±0	2.34 ±0	186.80 ±0
		US	21.00 ±0	5.71 ±0.58	4.52 ±0.79	51.00 ±0.28	2115.67 ±0.57	473.00 ±1.15	1.08 ±0	1067.00 ±3.47	0.01 ±0	6.87 ±0.04	13.58 ±0	1.03 ±0	1.08 ±0	1090.00 ±0
		DS	22.00 ±0	5.04 ±0.25	5.06 ±0.30	301.33 ±.66	621.00 ±2.65	1612.33 ±3.51	0.30 ±0	302.00 ±1.00	5.93 ±9.10	6.85 ±0.04	13.58 ±0	1.72 ±0	0.82 ±0	214.37 ±0
	June	BC	13.50 ±0	4.35 ±0.48	4.01 ±0.30	113.33 ±0.58	703.00 ±1.00	1423.33 ±2.57	0.34 ±0	342.67 ±0.5	0.02 ±0	7.26 ±0	7.08 ±0.30	5.98 ±0	12.38 ±0	150.16 ±0
		DP	12.67 ±0.29	5.13 ±0.03	4.31 ±0.21	207.17 ±0	757.33 ±2.08	1320.33 ±3.25	0.37 ±0	368.33 ±2.08	0.02 ±0	7.27 ±0	6.48 ±0.07	3.92 ±0	9.02 ±0	163.67 ±0
		US	13.00 ±0	4.48 ±0.25	9.61 ±0.44	113.83 ±0.58	1082.33 ±2.08	924.00 ±1.73	0.54 ±0	534.33 ±1.15	0.01 ±0	7.57 ±0	6.95 ±0.06	2.40 ±0	2.17 ±0	918.93 ±0
		DS	12.00 ±0	5.22 ±0.25	7.34 ±0.35	89.39 ±0.58	633.33 ±0.58	1580.00 ±1.73	0.31 ±0	308.00 ±0	0.01 ±0	7.76 ±0	10.89 ±0.21	1.78 ±0	1.44 ±0	164.90 ±0
WINTER	July	BC	14.80 ±0	4.10 ±0.40	2.13 ±0.38	116.56 ±0.58	724.67 ±6.57	1370.67 ±7.3	0.34 ±0	348.33 ±7.37	0.03 ±0.01	7.20 ±0.01	20.30 ±0.06	1.00 ±0	1.83 ±0	555.13 ±0
		DP	15.30 ±0.17	4.49 ±0.59	3.10 ±0.19	291.22 ±0.58	849.67 ±1.15	1177.00 ±1.00	0.42 ±0	416.33 ±0.58	0.03 ±0	7.00 ±0	25.67 ±0.37	0.89 ±0	0.75 ±0	202.80 ±0
		US	14.93 ±0.12	4.49 ±0.28	7.±80 ±1.03	311.72 ±0.52	1428.67 ±4.16	700.00 ±1.73	0.72 ±0	710.00 ±4.26	0.01 ±0	6.96 ±0.02	12.82 ±0.15	0.79 ±0	-0.03 ±0	210.33 ±0
		DS	15.83 ±0.29	4.53 ±0.21	6.75±0. 64	311.72 ±0.58	606.00 ±1.73	1650.33 ±4.04	0.29 ±0	294.67 ±0.58	0.02 ±0	6.93 ±0.02	23.18 ±0.12	1.35 ±0	0.09 ±0	191.87 ±0
	August	BC	21.00 ±0	4.10 ±0.03	1.54 ±0.19	310.11 ±0.58	754.66 ±3.79	1319.33 ±6.80	0.37 ±0	370.33 ±2.08	0.04 ±0	6.85 ±0.11	56.37 ±0.35	-	-	93.36 ±0
		DP	19.00 ±0	4.49 ±0.87	2.23 ±0.47	182.78 ±0.58	840.00 ±1	1190.33 ±1.52	0.41 ±0	411.67 ±0.57	0.05 ±0	7.09 ±0.04	68.53 ±0.56	-	-	43.08 ±0
		US	20.00 ±0	4.49 ±0.25	7.80 ±0.30	105.89±0 .±0.58	899.00 ±3.60	1112.33 ±4.72	0.44 ±0	441.33 ±1.58	0.02 ±0	7.12 ±0.02	28.73 ±0.05	-	-	46.14 ±0
		DS	19.00 ±0	4.53 ±0.25	5.74 ±0.14	309.56 ±0.58	676.00 ±3.25	1479.00 ±7.55	0.33 ±0	329.67 ±1.53	0.02 ±0	7.26 ±0.02	20.76 ±0.05	-	-	45.01 ±0

			Temperature	DO	BOD	COD	Conductivity	Resistivity	Salinity	TDS	TSS	pH	Turbidity	Nitrate	Phosphate	Residual Cl2
			°C	mg/L	mg/L	mg/L	(µS/cm)	Ώ.cm	%	mg/L	mg/L		Ntu	mg/L	mg/L	mg/L
SPRING	September	BC	25.67	6.41	1.91	308.67	864.00	1156.67	0.42	423.67	0.03	6.76	20.73	0.15	1.70	31.49
			±0.25	±0.12	±0.24	±0.58	±4.36	±6.35	±0	±0.30	±0.01	±0.02	±0.06	±0	±0	±0
		DP	26.00	6.61	2.52	309.00	789.67	1266.33	0.39	386.33	0.02	6.82	19.27	0.08	1.90	39.10
			±0	±0.05	±0.31	±0	±1.15	±2.08	±0.00	±0.57	±0.04	±0.04	±0.02	±0	±0	±0
		US	26.00	8.12	7.90	55.56	509.33	1963.00	0.25	246.67	0.01	6.41	10.67	0.17	0.10	29.58
			±0	±0.03	±0.21	±0	±1.15	±3.46	±0	±0.58	±0.01	±0.05	±0.06	±0	±0	±0
		DS	25.50	7.56	7.22	139.67	790.33	1265.33	0.39	386.33	0.01	6.52	11.50	1.47	0.16	40.10
			±0.25	±0.07	±0.25	±0.58	±1.52	±2.08	±0	±0.58	±0	±0.02	±0.1	±0	±0	±0
	October	BC	22.00	7.31	3.18	306.89	831.67	1202.33	0.41	407.33	0.03	6.60	30.53	0.88	1.88	39.46
			±0	±0.07	±0.32	±0	±1.15	±2.08	±0	±0.58	±0.04	±0.05	±0.2	±0	±0	±0
		DP	23.00	7.96	3.93	109.89	832.33	1200.67	0.41	408.00	0.02	6.75	28.50	0.82	1.57	34.37
			±0	±0.03	±0.17	±0	±0.57	±0.58	±0	±0	±0.01	±0.05	±0	±0	±0	±0
		US	24.00	7.70	7.08	195.33	499.33	2003.00	0.24	241.33	0.36	7.02	17.07	1.02	0.37	32.51
			±0	±0.04	±0.19	±0	±0.58	±1.73	±0	±0.57	±0.01	±0	±0.02	±0	±0	±0
		DS	24.00	7.64	6.93	148.00	689.67	1449.33	0.34	336.00	0.01	6.91	29.03	1.30	0.42	24.96
			±0	±0.09	±0.55	±0	±0.57	±0.58	±0	±0	±0	±0.01	±0.05	±0	±0	±0
	November	BC	23.00	6.87	3.49	123.78	907.67	1102.00	0.44	445.33	0.05	6.79	39.97	0.28	3.53	20.40
			±0	±0.04	±0.28	±0.58	±0	±1.73	±0	±0.58	±0.01	±0.01	±0.11	±0	±0	±0
		DP	22.00	6.86	3.35	287.22	970.00	1031.00	0.48	477.33	0.03	6.68	48.53	0.31	3.69	34.17
			±0	±0.05	±0.36	±0.58	±0	±4.36	±0.05	±2.08	±0	±0.02	±0.55	±0	±0	±0
		US	21.00	7.62	8.38	241.78	429.00	2330.00	0.20	206.87	0.01	6.86	21.33	0.97	0.64	33.62
			±0	±0.03	±0.43	±0.58	±0	±0	±0.05	±0.23	±0.6	±0.01	±0.76	±0	±0	±0
		DS	22.50	7.42	7.42	246.11	718.00	1392.33	0.35	350.00	0.01	6.72	14.10	3.38	0.45	28.17
			±0	±0.05	±0.28	±0.58	±0	±0.58	±0	±0	±0.1	±0.04	±0.45	±0	±0	±0
		BC	25.00	6.92	3.27	170.67	872.33	1760.33	0.43	428.00	0.05	6.78	36.13	0.24	1.82	24.17
			±0	±0.05	±0.03	±0.57	±0.58	±3.78	±0	±0	±0	±0.03	±0.40	±0	±0	±0
SUMMER	December	DP	21.00	7.60	3.54	153.56	961.67	1806.00	0.47	473.00	0.02	6.69	31.77	0.77	1.37	28.64
			±0	±0.02	±0.27	±1.154	±2.08	±2.64	±0	±1	±0.01	±0.01	±0.23	±0	±0	±0
		US	22.00	7.50	7.04	274.33	415.33	2082.00	0.20	200.03	0.01	6.85	12.20	0.39	0.03	24.66
			±0	±0.02	±0.47	±0.58	±0.58	±2.00	±0	±0.08	±0	±0.01	±0.26	±0	±0	±0
		DS	22.00	7.59	7.52	205.33	735.67	2010.33	0.36	359.33	0.01	6.64	10.33	1.95	0.35	29.12
			±0	±0.02	±0.09	±0.27	±1015	±0.57	±0	±0.58	±0	±0.06	±0.27	±0	±0	±0
	January	BC	24.00	7.60	3.72	306.89	804.00	1244.00	0.39	393.67	0.02	6.84	12.67	< 0.017	4.70	47.07
			±0	±0.03	±008	±2.08	±1	±4.36	±0	±0.58	±0.2	±0.01	±0.15	±0	±0	±0
		DP	23.00	7.66	3.77	109.89	841.33	1188.33	0.41	412.33	0.04	6.87	32.67	< 0.017	4.69	56.64
			±0	±0.03	±0.10	±0.58	±1.56	±2.08	±0	±0.58	±0.02	±0.03	±0.82	±0	±0	±0
		US	24.00	7.61	7.45	195.33	323.67	3093.33	0.15	155.10	0.03	7.04	11.40	0.58	0.23	25.04
			±0	±0.05	±0.11	±3.21	±0.5	±5.77	±0	±0.25	±0.04	±0.01	±0.26	±0	±0	±0
		DS	24.00	7.66	6.67	148.00	672.00	1488.33	0.33	327.33	0.01	6.92	8.72	1.22	0.12	36.18
			±0	±0.04	±0.16	±0	±1.00	±2.88	±0	±0.57	±0.03	±0.02	±0.04	±0	±0	±0
	February	BC	25.00	5.92	1.74	306.89	925.33	1080.67	0.45	454.33	0.07	7.80	40.37	< 0.017	3.51	40.02
			±0	±0.08	±0.15	±4.58	±1.52	±1.58	±0.01	±0.58	±0.01	±0.02	±0.21	±0	±0	±0
		DP	25.00	6.87	2.81	109.89	931.00	1188.33	0.46	457.67	0.03	7.88	44.07	< 0.017	2.71	65.13
			±0	±0.03	±0.08	±0.58	±1.73	±2.08	±0	±1.15	±0.02	±0.01	±0.25	±0	±0	±0
		US	25.00	7.48	7.56	195.33	319.33	3093.33	0.15	153.07	0.02	7.41	6.37	0.22	0.03	52.33
			±0	±0.01	±0.19	±0.58	±0.57	±5.77	±0	±0.30	±0.01	±0.01	±0.05	±0	±0	±0
		DS	27.00	7.51	8.19	148.00	641.33	1488.33	0.31	312.33	0.01	7.77	5.94	0.16	0.53	56.61
			±0	±0.02	±0.78	±0	±1.154	±2.89	±0	±0.58	±0	±0.01	±0.09	±0	±0	±0

			Temperature	DO	BOD	COD	Conductivity	Resistivity	Salinity	TDS	TSS	pH	Turbidity	Nitrate	Phosphate	Residual Cl_2
			°C	mg/L	mg/L	mg/L	(µS/cm)	Ώ.cm	%	mg/L	mg/L		Ntu	mg/L	mg/L	mg/L
AUTUM N	March	BC	25.67 ±0	8.16 ±0.25	2.20 ±0.13	154.33 ±0.33	878.33 ±4.5	1125.67 ±5.51	0.44 ±0.01	436.00 ±2	0.01 ±0	7.12 ±0.21	6.65 ±0.22	-	-	
		DP	26.00 ±0	8.16 ±0.25	3.12 ±0.27	239.06 ±0	970.33 ±11.5	1023.67 ±1.58	0.48 ±0.01	477.33 ±5.5	0.02 ±0	7.18 ±0.12	5.71 ±0.58	-	-	
		US	26.00 ±0	8.58 ±0.58	7.79 ±0.83	141.67 ±0.66	431.33 ±2.88	2320.00 ±17.32	0.21 ±0	207.97 ±5.5	0.00 ±0	7.52 ±0.08	5.16 ±0.05	-	-	
		DS	25.50 ±0	8.62 ±0.61	4.97 ±0.59	313.61 ±0	615.00 ±1.72	1622.67 ±3.60	0.30 ±0	299.00 ±1.7	0.01 ±0	7.51 ±0.08	7.32 ±0.32	-	-	
	April	BC	20. ±0	8.40 ±0.51	3.53 ±0.29	199.28 ±0.5	790.33 ±3.05	1265.33 ±4.509	0.39 ±0.	386.67 ±1.52	0.05 ±0	6.82 ±0.03	1.34 ±0	2.81 ±0	1.09 ±0	191.79 ±0
		DP	20.17 ±0.29	8.51 ±0.60	3.58 ±0.14	180.33 ±0.33	884.00 ±4.58	1131.33 ±6.02	0.43 ±0	433.67 ±2.08	0.03 ±0	6.70 ±0.05	1.44 ±0.018	2.35 ±0	0.86 ±0	188.63 ±0
		US	17.67 ±0.28	8.69 ±0.31	8.87 ±0.24	102.28 ±0.5	328.67 ±1.57	304.33 ±1.53	0.16 ±0	157.67 ±1.58	0.01 ±0	6.92 ±0.02	8.75 ±0	1.29 ±0	0.17 ±0	55.86 ±0
		DS	19.00 ±0	8.69 ±0.58	7.84 ±0.83	115.39 ±0	627.33 ±0.57	1594.33 ±2.08	0.30 ±0	305.33 ±0.57	0.06 ±0	7.01 ±0.04	17.10 ±	0.82 ±0	0.28 ±0	132.50 ±0
	May	BC	19.00 ±0	8.29 ±0.01	3.29 ±0.25	312.78 ±0.89	1148.33 ±5.50	871.00 ±4.00	0.57 ±0	567.33 ±2.51	0.04 ±0	6.93 ±0.09	28.70 ±0	<0.057 ±0	0.05 ±0	343.10 ±0
		DP	18.50 ±0	8.19 ±0.30	4.10 ±0.16	245.33 ±1.15	1253.67 ±3.05	797.67 ±2.51	0.62 ±0.01	621.33 ±1.53	0.03 ±0	6.93 ±0.05	30.30 ±0	<0.057 ±0	<0.025 ±0	308.53 ±0
		US	13.67 ±0.57	8.78 ±0.02	11.04 ±0.97	305.61 ±0.33	363.67 ±1.154	2.75 ±11.54	0.17 ±0	174.67 ±0.25	0.00 ±0	6.63 ±0.06	3.18 ±0	0.84 ±0	<0.025 ±0	108.67 ±0
		DS	16.07 ±0.16	8.59 ±0.21	9.27 ±0.55	302.67 0.3	939.67 ±1.154	1064.33 ±1.15	0.46 ±0	462.00 ±1	0.02 ±0.1	6.83 ±0	17.80 ±0	0.51 ±0	<0.025 ±0	249.60 ±0
WINTER	June	BC	18.00 ±0	7.89 ±0.13	4.18 ±0.39	307.17 ±0	1084.33 ±2.08	925.33 ±1.154	0.53 ±0.01	534.33 ±1.53	0.01 ±0	7.60 ±0.01	7.68 ±0.02	3.76 ±0	1.05 ±0	381.50 ±0
		DP	17.50 ±0	8.10 ±0.61	4.36 ±0.18	124.17 ±0	1166.33 ±2.08	857.33 ±1.58	0.58 ±0	577.33 ±1.15	0.01 ±0	7.61 ±0.01	7.47 ±0.5	1.99 ±0	0.88 ±0	677.37 ±0
		US	16.00 ±0	8.22 ±0.02	9.85 ±0.63	26.33 ±0	383.67 ±0.27	2610.00 ±0	0.18 ±0	184.53 ±0.30	0.00 ±0	7.93 ±0.01	6.87 ±0.12	2.38 ±0	0.66 ±0	114.57 ±0
		DS	13.87 ±0.16	8.06 ±0.03	6.95 ±0.46	72.28 ±0	826.67 ±1.52	1209.00 ±1.154	0.40 ±0	404.67 ±0.58	0.01 ±0	7.81 ±0	9.13 ±0.11	2.15 ±0	0.33 ±0	298.43 ±0
	July	BC	16.50 ±0	7.19 ±0.59	2.38 ±0.32	193.67 ±0.33	974.67 ±0.28	1026.67 ±0.58	0.47 ±0.01	467.30 ±3.00	0.02 ±0.01	6.73 ±0.1	20.78 ±0.15	0.47 ±0	-0.03 ±0	273.47 ±0
		DP	17.00 ±0	8.71 ±0.16	4.45 ±0.26	308.67 ±0.78	1069.00 ±1.00	935.67 ±0.28	0.53 ±0.01	523.33 ±2.31	0.02 ±0.01	6.60 ±0.01	24.58 ±0.15	0.57 ±0	0.09 ±0	261.23 ±0
		US	13.50 ±0	8.58 ±0.80	8.14 ±0.71	309.39 ±1.49	321.67 ±0.58	3.11 ±0	0.15 ±0	154.30 ±0.24	0.00 ±0	6.34 ±0.01	7.39 ±0.01	1.03 ±0	-0.03 ±0	97.67 ±0
		DS	14.50 ±0	8.46 ±0.01	8.12 ±0.55	298.06 ±0	706.67 ±1.53	1415.00 ±2.64	0.34 ±0	345.00 ±0.28	0.02 ±0.01	6.47 ±0.02	19.77 ±0	0.71 ±0	0.07 ±0	196.00 ±0
	August	BC	16.80 ±0	7.19 ±0.01	4.10 ±0.21	139.56 ±0.33	849.33 ±1.58	1187.00 ±16.6	0.42 ±0.05	416.67 ±0.28	0.02 ±010	6.85 ±0.11	19.73 ±0.05	1.42 ±0	0.93 ±0	38.32 ±0
		DP	16.50 ±0	8.71 ±0.30	4.49 ±0.58	309.00 ±0.24	958.00 ±2.00	1044.00 ±2.00	0.47 ±0	471.00 ±1.00	0.01 ±0	7.09 ±0.04	16.80 ±0.17	1.96 ±0	1.47 ±0	31.77 ±0
		US	14.50 ±0	8.58 ±0.58	10.61 ±0.20	207.56 ±0.58	706.67 ±11.28	1419.00 ±11.36	0.34 ±0	344.67 ±3.5	0.03 ±0.01	7.12 ±0.03	40.40 ±0.36	0.80 ±0	1.89 ±0	33.79 ±0
		DS	12.00 ±0	8.46 ±0.25	8.84 ±0.71	311.78 ±0.25	757.33 ±4.04	1320.67 ±4.04	0.37 ±0	369.67 ±1.154	0.02 ±0.01	7.26 ±7.26	14.10 ±0.1	2.29 ±0	0.59 ±0	32.90 ±0

			Temperature	DO	BOD	COD	Conductivity	Resistivity	Salinity	TDS	TSS	pH	Turbidity	Nitrate	Phosphate	Residual CL_2
			°C	mg/L	mg/L	mg/L	(µS/cm)	Ώ.cm	%	mg/L	mg/L		Ntu	mg/L	mg/L	mg/L
SPRING	September	BC	22.00	7.97	4.76	98.22	672.00	1488.33	0.33	327.33	0.01	6.75	5.84	8.22	38.90	31.27
			±0	±0.49	±1.20	±0.57	±1.00	±2.08	±0	±0.57	±0.01	±0.08	±0.01	±0	±0	±0
		DP	20.00	8.26	4.57	310.44	778.67	1284.00	0.38	380.67	0.00	6.37	16.63	0.23	17.77	22.31
			±0	±0.02	±0.27	±0.57	±0.58	±1.00	±0	±0.57	±0	±0.03	±0.06	±0	±0	±0
		US	20.00	7.74	8.22	310.33	528.67	1890.67	0.25	256.00	0.01	6.48	15.83	2.65	14.90	35.76
			±0	±0.05	±0.25	±0.57	±1.15	±4.04	±0	±0	±0	±0.06	±0.15	±0	±0	±0
		DS	20.00	7.70	7.46	189.89	542.00	1841.67	0.26	261.67	0.02	6.59	6.98	0.72	18.62	28.35
			±0	±0.51	±0.97	±0.57	±6.55	±6.81	±0	±0	±0.01	±0	±0.015	±0	±0	±0
	October	BC	20.00	8.11	4.17	311.89	733.67	1363.00	0.36	358.67	0.00	6.91	20.00	1.23	45.00	36.44
			±0	±0.02	±0.36	±0.58	±0.58	±1.73	±0	±0.58	±0	±0.09	±0.1	±0	±0	±0
		DP	20.00	8.36	4.25	239.33	697.67	1433.33	0.34	340.33	0.00	6.85	16.33	1.64	43.30	38.37
			±0	±0.02	±0.21	±0.57	±0.58	±1.154	±0	±0.58	±	±0.14	±0.06	±0	±0	±0
		US	17.00	8.44	7.98	35.67	458.33	2180.67	0.22	221.33	0.00	6.97	3.68	2.32	25.50	24.19
			±0	±0.02	±0.24	±0.57	±0.58	±7.57	±0	±0.58	±0	±0.02	±0.01	±0	±0	±0
		DS	19.00	8.50	8.92	54.11	545.67	1831.67	0.26	264.67	0.01	6.98	5.10	2.47	35.90	30.45
			±0	±0.02	±0.32	±0.23	±.58	±2.08	±0	±0.58	±0	±0.03	±0.01	±0	±0	±0
	November	BC	20.00	7.61	4.05	69.11	892.33	1116.33	0.43	431.67	-0.15	6.82	5.51	0.50	61.20	25.57
			±0	±0.07	±0.19	±0.58	±0.58	±1.53	±	±4.51	±0	±0.02	±0.08	±0	±0	±0
		DP	20.00	7.64	4.22	108.56	1087.67	914.67	0.54	534.67	0.01	7.14	6.48	0.33	56.40	33.42
			±0	±0.03	±0.09	±0.58	±6.68	±2.31	±0.01	±5.13	±0	±0.04	±0.04	±0	±0	±0
		US	17.00	7.84	8.27	306.78	472.00	2120.33	0.23	228.00	0.01	7.12	8.11	2.11	29.10	56.91
			±0	±0.01	±0.23	±0.58	±3.05	±0.28	±0	±0	±0	±0.01	±0.05	±0	±0	±0
		DS	18.00	7.60	7.96	257.56	715.00	1397.33	0.35	349.00	0.03	7.16	16.53	1.27	55.90	28.07
			±0	±0.02	±0.19	±0.58	±0	±1.54	±0	±1.00	±0	±0.01	±0.26	±0	±0	±0
	December	BC	22.00	7.07	2.88	93.33	552.67	1760.33	0.27	267.67	0.05	6.55	4.41	0.11	17.80	28.40
			±0	±0.07	±0.24	±0.57	±1.00	±3.79	±0	±0.58	±0	±0.29	±0.29	±0	±0	±0
		DP	22.00	7.43	3.38	300.44	568.00	1806.00	0.27	275.33	0.04	6.45	29.43	< 0.017	21.50	30.42
			±0	±0.02	±0.18	±0.58	±1.23	±2.65	±0	±0.58	±0	±0.01	±0.05	±0	±0	±0
		US	20.00	7.88	7.20	24.33	480.33	2082.00	0.23	232.00	0.05	6.47	32.10	< 0.017	1.58	40.31
			±0	±0.01	±0.46	±0	±1.00	±2.00	±0	±0	±0	±0.01	±0.1	±0	±0	±0
		DS	20.00	7.70	6.96	35.33	497.00	2010.33	0.24	241.00	0.05	6.51	28.10	0.51	8.40	40.40
			±0	±0.02	±0.47	±0.58	±0.58	±0.58	±0	±0	±0	±0.04	±0.1	±0	±0	±0
SUMMER	January	BC	23.00	7.24	3.52	311.89	848.00	1176.67	0.42	417.67	1.12	6.59	9.43	< 0.017	31.80	50.82
			±0	±0.03	±0.13	±0.58	±0	±1.16	±0	±1.53	±0	±0.02	±0.04	±0	±0	±0
		DP	23.00	7.71	3.80	239.33	849.33	1177.33	0.42	416.33	0.00	6.61	9.29	< 0.017	39.70	68.97
			±	±0.03	±0.21	±0.58	±1.00	±0.58	±0	±0.58	±0	±0.02	±0.04	±0	±0	±0
		US	22.00	7.71	7.11	35.67	410.33	2440.00	0.20	197.63	-0.11±	6.71	10.80	1.21	26.00	25.75
			±0	±0.02	±0.37	±0.58	±0.58	±0	±0	±0.23	±0	±0.02	±0	±0	±0	±0
		DS	22.00	8.03	8.23	54.11	522.00	1915.00	0.25	253.00	0.01	6.73	10.60	0.84	29.50	24.37
			±0	±0.02	±0.60	±0.58	±0	±1.73	±0	±0	±0	±0	±0	±0	±0	±0
	February	BC	24.00	7.54	4.30	311.89	925.33	1080.67	0.45	454.33	0.01	7.72	3.93	< 0.017	54.90	31.71
			±0	±.0.02	±0.02	±0.58	±1.53	±1.53	±0	±0.58	±0	±0.03	±0.01	±0	±0	±0
		DP	24.00	7.75	4.24	239.33	931.00	1188.33	0.46	457.67	0.00	7.87	4.02	< 0.017	55.30	42.00
			±0	±0.01	±0.11	±0.28	±0.57	±2.08	±0	±1.15	±0	±0.02	±0.03	±0	±0	±0
		US	21.0	7.22	7.64	35.67	319.33	3093.33	0.15	153.07	0.00	7.50	8.83	0.44	28.10	35.99
			±0	±0.04	±0.41	±0.58	±0.58	±5.77	±0	±0.30	±	±0.05	±0.04	±0	±0	±0
		DS	23.00	7.94	8.71	54.11	641.33	1488.33	0.31	312.33	0.01	8.08	5.80	0.39	41.50	25.18
			±0	±0.01	±0.17	±0.58	±1.154	±2.88	±0	±0.58	±0	±0	±0.02	±0	±0	±0

APPENDIX II

Original Statistics tables

NGWWTP: BC

Correlations

		REPLICATES	pH	TEMP	TURBIDITY	TDS	COD	AEROMONAS	PSEUDOMONAS_log10	LISTERIA_log10	YERSINIA_log10	LEGIONELLA_log10
REPLICATES	Pearson Correlation	1	-.092	.225	-.239	-.388*	-.582**	-.056	.007	-.683**	.037	-.420*
	Sig. (2-tailed)		.592	.186	.160	.019	.000	.746	.966	.000	.830	.011
	N	36	36	36	36	36	36	36	36	36	36	36
pH	Pearson Correlation	-.092	1	.183	-.236	.439**	.414*	.266	.202	.297	-.130	.355*
	Sig. (2-tailed)	.592		.286	.165	.007	.012	.116	.238	.078	.450	.033
	N	36	36	36	36	36	36	36	36	36	36	36
TEMP	Pearson Correlation	.225	.183	1	-.582**	-.307	-.334*	.374*	.023	-.291	-.008	-.121
	Sig. (2-tailed)	.186	.286		.000	.068	.047	.025	.893	.085	.962	.481
	N	36	36	36	36	36	36	36	36	36	36	36
TURBIDITY	Pearson Correlation	-.239	-.236	-.582**	1	.474**	.300	-.192	.122	.159	.110	-.050
	Sig. (2-tailed)	.160	.165	.000		.003	.076	.262	.477	.356	.523	.772
	N	36	36	36	36	36	36	36	36	36	36	36
TDS	Pearson Correlation	-.388*	.439**	-.307	.474**	1	.786**	.096	.362*	.465**	-.084	.377*
	Sig. (2-tailed)	.019	.007	.068	.003		.000	.579	.030	.004	.627	.023
	N	36	36	36	36	36	36	36	36	36	36	36
COD	Pearson Correlation	-.582**	.414*	-.334*	.300	.786**	1	-.212	-.088	.537**	-.258	.287
	Sig. (2-tailed)	.000	.012	.047	.076	.000		.214	.610	.001	.129	.090
	N	36	36	36	36	36	36	36	36	36	36	36
AEROMONAS	Pearson Correlation	-.056	.266	.374*	-.192	.096	-.212	1	.727**	.323	.448**	.367*
	Sig. (2-tailed)	.746	.116	.025	.262	.579	.214		.000	.055	.006	.028
	N	36	36	36	36	36	36	36	36	36	36	36
PSEUDOMONAS_log10	Pearson Correlation	.007	.202	.023	.122	.362*	-.088	.727**	1	.280	.709**	.701**
	Sig. (2-tailed)	.966	.238	.893	.477	.030	.610	.000		.098	.000	.000
	N	36	36	36	36	36	36	36	36	36	36	36
LISTERIA_log10	Pearson Correlation	-.683**	.297	-.291	.159	.465**	.537**	.323	.280	1	-.023	.608**
	Sig. (2-tailed)	.000	.078	.085	.356	.004	.001	.055	.098		.895	.000
	N	36	36	36	36	36	36	36	36	36	36	36
YERSINIA_log10	Pearson Correlation	.037	-.130	-.008	.110	-.084	-.258	.448**	.709**	-.023	1	.525**
	Sig. (2-tailed)	.830	.450	.962	.523	.627	.129	.006	.000	.895		.001
	N	36	36	36	36	36	36	36	36	36	36	36
LEGIONELLA_log10	Pearson Correlation	-.420*	.355*	-.121	-.050	.377*	.287	.367*	.701**	.608**	.525**	1
	Sig. (2-tailed)	.011	.033	.481	.772	.023	.090	.028	.000	.000	.001	
	N	36	36	36	36	36	36	36	36	36	36	36

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Original Statistics tables

NGWWTP: DP

		REPLICATES	pH	TEMP	TURBIDITY	TDS	COD	AEROMONAS_log10	PSEUDOMONAS_log10	LISTERIA_log10	YERSINIA_log10	LEGIONELLA_log10
REPLICATES	Pearson Correlation	1	-.037	.230	.002	-.631**	.280	-.174	-.148	-.119	-.054	-.479**
	Sig. (2-tailed)		.830	.178	.989	.000	.099	.309	.388	.491	.754	.003
	N	36	36	36	36	36	36	36	36	36	36	36
pH	Pearson Correlation	-.037	1	.068	-.434**	.208	.141	-.315	-.134	.089	-.316	-.353*
	Sig. (2-tailed)	.830		.694	.008	.224	.411	.061	.434	.606	.060	.035
	N	36	36	36	36	36	36	36	36	36	36	36
TEMP	Pearson Correlation	.230	.068	1	-.385*	-.507**	.221	-.493**	-.278	-.456**	-.260	-.501**
	Sig. (2-tailed)	.178	.694		.020	.002	.196	.002	.100	.005	.126	.002
	N	36	36	36	36	36	36	36	36	36	36	36
TURBIDITY	Pearson Correlation	.002	-.434**	-.385*	1	.086	.121	.656**	.582**	.309	.461**	.548**
	Sig. (2-tailed)	.989	.008	.020		.619	.483	.000	.000	.066	.005	.001
	N	36	36	36	36	36	36	36	36	36	36	36
TDS	Pearson Correlation	-.631**	.208	-.507**	.086	1	-.350*	.145	.085	.218	.150	.198
	Sig. (2-tailed)	.000	.224	.002	.619		.036	.399	.624	.202	.381	.247
	N	36	36	36	36	36	36	36	36	36	36	36
COD	Pearson Correlation	.280	.141	.221	.121	-.350*	1	.406*	.570**	.296	.184	.314
	Sig. (2-tailed)	.099	.411	.196	.483	.036		.014	.000	.080	.282	.062
	N	36	36	36	36	36	36	36	36	36	36	36
AEROMONAS_log10	Pearson Correlation	-.174	-.315	-.493**	.656**	.145	.406*	1	.951**	.558**	.820**	.912**
	Sig. (2-tailed)	.309	.061	.002	.000	.399	.014		.000	.000	.000	.000
	N	36	36	36	36	36	36	36	36	36	36	36
PSEUDOMONAS_log10	Pearson Correlation	-.148	-.134	-.278	.582**	.085	.570**	.951**	1	.546**	.794**	.838**
	Sig. (2-tailed)	.388	.434	.100	.000	.624	.000	.000		.001	.000	.000
	N	36	36	36	36	36	36	36	36	36	36	36
LISTERIA_log10	Pearson Correlation	-.119	.089	-.456**	.309	.218	.296	.558**	.546**	1	.383*	.538**
	Sig. (2-tailed)	.491	.606	.005	.066	.202	.080	.000	.001		.021	.001
	N	36	36	36	36	36	36	36	36	36	36	36
YERSINIA_log10	Pearson Correlation	-.054	-.316	-.260	.461**	.150	.184	.820**	.794**	.383*	1	.635**
	Sig. (2-tailed)	.754	.060	.126	.005	.381	.282	.000	.000	.021		.000
	N	36	36	36	36	36	36	36	36	36	36	36
LEGIONELLA_log10	Pearson Correlation	-.479**	-.353*	-.501**	.548**	.198	.314	.912**	.838**	.538**	.635**	1
	Sig. (2-tailed)	.003	.035	.002	.001	.247	.062	.000	.000	.001	.000	
	N	36	36	36	36	36	36	36	36	36	36	36

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Original Statistics tables

NGWWTP: US

Correlations

		REPLICATES	pH	TDS	BOD	AEROMONAS_log10	PSEUDOMONAS	LISTERIA_log10	YESINIA	LEGIONELLA_log10
REPLICATES	Pearson Correlation	1	-.145	.112	-.351*	.424**	.274	-.333*	.117	.123
	Sig. (2-tailed)		.399	.516	.036	.010	.105	.048	.496	.474
	N	36	36	36	36	36	36	36	36	36
pH	Pearson Correlation	-.145	1	-.135	-.068	-.444**	-.172	.509**	-.424**	-.496**
	Sig. (2-tailed)	.399		.431	.694	.007	.315	.002	.010	.002
	N	36	36	36	36	36	36	36	36	36
TDS	Pearson Correlation	.112	-.135	1	-.305	.318	.742**	.461**	.601**	.308
	Sig. (2-tailed)	.516	.431		.071	.059	.000	.005	.000	.068
	N	36	36	36	36	36	36	36	36	36
BOD	Pearson Correlation	-.351*	-.068	-.305	1	-.248	-.293	-.041	-.093	-.068
	Sig. (2-tailed)	.036	.694	.071		.145	.083	.815	.588	.694
	N	36	36	36	36	36	36	36	36	36
AEROMONAS_log10	Pearson Correlation	.424**	-.444**	.318	-.248	1	.776**	-.122	.663**	.603**
	Sig. (2-tailed)	.010	.007	.059	.145		.000	.477	.000	.000
	N	36	36	36	36	36	36	36	36	36
PSEUDOMONAS	Pearson Correlation	.274	-.172	.742**	-.293	.776**	1	.381*	.785**	.565**
	Sig. (2-tailed)	.105	.315	.000	.083	.000		.022	.000	.000
	N	36	36	36	36	36	36	36	36	36
LISTERIA_log10	Pearson Correlation	-.333*	.509**	.461**	-.041	-.122	.381*	1	.047	.167
	Sig. (2-tailed)	.048	.002	.005	.815	.477	.022		.785	.331
	N	36	36	36	36	36	36	36	36	36
YESINIA	Pearson Correlation	.117	-.424**	.601**	-.093	.663**	.785**	.047	1	.495**
	Sig. (2-tailed)	.496	.010	.000	.588	.000	.000	.785		.002
	N	36	36	36	36	36	36	36	36	36
LEGIONELLA_log10	Pearson Correlation	.123	-.496**	.308	-.068	.603**	.565**	.167	.495**	1
	Sig. (2-tailed)	.474	.002	.068	.694	.000	.000	.331	.002	
	N	36	36	36	36	36	36	36	36	36

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Original Statistics tables

NGWWTP: DS

Correlations

		REPLICATES	pH	TEMP	TURBIDITY	TDS	COD	BOD	AEROMONAS_log10	PSEUDOMONAS_log10	LISTERIA_log10	YERSINIA_log10	LEGIONELLA_log10
REPLICATES	Pearson Correlation	1	-.126	.254	-.057	-.471**	-.059	.074	-.284	-.379*	-.623**	-.294	-.384*
	Sig. (2-tailed)		.463	.135	.740	.004	.732	.670	.094	.023	.000	.082	.021
	N	36	36	36	36	36	36	36	36	36	36	36	36
pH	Pearson Correlation	-.126	1	.017	-.379*	.461**	.236	-.025	-.334*	-.398*	.503**	-.398*	-.359*
	Sig. (2-tailed)	.463		.923	.023	.005	.165	.887	.047	.016	.002	.016	.032
	N	36	36	36	36	36	36	36	36	36	36	36	36
TEMP	Pearson Correlation	.254	.017	1	-.391*	-.707**	-.420*	-.336*	-.538**	-.268	.006	-.376*	-.596**
	Sig. (2-tailed)	.135	.923		.018	.000	.011	.045	.001	.114	.973	.024	.000
	N	36	36	36	36	36	36	36	36	36	36	36	36
TURBIDITY	Pearson Correlation	-.057	-.379*	-.391*	1	.179	-.238	.056	.580**	.515**	-.172	.563**	.644**
	Sig. (2-tailed)	.740	.023	.018		.295	.162	.747	.000	.001	.317	.000	.000
	N	36	36	36	36	36	36	36	36	36	36	36	36
TDS	Pearson Correlation	-.471**	.461**	-.707**	.179	1	.501**	.152	.381*	.179	.429**	.209	.412*
	Sig. (2-tailed)	.004	.005	.000	.295		.002	.377	.022	.297	.009	.220	.013
	N	36	36	36	36	36	36	36	36	36	36	36	36
COD	Pearson Correlation	-.059	.236	-.420*	-.238	.501**	1	.215	.253	.200	-.175	.223	.195
	Sig. (2-tailed)	.732	.165	.011	.162	.002		.208	.136	.242	.308	.192	.254
	N	36	36	36	36	36	36	36	36	36	36	36	36
BOD	Pearson Correlation	.074	-.025	-.336*	.056	.152	.215	1	.084	-.021	-.251	.036	.105
	Sig. (2-tailed)	.670	.887	.045	.747	.377	.208		.626	.903	.140	.834	.543
	N	36	36	36	36	36	36	36	36	36	36	36	36
AEROMONAS_log10	Pearson Correlation	-.284	-.334*	-.538**	.580**	.381*	.253	.084	1	.927**	-.027	.966**	.971**
	Sig. (2-tailed)	.094	.047	.001	.000	.022	.136	.626		.000	.877	.000	.000
	N	36	36	36	36	36	36	36	36	36	36	36	36
PSEUDOMONAS_log10	Pearson Correlation	-.379*	-.398*	-.268	.515**	.179	.200	-.021	.927**	1	-.025	.975**	.906**
	Sig. (2-tailed)	.023	.016	.114	.001	.297	.242	.903	.000		.883	.000	.000
	N	36	36	36	36	36	36	36	36	36	36	36	36
LISTERIA_log10	Pearson Correlation	-.623**	.503**	.006	-.172	.429**	-.175	-.251	-.027	-.025	1	-.106	-.039
	Sig. (2-tailed)	.000	.002	.973	.317	.009	.308	.140	.877	.883		.540	.819
	N	36	36	36	36	36	36	36	36	36	36	36	36
YERSINIA_log10	Pearson Correlation	-.294	-.398*	-.376*	.563**	.209	.223	.036	.966**	.975**	-.106	1	.937**
	Sig. (2-tailed)	.082	.016	.024	.000	.220	.192	.834	.000	.000	.540		.000
	N	36	36	36	36	36	36	36	36	36	36	36	36
LEGIONELLA_log10	Pearson Correlation	-.384*	-.359*	-.596**	.644**	.412*	.195	.105	.971**	.906**	-.039	.937**	1
	Sig. (2-tailed)	.021	.032	.000	.000	.013	.254	.543	.000	.000	.819	.000	
	N	36	36	36	36	36	36	36	36	36	36	36	36

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Original Statistics tables

NWWTP: BC

		Months_replicates	pH	TURBIDITY	COD	AEROMONAS_log10	PSEUDOMONAS_log10	LISTERIA_log10	YERSINIA	LEGIONELLA
Months_replicates	Pearson Correlation	1	-.195	.184	.315	.842**	.799**	.799**	.677**	.369*
	Sig. (2-tailed)		.255	.284	.062	.000	.000	.000	.000	.027
	N	36	36	36	36	36	36	36	36	36
pH	Pearson Correlation	-.195	1	.164	-.078	-.220	-.244	-.244	-.075	-.217
	Sig. (2-tailed)	.255		.340	.651	.197	.152	.152	.664	.203
	N	36	36	36	36	36	36	36	36	36
TURBIDITY	Pearson Correlation	.184	.164	1	.486**	.296	.213	.213	.605**	.551**
	Sig. (2-tailed)	.284	.340		.003	.079	.213	.213	.000	.000
	N	36	36	36	36	36	36	36	36	36
COD	Pearson Correlation	.315	-.078	.486**	1	.501**	.398*	.398*	.563**	.520**
	Sig. (2-tailed)	.062	.651	.003		.002	.016	.016	.000	.001
	N	36	36	36	36	36	36	36	36	36
AEROMONAS_log10	Pearson Correlation	.842**	-.220	.296	.501**	1	.978**	.978**	.694**	.613**
	Sig. (2-tailed)	.000	.197	.079	.002		.000	.000	.000	.000
	N	36	36	36	36	36	36	36	36	36
PSEUDOMONAS_log10	Pearson Correlation	.799**	-.244	.213	.398*	.978**	1	1.000**	.613**	.571**
	Sig. (2-tailed)	.000	.152	.213	.016	.000		0.000	.000	.000
	N	36	36	36	36	36	36	36	36	36
LISTERIA_log10	Pearson Correlation	.799**	-.244	.213	.398*	.978**	1.000**	1	.613**	.571**
	Sig. (2-tailed)	.000	.152	.213	.016	.000	0.000		.000	.000
	N	36	36	36	36	36	36	36	36	36
YERSINIA	Pearson Correlation	.677**	-.075	.605**	.563**	.694**	.613**	.613**	1	.793**
	Sig. (2-tailed)	.000	.664	.000	.000	.000	.000	.000		.000
	N	36	36	36	36	36	36	36	36	36
LEGIONELLA	Pearson Correlation	.369*	-.217	.551**	.520**	.613**	.571**	.571**	.793**	1
	Sig. (2-tailed)	.027	.203	.000	.001	.000	.000	.000	.000	
	N	36	36	36	36	36	36	36	36	36

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Original Statistics tables

NWWTP: DP

Correlations

		TURBIDITY	COD	BOD	TSS_log10	AEROMONAS_log10	PSEUDOMONAS_log10	LISTERIA_log10	YERSINIA_log10	LEGIONELLA
TURBIDITY	Pearson Correlation	1	.011	-.288	.625**	.203	.271	.009	.276	.537**
	Sig. (2-tailed)		.947	.089	.000	.235	.110	.959	.103	.001
	N	36	36	36	36	36	36	36	36	36
COD	Pearson Correlation	.011	1	-.222	.058	.341*	.437**	.357*	.331*	.189
	Sig. (2-tailed)	.947		.193	.735	.042	.008	.033	.049	.270
	N	36	36	36	36	36	36	36	36	36
BOD	Pearson Correlation	-.288	-.222	1	-.370*	-.066	-.026	-.015	-.142	-.280
	Sig. (2-tailed)	.089	.193		.026	.700	.880	.930	.407	.098
	N	36	36	36	36	36	36	36	36	36
TSS_log10	Pearson Correlation	.625**	.058	-.370*	1	.109	.149	.116	.145	.200
	Sig. (2-tailed)	.000	.735	.026		.528	.385	.499	.398	.241
	N	36	36	36	36	36	36	36	36	36
AEROMONAS_log10	Pearson Correlation	.203	.341*	-.066	.109	1	.966**	.266	.976**	.545**
	Sig. (2-tailed)	.235	.042	.700	.528		.000	.117	.000	.001
	N	36	36	36	36	36	36	36	36	36
PSEUDOMONAS_log10	Pearson Correlation	.271	.437**	-.026	.149	.966**	1	.265	.946**	.626**
	Sig. (2-tailed)	.110	.008	.880	.385	.000		.118	.000	.000
	N	36	36	36	36	36	36	36	36	36
LISTERIA_log10	Pearson Correlation	.009	.357*	-.015	.116	.266	.265	1	.369*	.240
	Sig. (2-tailed)	.959	.033	.930	.499	.117	.118		.027	.159
	N	36	36	36	36	36	36	36	36	36
YERSINIA_log10	Pearson Correlation	.276	.331*	-.142	.145	.976**	.946**	.369*	1	.615**
	Sig. (2-tailed)	.103	.049	.407	.398	.000	.000	.027		.000
	N	36	36	36	36	36	36	36	36	36
LEGIONELLA	Pearson Correlation	.537**	.189	-.280	.200	.545**	.626**	.240	.615**	1
	Sig. (2-tailed)	.001	.270	.098	.241	.001	.000	.159	.000	
	N	36	36	36	36	36	36	36	36	36

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Original Statistics tables

NWWTP: US

Correlations

		REPLICATES	pH	TEMP	COD	BOD	AEROMONAS_log10	PSEUDOMONAS_log10	LISTERIA_log10	YERSINIA_log10	LEGIONELLA_log10
REPLICATES	Pearson Correlation	1	-.160	.369*	.401*	.231	.021	.089	-.267	.233	-.212
	Sig. (2-tailed)		.350	.027	.015	.174	.902	.606	.115	.172	.215
	N	36	36	36	36	36	36	36	36	36	36
pH	Pearson Correlation	-.160	1	-.099	.076	.274	-.222	-.321	.479**	-.317	-.228
	Sig. (2-tailed)	.350		.566	.661	.106	.194	.056	.003	.060	.181
	N	36	36	36	36	36	36	36	36	36	36
TEMP	Pearson Correlation	.369*	-.099	1	.060	-.153	-.560**	-.488**	-.793**	-.542**	-.331*
	Sig. (2-tailed)	.027	.566		.728	.373	.000	.003	.000	.001	.049
	N	36	36	36	36	36	36	36	36	36	36
COD	Pearson Correlation	.401*	.076	.060	1	.376*	.009	-.008	-.298	.038	-.469**
	Sig. (2-tailed)	.015	.661	.728		.024	.957	.964	.078	.827	.004
	N	36	36	36	36	36	36	36	36	36	36
BOD	Pearson Correlation	.231	.274	-.153	.376*	1	-.146	-.150	.092	-.116	-.539**
	Sig. (2-tailed)	.174	.106	.373	.024		.395	.383	.594	.500	.001
	N	36	36	36	36	36	36	36	36	36	36
AEROMONAS_log10	Pearson Correlation	.021	-.222	-.560**	.009	-.146	1	.892**	.369*	.855**	.567**
	Sig. (2-tailed)	.902	.194	.000	.957	.395		.000	.027	.000	.000
	N	36	36	36	36	36	36	36	36	36	36
PSEUDOMONAS_log10	Pearson Correlation	.089	-.321	-.488**	-.008	-.150	.892**	1	.361*	.908**	.410*
	Sig. (2-tailed)	.606	.056	.003	.964	.383	.000		.030	.000	.013
	N	36	36	36	36	36	36	36	36	36	36
LISTERIA_log10	Pearson Correlation	-.267	.479**	-.793**	-.298	.092	.369*	.361*	1	.411*	.330*
	Sig. (2-tailed)	.115	.003	.000	.078	.594	.027	.030		.013	.049
	N	36	36	36	36	36	36	36	36	36	36
YERSINIA_log10	Pearson Correlation	.233	-.317	-.542**	.038	-.116	.855**	.908**	.411*	1	.490**
	Sig. (2-tailed)	.172	.060	.001	.827	.500	.000	.000	.013		.002
	N	36	36	36	36	36	36	36	36	36	36
LEGIONELLA_log10	Pearson Correlation	-.212	-.228	-.331*	-.469**	-.539**	.567**	.410*	.330*	.490**	1
	Sig. (2-tailed)	.215	.181	.049	.004	.001	.000	.013	.049	.002	
	N	36	36	36	36	36	36	36	36	36	36

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Original Statistics tables

NWWTP: US

		REPLICATES	pH	TEMP	TURBIDITY	COD	BOD_log10	AEROMONAS_log10	PSEUDOMONAS_log10	LISTERIA_log10	YERSINIA_log10	LEGIONELLA
REPLICATES	Pearson Correlation	1	-.170	.297	-.202	.176	.039	-.096	.028	-.317	.077	-.371*
	Sig. (2-tailed)		.320	.079	.238	.304	.822	.578	.872	.060	.654	.026
	N	36	36	36	36	36	36	36	36	36	36	36
pH	Pearson Correlation	-.170	1	-.004	-.456**	-.092	-.128	-.651**	-.594**	.548**	-.657**	-.405*
	Sig. (2-tailed)	.320		.982	.005	.593	.456	.000	.000	.001	.000	.014
	N	36	36	36	36	36	36	36	36	36	36	36
TEMP	Pearson Correlation	.297	-.004	1	-.007	.043	-.398*	-.409*	-.519**	-.679**	-.359*	-.619**
	Sig. (2-tailed)	.079	.982		.966	.805	.016	.013	.001	.000	.031	.000
	N	36	36	36	36	36	36	36	36	36	36	36
TURBIDITY	Pearson Correlation	-.202	-.456**	-.007	1	.007	-.022	.013	.118	-.357*	.029	-.012
	Sig. (2-tailed)	.238	.005	.966		.969	.899	.940	.492	.032	.868	.945
	N	36	36	36	36	36	36	36	36	36	36	36
COD	Pearson Correlation	.176	-.092	.043	.007	1	-.109	-.256	-.340*	-.474**	-.387*	-.229
	Sig. (2-tailed)	.304	.593	.805	.969		.526	.131	.043	.003	.020	.178
	N	36	36	36	36	36	36	36	36	36	36	36
BOD_log10	Pearson Correlation	.039	-.128	-.398*	-.022	-.109	1	.190	.365*	.285	.274	.305
	Sig. (2-tailed)	.822	.456	.016	.899	.526		.266	.029	.092	.106	.071
	N	36	36	36	36	36	36	36	36	36	36	36
AEROMONAS_log10	Pearson Correlation	-.096	-.651**	-.409*	.013	-.256	.190	1	.860**	.084	.900**	.769**
	Sig. (2-tailed)	.578	.000	.013	.940	.131	.266		.000	.628	.000	.000
	N	36	36	36	36	36	36	36	36	36	36	36
PSEUDOMONAS_log10	Pearson Correlation	.028	-.594**	-.519**	.118	-.340*	.365*	.860**	1	.202	.901**	.748**
	Sig. (2-tailed)	.872	.000	.001	.492	.043	.029	.000		.237	.000	.000
	N	36	36	36	36	36	36	36	36	36	36	36
LISTERIA_log10	Pearson Correlation	-.317	.548**	-.679**	-.357*	-.474**	.285	.084	.202	1	.134	.329*
	Sig. (2-tailed)	.060	.001	.000	.032	.003	.092	.628	.237		.437	.050
	N	36	36	36	36	36	36	36	36	36	36	36
YERSINIA_log10	Pearson Correlation	.077	-.657**	-.359*	.029	-.387*	.274	.900**	.901**	.134	1	.709**
	Sig. (2-tailed)	.654	.000	.031	.868	.020	.106	.000	.000	.437		.000
	N	36	36	36	36	36	36	36	36	36	36	36
LEGIONELLA	Pearson Correlation	-.371*	-.405*	-.619**	-.012	-.229	.305	.769**	.748**	.329*	.709**	1
	Sig. (2-tailed)	.026	.014	.000	.945	.178	.071	.000	.000	.050	.000	
	N	36	36	36	36	36	36	36	36	36	36	36

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

APPENDIX III

TABLE: Antimicrobial resistance/susceptibility determination of *Listeria* spp. Isolates (Isolates 1-19)

AB	***Listeria* spp.**																																							
	1		**2**		**3**		**4**		**5**		**6**		**7**		**8**		**9**		**10**		**11**		**12**		**13**		**14**		**15**		**16**		**17**		**18**		**19**	
W	22	S	0	R	20	S	23	S	22	S	23	S	0	R	14	I	15	I	0	R	0	R	23	S	0	R	0	R	0	R	0	R	0	R	23	S	20	S
C	20	S	24	S	24	S	20	S	21	S	24	S	20	S	20	S	20	S	22	S	20	S	24	S	25	S	24	S	30	S	26	S	25	S	24	S	20	S
FOS	23	S	23	S	22	S	21	S	23	S	23	S	22	S	23	S	23	S	23	S	22	S	23	S	23	S	23	S	22	S	23	S	23	S	22	S	23	S
TE	16	R	16	R	18	R	16	R	18	R	20	I	20	I	0	R	0	R	23	S	17	R	20	I	27	S	33	S	30	S	20	I	18	R	20	I	20	I
S	20	S	20	S	20	S	22	S	20	S	20	S	20	S	20	S	20	S	20	S	22	S	20	S	20	S	24	S	25	S	25	S	20	S	20	S	20	S
MXF	16	I	20	S	20	S	20	S	26	S	26	S	20	S	20	S	24	S	23	S	23	S	26	S	35	S	32	S	32	S	30	S	26	S	26	S	20	S
CIP	21	S	21	S	21	S	21	S	22	S	20	I	22	S	24	S	24	S	12	R	12	R	20	I	32	S	34	S	32	S	37	S	22	S	20	I	22	S
AMP	12	R	16	R	12	R	15	R	9	R	0	R	10	R	0	R	0	R	13	R	16	R	0	R	16	R	15	R	15	R	16	R	9	R	0	R	10	R
FD	26	S	24	S	26	S	26	S	26	S	24	S	26	S	26	S	26	S	24	S	26	S	26	S	26	S	24	S	26	S	26	S	26	S	24	S	26	S
F	14	R	15	I	13	R	13	R	15	I	14	R	13	R	12	R	15	I	0	R	0	R	15	I	20	S	18	S	15	I	27	S	14	R	14	R	13	R
K	15	I	15	I	15	I	15	I	10	R	11	R	13	R	15	I	19	S	0	R	0	R	11	R	31	S	30	S	32	S	33	S	10	R	11	R	15	I
NA	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
CT	12	S	12	S	12	S	11	S	12	S	11	S	12	S	13	S	12	S	10	R	17	S	11	S	15	S	15	S	15	S	15	S	12	S	11	S	12	S
CN	24	S	24	S	20	S	21	S	16	S	15	I	22	S	24	S	24	S	24	S	23	S	15	S	30	S	25	S	26	S	25	S	16	S	15	I	22	S
KF	0	R	0	R	0	R	0	R	21	S	22	S	21	S	14	I	14	I	0	R	0	R	0	R	0	R	0	R	0	R	0	R	21	S	22	S	21	S
P	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
CRO	21	S	22	S	22	S	20	S	22	S	20	S	22	S	22	S	24	S	13	R	20	S	20	S	25	S	20	S	20	S	21	S	22	S	20	S	22	S
VA	28	S	31	S	26	S	26	S	24	S	25	R	0	R	0	R	0	R	28	S	30	S	27	S	26	S	24	S	25	S	24	S	0	R	0	R	0	R
DA	29	S	29	S	28	S	28	S	27	S	25	S	0	R	0	R	26	S	25	S	29	S	28	S	26	S	26	S	28	S	26	S	26	S	29	S	27	S
CTX	30	S	29	S	27	S	25	I	26	S	36	S	23	S	30	S	30	S	23	R	28	S	36	S	32	S	30	S	30	S	30	S	26	S	36	S	23	S
ETP	25	S	25	S	25	S	25	S	17	I	22	S	22	S	22	S	24	S	19	S	21	S	22	S	34	S	32	S	31	S	31	S	17	I	22	S	22	S
AK	24	S	24	S	25	S	25	S	26	S	25	S	26	S	26	S	27	S	25	S	27	S	28	S	32	S	26	S	40	S	31	S	30	S	30	S	36	S
MEM	25	S	24	S	25	S	21	S	15	I	19	S	19	S	20	S	24	S	30	S	33	S	19	S	37	S	32	S	32	S	33	S	15	I	19	S	19	S
E	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R

R-resistant S-Sensitive I-Intermediate

Table: Antimicrobial resistance/susceptibility determination of *Listeria* spp. Isolates (Isolates 20-38)

AB	***Listeria* spp.**																																							
	20		**21**		**22**		**23**		**24**		**25**		**26**		**27**		**28**		**29**		**30**		**31**		**32**		**33**		**34**		**35**		**36**		**37**		**38**	
W	0	R	0	R	24	S	24	S	24	S	12	R	10	R	14	I	15	I	16	S	15	I	16	S	10	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
C	21	S	24	S	23	S	23	S	30	S	22	S	22	S	20	S	20	S	19	S	20	S	25	S	24	S	21	S	21	S	25	S	24	S	22	S	20	S	20	S	20	S
FOS	23	S	22	S	23	S	22	S	23	S	23	S	22	S	23	S	22	S	23	S	22	S	23	S	22	S	22	S	23	S	22	S	23	S	22	S	23	S	23	S	23	S
TE	22	I	17	R	18	R	20	I	18	R	0	R	0	R	0	R	0	R	10	R	15	R	16	R	16	R	23	S	24	S	21	I	24	S	24	S	24	S	24	S	24	S
S	20	S	20	S	24	S	25	S	20	S	21	S	20	S	20	S	20	S	20	S	20	S	20	S	24	S	25	S	20	S	20	S	24	S	25	S	25	S	20	S	20	S
MXF	20	S	18	I	20	S	19	S	20	S	25	S	22	S	20	S	24	S	15	R	16	I	24	S	20	S	24	S	26	S	25	S	24	S	25	S	25	S	25	S	25	S
CIP	24	S	21	S	25	S	24	S	21	S	25	S	22	S	24	S	24	S	22	S	20	I	21	S	20	I	30	S	34	S	35	S	30	S	45	S	30	S	30	S	30	S
AMP	9	R	15	R	16	R	13	R	15	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	9	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
FD	26	S	24	S	26	S	26	S	26	S	24	S	26	S	26	S	26	S	24	S	26	S	26	S	26	S	24	S	26	S	26	S	26	S	24	S	26	S	26	S	26	S
F	13	R	24	S	15	I	24	S	15	I	15	I	10	R	12	R	15	I	0	R	9	R	12	R	10	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
K	13	R	14	R	15	I	16	I	16	I	19	S	17	S	15	I	19	S	10	R	15	I	11	R	15	I	9	R	11	R	9	R	15	I	21	S	9	R	0	R	0	R
NA	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
CT	11	S	12	S	16	S	12	S	11	S	13	S	9	R	13	S	12	S	10	R	11	S	11	S	10	R	15	S	15	S	15	S	0	R	18	S	15	S	15	S	15	S
CN	20	S	23	S	21	S	20	S	20	S	24	S	24	S	24	S	24	S	20	S	21	S	15	I	20	S	35	S	35	S	35	S	30	S	27	S	30	S	30	S	30	S
KF	20	S	10	R	12	I	0	R	0	R	15	I	16	I	14	I	14	I	0	R	20	S	20	S	20	S	0	R	0	R	10	R	0	R	0	R	0	R	0	R	0	R
P	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
CRO	21	S	24	S	24	S	24	S	22	S	25	S	10	R	22	S	24	S	22	S	22	S	22	S	23	S	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
VA	28	S	30	S	0	R	26	S	24	S	25	S	24	S	28	S	30	S	0	R	26	S	24	S	25	S	24	S	0	R	0	R	0	R	0	R	0	R	0	R	0	R
DA	25	S	26	S	25	S	27	S	27	S	27	S	26	S	27	S	29	S	0	R	26	S	28	S	27	S	27	S	0	R	27	S	26	S	25	S	0	R	0	R	0	R
CTX	24	I	28	S	30	S	26	S	25	I	26	S	18	R	30	S	30	S	25	I	26	S	27	S	25	S	13	R	14	R	11	R	11	R	13	R	13	R	13	R	13	R
ETP	22	S	20	S	22	S	22	S	22	S	24	S	15	R	22	S	24	S	23	S	22	S	16	I	15	R	9	R	14	R	19	S	18	I	17	I	19	S	19	S	19	S
AK	24	S	24	S	25	S	25	S	26	S	20	S	22	S	18	S	22	S	24	S	24	S	25	S	25	S	34	S	37	S	35	S	32	S	42	S	36	S	36	S	36	S
MEM	18	S	24	S	22	S	20	S	24	S	22	S	20	S	20	S	24	S	27	S	15	I	16	S	15	I	32	S	32	S	24	S	30	S	25	S	32	S	32	S	32	S
E	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R

R-resistant S-Sensitive I-Intermediate

Table: Antimicrobial resistance/susceptibility determination of *Listeria* spp. Isolates (Isolates 39-57)

AB	*Listeria spp.*																																							
	39		**40**		**41**		**42**		**43**		**44**		**45**		**46**		**47**		**48**		**49**		**50**		**51**		**52**		**53**		**54**		**55**		**56**		**57**	
W	0	R	0	R	16	S	0	R	0	R	0	R	0	R	0	R	31	S	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	20	S	19	S
C	21	S	21	S	20	S	21	S	21	S	21	S	25	S	24	S	25	S	20	S	20	S	21	S	20	S	20	S	19	S	30	S	22	S	26	S	26	S
FOS	22	S	23	S	22	S	23	S	22	S	23	S	22	S	23	S	23	S	22	S	23	S	22	S	23	S	22	S	23	S	23	S	22	R	23	S	22	S
TE	23	S	25	S	25	S	25	S	23	S	13	R	20	I	20	I	25	S	18	R	20	I	17	R	19	R	20	I	24	S	25	S	22	I	20	I	19	R
S	20	S	21	S	20	S	21	S	20	S	20	S	18	S	18	S	26	S	20	S	16	S	16	S	16	S	18	S	18	S	20	S	21	S	20	S	20	S
MXF	25	S	24	S	26	S	26	S	21	S	24	S	27	S	39	S	30	S	26	S	25	S	20	S	14	R	26	S	20	S	27	S	24	S	13	R	22	S
CIP	35	S	40	S	40	S	42	S	37	S	37	S	35	S	35	S	30	S	35	S	34	S	35	S	35	S	37	S	35	S	31	S	35	S	24	S	24	S
AMP	0	R	0	R	0	R	0	R	0	R	0	R	15	R	18	I	20	S	16	R	18	I	10	R	15	R	15	R	15	R	17	R	15	R	17	R	16	R
FD	26	S	24	S	26	S	26	S	26	S	24	S	26	S	26	S	26	S	24	S	26	S	26	S	26	S	24	S	26	S	26	S	26	S	24	S	26	S
F	0	R	0	R	0	R	0	R	0	R	15	I	15	I	15	I	15	I	15	I	15	I	11	R	10	R	9	R	10	R	17	S	0	R	9	R	9	R
K	9	R	10	R	18	S	15	I	17	S	20	S	15	I	10	R	20	S	10	R	0	R	0	R	0	R	0	R	0	R	23	S	0	S	15	I	15	I
NA	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
CT	16	S	16	S	16	S	17	S	17	S	16	S	12	S	11	S	11	S	13	S	15	S	16	S	10	R	12	S	11	S	14	S	11	S	9	R	11	S
CN	30	S	34	S	38	S	40	S	40	S	40	S	21	S	20	S	28	S	20	S	20	S	23	S	21	S	20	S	20	S	30	S	24	S	20	S	16	S
KF	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	S	21	S	20	S
P	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
CRO	0	R	0	R	0	R	0	R	0	R	0	R	14	I	10	R	14	I	14	I	13	I	12	I	14	I	14	I	11	R	30	S	14	I	22	S	24	S
VA	0	R	0	R	0	R	0	R	0	R	0	R	25	S	25	S	20	S	27	S	29	S	25	S	26	S	27	S	24	S	20	S	27	S	0	R	0	R
DA	0	R	25	S	25	S	29	S	27	S	27	S	22	S	25	S	25	S	0	R	28	S	28	S	29	S	28	S	28	S	29	S	29	S	29	S	29	S
CTX	10	R	10	R	13	R	12	R	10	R	10	R	25	I	22	R	20	R	25	I	22	R	20	R	24	I	22	R	24	I	30	S	24	I	29	S	30	S
ETP	22	S	12	R	15	R	12	R	11	R	14	R	13	R	21	S	25	S	9	R	10	R	14	R	12	R	15	R	12	R	27	S	9	R	15	R	17	I
AK	37	S	40	S	39	S	35	S	34	S	40	S	25	S	20	S	27	S	24	S	22	S	24	S	24	S	21	S	21	S	30	S	22	S	24	S	24	S
MEM	35	S	30	S	26	S	30	S	32	S	35	S	25	S	15	I	32	S	21	S	20	S	20	S	20	S	20	S	18	S	28	S	20	S	16	S	18	S
E	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R

Table: Antimicrobial resistance/susceptibility determination of *Listeria* spp. Isolates (Isolates 58-75)

AB	***Listeria SPP.***																																			
	58		**59**		**60**		**61**		**62**		**63**		**64**		**65**		**66**		**67**		**68**		**69**		**70**		**71**		**72**		**73**		**74**		**75**	
W	0	R	20	S	0	R	0	R	30	S	10	R	9	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
C	30	S	30	S	25	S	24	S	30	S	15	R	25	S	24	S	30	S	26	S	25	S	24	S	30	S	26	S	20	S	25	S	24	S	30	S
FOS	23	S	22	S	23	S	23	S	22	S	23	S	23	S	23	S	23	S	23	S	23	S	23	S	23	S	23	S	23	S	23	S	23	S	23	S
TE	18	R	17	R	30	S	23	S	22	I	30	S	18	R	16	R	16	R	17	R	24	S	22	I	21	I	23	S	17	R	18	R	16	R	20	I
S	20	S	21	S	20	S	24	S	26	S	24	S	20	S	21	S	20	S	20	S	20	S	21	S	18	S	20	S	22	S	20	S	19	S	18	S
MXF	20	S	20	S	22	S	25	S	30	S	25	S	20	S	19	S	24	S	24	S	20	S	26	S	24	S	23	S	23	S	20	S	23	S	24	S
CIP	20	I	26	S	31	S	30	S	30	S	32	S	25	S	23	S	32	S	32	S	29	S	25	S	34	S	32	S	32	S	35	S	34	S	34	S
AMP	17	R	18	I	10	R	10	R	22	S	20	S	17	I	16	R	21	S	24	S	16	R	10	R	21	S	23	S	16	R	0	R	18	I	20	S
FD	26	S	24	S	26	S	26	S	26	S	24	S	26	S	26	S	26	S	24	S	26	S	26	S	26	S	24	S	26	S	26	S	26	S	24	S
F	15	I	15	I	25	S	15	I	15	I	21	S	15	I	12	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
K	12	R	11	R	34	S	15	I	21	S	33	S	11	R	10	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
NA	0	R	0	R	0	R	10	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
CT	12	S	11	S	12	S	9	R	9	R	10	R	11	S	11	S	10	R	10	R	9	R	13	S	9	R	10	R	17	S	11	S	11	S	10	R
CN	20	S	14	I	30	S	20	S	29	S	30	S	15	I	15	I	24	S	22	S	23	S	25	S	24	S	24	S	23	S	22	S	24	S	22	S
KF	20	S	22	S	35	S	17	S	36	S	36	S	20	S	21	S	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
P	0	R	0	R	0	R	12	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
CRO	25	S	21	S	20	S	11	I	24	S	24	S	23	S	23	S	16	I	16	I	11	R	12	R	11	R	13	I	20	S	12	R	14	S	11	R
VA	30	S	27	S	24	S	25	S	20	S	25	S	0	R	0	R	25	S	30	S	26	S	28	S	24	S	28	S	30	S	26	S	28	S	26	S
DA	29	S	28	S	28	S	27	S	25	S	0	R	28	S	28	S	22	S	29	S	29	S	29	S	29	S	25	S	29	S	29	S	28	S	28	S
CTX	30	S	30	S	32	S	0	R	20	R	34	S	26	S	26	S	25	I	26	S	25	I	27	S	21	R	23	R	28	S	22	R	22	R	24	I
ETP	20	S	18	I	35	S	29	S	23	S	34	S	20	S	20	S	20	S	20	S	9	R	9	R	16	I	19	S	21	S	20	S	9	R	10	R
AK	24	S	24	S	25	S	22	S	24	S	26	S	19	S	19	S	23	S	24	S	24	S	26	S	24	S	24	S	23	S	24	S	24	S	24	S
MEM	15	I	15	I	15	I	16	S	26	S	22	S	24	S	24	S	36	S	36	S	20	S	20	S	30	S	30	S	33	S	20	S	21	S	22	S
E	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R

R-resistant S-Sensitive I-Intermediate

Tables: Antibiotic Resistance Index (ARI) OF *Listeria* spp (isolates 1-78).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**
No. R	7	7	7	7	6	7	9	8	6	12	10	6	6	6	6	6	9	7	6	7	8	6	5	6	6	11	6	5
No. T	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24
ARI	0.29	0.29	0.29	0.29	0.25	0.29	0.38	0.33	0.25	0.5	0.4	0.25	0.25	0.25	0.25	0.25	0.38	0.29	0.25	0.29	0.33	0.25	0.21	0.25	0.25	0.46	0.25	0.21

	29	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**46**	**47**	**48**	**49**	**50**	**51**	**52**	**53**	**54**	**55**	**56**
No. R	12	6	7	9	11	13	11	11	10	12	11	11	10	11	11	11	7	8	3	10	8	11	12	10	10	6	7	9
No. T	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24
ARI	0.5	0.25	0.29	0.38	0.46	0.54	0.46	0.46	0.4	0.5	0.46	0.46	0.4	0.46	0.46	0.46	0.29	0.33	0.13	0.4	0.33	0.46	0.5	0.4	0.4	0.25	0.29	0.38

	57	**58**	**59**	**60**	**61**	**62**	**63**	**64**	**65**	**66**	**67**	**68**	**69**	**70**	**71**	**72**	**73**	**74**	**75**	**76**	**77**	**78**
No. R	7	8	6	5	7	5	5	6	8	9	9	11	10	10	9	9	11	9	10	8	9	7
No. T	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24
ARI	0.29	0.33	0.25	0.21	0.29	0.21	0.21	0.25	0.33	0.38	0.38	0.46	0.4	0.4	0.38	0.38	0.46	0.38	0.4	0.33	0.38	0.29

APENDIX IV

Table: Antimicrobial resistance/susceptibility determination of *Aeromonas* spp. isolates (1-19)

AB	***Aeromonas* spp.**																																							
	1		**2**		**3**		**4**		**5**		**6**		**7**		**8**		**9**		**10**		**11**		**12**		**13**		**14**		**15**		**16**		**17**		**18**		**19**	
W	25	S	25	S	26	S	26	S	0	R	0	R	0	R	0	R	0	R	0	R	25	S	25	S	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
C	24	S	24	S	28	S	28	S	26	S	30	S	30	S	29	S	30	S	30	S	24	S	25	S	35	S	35	S	30	S	30	S	31	S	30	S	0	R		
FOS	18	S	21	S	25	S	25	S	18	S	18	S	0	S	11	R	26	S	25	S	21	S	21	S	18	S	20	S	15	I	15	I	20	S	25	S	0	R		
TE	16	R	17	R	20	I	20	I	25	S	25	S	24	S	22	I	25	S	23	S	17	R	17	R	27	S	30	S	25	S	24	S	13	R	21	I	20	I		
S	22	S	25	S	25	S	25	S	20	S	30	S	15	S	16	S	17	S	16	S	25	S	24	S	28	S	20	S	20	S	17	S	18	S	20	S	15	S		
MXF	18	I	20	S	22	S	22	S	14	R	25	S	25	S	25	S	20	S	25	S	20	S	20	S	30	S	30	S	26	S	27	S	24	S	24	S	18	S		
CIP	24	S	25	S	29	S	29	S	24	S	33	S	35	S	30	S	30	S	31	S	25	S	26	S	35	S	35	S	29	S	30	S	27	S	30	S	24	S		
AMP	8	R	0	R	11	R	11	R	0	R	0	R	0	R	0	R	10	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R		
FD	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R		
F	18	S	17	S	20	S	20	S	17	S	18	S	18	S	18	S	20	S	17	S	17	S	16	S	20	S	24	S	18	S	17	S	20	S	20	S	18	S		
K	21	S	21	S	25	S	25	S	21	S	21	S	20	S	20	S	30	S	21	S	21	S	21	S	20	S	18	S	20	S	17	I	22	S	20	S	22	S		
NA	8	R	7	R	9	R	9	R	0	R	31	S	30	S	26	S	0	R	27	S	7	R	7	R	0	R	0	R	0	R	0	R	28	S	27	S	0	R		
CT	14	S	14	S	15	S	15	S	11	S	14	S	13	S	16	S	15	S	14	S	14	S	14	S	15	S	16	S	14	S	12	S	14	S	15	S	15	S		
CN	22	S	22	S	30	S	30	S	21	S	22	S	20	S	20	S	30	S	21	S	22	S	21	S	26	S	27	S	25	S	20	S	20	S	21	S	20	S		
KF	20	S	21	S	25	S	25	S	0	R	0	R	0	R	0	R	0	R	0	R	21	S	21	S	25	S	25	S	20	S	16	I	0	R	0	R	0	R		
P	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R		
CRO	26	S	26	S	32	S	32	S	25	S	32	S	28	S	27	S	33	S	30	S	26	S	26	S	40	S	40	S	40	S	40	S	30	S	30	S	27	S		
VA	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R		
DA	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R		
CTX	25	I	26	S	30	S	30	S	25	S	33	S	30	S	25	I	30	S	28	S	26	S	26	S	40	S	40	S	39	S	39	S	29	S	32	S	30	S		
ETP	26	S	25	S	32	S	32	S	20	S	30	S	22	S	23	S	27	S	25	S	25	S	25	S	25	S	30	S	20	S	16	S	24	S	25	S	24	S		
AK	20	S	26	S	25	S	25	S	20	S	23	S	20	S	24	S	25	S	21	S	26	S	26	S	25	S	30	S	22	S	20	S	20	S	20	S	18	S		
MEM	25	S	18	S	30	S	30	S	21	S	30	S	26	S	27	S	30	S	26	S	18	S	18	S	30	S	35	S	25	S	21	S	25	S	26	S	28	S		
E	12	R	10	R	10	R	10	R	14	R	21	S	16	I	15	R	16	I	20	S	10	R	10	R	16	I	20	S	16	I	17	I	21	S	20	I	20	I		

R-resistant S-Sensitive I-Intermediate

Table: Antimicrobial resistance/susceptibility determination of *Aeromonas* spp. isolates (1-19)

AB	***Aeromonas* spp.**																																							
	1		**2**		**3**		**4**		**5**		**6**		**7**		**8**		**9**		**10**		**11**		**12**		**13**		**14**		**15**		**16**		**17**		**18**		**19**	
W	25	S	25	S	26	S	26	S	0	R	0	R	0	R	0	R	0	R	0	R	25	S	25	S	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
C	24	S	24	S	28	S	28	S	26	S	30	S	30	S	29	S	30	S	30	S	24	S	25	S	35	S	35	S	30	S	30	S	31	S	30	S	0	R	0	R	0	R
FOS	18	S	21	S	25	S	25	S	18	S	18	S	0	S	11	R	26	S	25	S	21	S	21	S	18	S	20	S	15	I	15	I	20	S	25	S	0	R	0	R	0	R
TE	16	R	17	R	20	I	20	I	25	S	25	S	24	S	22	I	25	S	23	S	17	R	17	R	27	S	30	S	25	S	24	S	13	R	21	I	20	I	20	I	20	I
S	22	S	25	S	25	S	25	S	20	S	30	S	15	S	16	S	17	S	16	S	25	S	24	S	28	S	20	S	20	S	17	S	18	S	20	S	15	S	15	S	15	S
MXF	18	I	20	S	22	S	22	S	14	R	25	S	25	S	25	S	20	S	25	S	20	S	20	S	30	S	30	S	26	S	27	S	24	S	24	S	18	S	18	S	18	S
CIP	24	S	25	S	29	S	29	S	24	S	33	S	35	S	30	S	30	S	31	S	25	S	26	S	35	S	35	S	29	S	30	S	27	S	30	S	24	S	24	S	24	S
AMP	8	R	0	R	11	R	11	R	0	R	0	R	0	R	0	R	10	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
FD	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
F	18	S	17	S	20	S	20	S	17	S	18	S	18	S	18	S	20	S	17	S	17	S	16	S	20	S	24	S	18	S	17	S	20	S	20	S	18	S	18	S	18	S
K	21	S	21	S	25	S	25	S	21	S	21	S	20	S	20	S	30	S	21	S	21	S	21	S	20	S	18	S	20	S	17	I	22	S	20	S	22	S	22	S	22	S
NA	8	R	7	R	9	R	9	R	0	R	31	S	30	S	26	S	0	R	27	S	7	R	7	R	0	R	0	R	0	R	0	R	28	S	27	S	0	R	0	R	0	R
CT	14	S	14	S	15	S	15	S	11	S	14	S	13	S	16	S	15	S	14	S	14	S	14	S	15	S	16	S	14	S	12	S	14	S	15	S	15	S	15	S	15	S
CN	22	S	22	S	30	S	30	S	21	S	22	S	20	S	20	S	30	S	21	S	22	S	21	S	26	S	27	S	25	S	20	S	20	S	21	S	20	S	20	S	20	S
KF	20	S	21	S	25	S	25	S	0	R	0	R	0	R	0	R	0	R	0	R	21	S	21	S	25	S	25	S	20	S	16	I	0	R	0	R	0	R	0	R	0	R
P	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
CRO	26	S	26	S	32	S	32	S	25	S	32	S	28	S	27	S	33	S	30	S	26	S	26	S	40	S	40	S	40	S	40	S	30	S	30	S	27	S	27	S	27	S
VA	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
DA	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
CTX	25	I	26	S	30	S	30	S	25	S	33	S	30	S	25	I	30	S	28	S	26	S	26	S	40	S	40	S	39	S	39	S	29	S	32	S	30	S	30	S	30	S
ETP	26	S	25	S	32	S	32	S	20	S	30	S	22	S	23	S	27	S	25	S	25	S	25	S	25	S	30	S	20	S	16	S	24	S	25	S	24	S	24	S	24	S
AK	20	S	26	S	25	S	25	S	20	S	23	S	20	S	24	S	25	S	21	S	26	S	26	S	25	S	30	S	22	S	20	S	20	S	20	S	18	S	18	S	18	S
MEM	25	S	18	S	30	S	30	S	21	S	30	S	26	S	27	S	30	S	26	S	18	S	18	S	30	S	35	S	25	S	21	S	25	S	26	S	28	S	28	S	28	S
E	12	R	10	R	10	R	10	R	14	R	21	S	16	I	15	R	16	I	20	S	10	R	10	R	16	I	20	S	16	I	17	I	21	S	20	I	20	I	20	I	20	I

R-resistant S-Sensitive I-Intermediate

Table: Antimicrobial resistance/susceptibility determination of *Aeromonas* spp. isolates (39-57)

AB	***Aeromonas* spp.**																																							
	39		**40**		**41**		**42**		**43**		**44**		**45**		**46**		**47**		**48**		**49**		**50**		**51**		**52**		**53**		**54**		**55**		**56**		**57**	
W	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	16	R	16	R	19	R	16	R	20	S	18	S	15	I
C	30	S	30	S	30	S	30	S	34	S	35	S	35	S	35	S	30	S	30	S	30	S	30	S	20	S	19	S	20	S	29	S	15	R	15	R	27	S
FOS	29	S	29	S	30	S	30	S	35	S	35	S	35	S	40	S	24	S	25	S	25	S	26	S	23	S	21	S	25	S	23	S	19	S	20	S	27	S
TE	25	S	25	S	25	S	25	S	24	S	26	S	25	S	25	S	21	I	24	S	21	I	21	I	9	R	11	R	10	R	20	I	14	R	17	R	21	I
S	23	S	23	S	24	S	24	S	24	S	25	S	23	S	22	S	21	S	24	S	21	S	20	S	14	I	12	I	13	I	20	S	9	R	10	R	17	S
MXF	20	S	18	I	21	S	16	I	35	S	35	S	32	S	35	S	30	S	30	S	30	S	25	S	19	S	17	I	21	S	17	I	17	I	19	S	15	R
CIP	26	S	30	S	25	S	32	S	35	S	35	S	31	S	35	S	30	S	30	S	30	S	30	S	21	S	24	S	25	S	21	S	21	S	20	I	20	S
AMP	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
FD	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
F	21	S	21	S	21	S	21	S	25	S	30	S	30	S	31	S	17	S	15	I	16	I	20	S	13	R	15	I	14	R	16	I	16	I	16	I	14	R
K	26	S	26	S	26	S	25	S	25	S	25	S	25	S	25	S	22	S	25	S	20	S	20	S	16	I	20	S	19	S	16	I	10	R	9	R	14	R
NA	0	R	0	R	0	R	0	R	30	S	37	S	37	S	35	S	25	S	26	S	26	S	27	S	0	R	0	R	0	R	0	R	0	R	0	R	0	R
CT	15	S	15	S	15	S	15	S	16	S	16	S	16	S	20	S	14	S	15	S	11	S	11	S	10	R	11	S	11	S	11	S	11	S	11	S	10	R
CN	26	S	26	S	26	S	26	S	27	S	29	S	29	S	30	S	20	S	20	S	20	S	21	S	19	S	21	S	20	S	20	S	22	S	21	S	20	S
KF	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
P	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
CRO	30	S	33	S	30	S	34	S	32	S	35	S	34	S	35	S	24	S	25	S	25	S	30	S	27	S	26	S	30	S	30	S	29	S	27	S	24	S
VA	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
DA	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
CTX	30	S	31	S	30	S	32	S	30	S	36	S	35	S	35	S	25	S	26	S	26	S	31	S	24	S	25	I	24	I	27	S	18	R	26	S	30	S
ETP	30	S	31	S	30	S	31	S	30	S	21	S	25	S	21	S	20	S	20	S	20	S	17	I	9	R	7	R	10	R	9	R	12	R	10	R	11	R
AK	24	S	34	S	25	S	35	S	25	S	30	S	24	S	31	S	22	S	22	S	22	S	22	S	16	I	17	S	17	S	17	S	20	S	21	S	15	I
MEM	34	S	34	S	35	S	33	S	30	S	30	S	30	S	30	S	20	S	22	S	22	S	26	S	12	R	14	I	11	R	15	I	15	I	10	R	15	I
E	12	R	12	R	13	R	14	R	19	I	20	I	20	I	11	R	14	R	14	R	15	R	14	R	15	R	15	R	15	R	14	R	10	R	10	R	15	R

R-resistant S-Sensitive I-Intermediate

Table: Antimicrobial resistance/susceptibility determination of *Aeromonas* spp. isolates (58-75)

AB	***Aeromonas* spp.**																																							
	58		**59**		**60**		**61**		**62**		**63**		**64**		**65**		**66**		**67**		**68**		**69**		**70**		**71**		**72**		**73**		**74**		**75**							
W	16	S	19	S	18	S	17	S	15	I	0	R	17	S	0	R	0	R	0	R	0	R	20	S	0	R	17	S	0	R	0	R	0	R	0	R						
C	26	S	25	S	27	S	25	S	26	S	30	S	30	S	30	S	30	S	30	S	25	S	25	S	30	S	30	S	25	S	25	S	25	S	29	S						
FOS	25	S	25	S	23	S	25	S	24	S	25	S	25	S	25	S	24	S	25	S	21	S	28	S	30	S	22	S	25	S	25	S	25	S	26	S						
TE	20	I	21	I	20	I	21	I	20	I	22	I	21	I	25	S	25	S	25	S	20	I	24	S	20	I	25	S	20	I	22	I	21	I	22	I						
S	17	S	20	S	19	S	20	S	18	S	11	I	9	R	10	R	9	R	10	R	10	R	11	I	10	R	9	R	10	R	15	S	18	S	18	S						
MXF	16	I	18	I	16	I	17	I	16	I	25	S	24	S	24	S	22	S	24	S	20	S	25	S	30	S	28	S	19	S	22	S	16	I	20	S						
CIP	20	I	22	S	21	S	20	I	24	S	27	S	27	S	30	S	30	S	29	S	25	S	30	S	33	S	30	S	20	I	21	S	21	S	22	S						
AMP	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R						
FD	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R						
F	15	I	15	I	15	I	16	I	15	I	26	S	19	S	19	S	20	S	20	S	16	I	20	S	22	S	19	S	19	S	15	I	19	S	20	S						
K	14	R	15	I	14	R	14	R	15	I	16	I	14	R	15	I	16	I	20	S	20	S	21	S	22	S	24	S	15	S	15	S	14	R	18	S						
NA	0	R	0	R	0	R	0	R	0	R	30	S	30	S	30	S	32	S	30	S	25	S	32	S	30	S	30	S	0	R	0	R	0	R	0	R						
CT	10	R	10	R	10	R	11	S	10	R	13	S	12	S	15	S	15	S	14	S	11	S	11	S	15	S	15	S	11	S	10	R	10	R	10	R						
CN	20	S	18	S	20	S	20	S	20	S	22	S	19	S	24	S	24	S	21	S	20	S	22	S	20	S	20	S	21	S	20	S	20	S	20	S						
KF	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R						
P	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R						
CRO	20	R	22	R	22	R	19	R	21	R	26	S	24	S	24	S	26	S	25	S	24	S	25	S	32	S	30	S	25	S	21	S	20	R	20	R						
VA	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R						
DA	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R						
CTX	25	I	22	R	30	S	30	S	21	R	30	S	26	S	30	S	28	S	30	S	26	S	30	S	35	S	33	S	25	I	26	S	25	S	27	S						
ETP	14	R	13	R	9	R	12	R	10	R	21	S	24	S	25	S	25	S	24	S	20	S	27	S	25	S	26	S	11	R	11	R	16	I	11	R						
AK	16	I	17	S	17	S	17	S	17	S	19	S	20	S	20	S	20	S	21	S	16	I	25	S	22	S	19	S	20	S	19	S	19	S	17	S						
MEM	13	R	15	I	12	R	16	S	14	I	28	S	28	S	30	S	30	S	28	S	22	S	25	S	27	S	26	S	14	S	11	R	10	R	10	R						
E	15	R	14	R	14	R	12	R	12	R	18	I	23	S	15	R	15	R	17	I	15	R	20	S	15	R	15	R	13	R	12	R	12	R	10	R						

R-resistant S-Sensitive I-Intermediate

Table: Antimicrobial resistance/susceptibility determination of *Aeromonas* spp. isolates (76- 94)

AB	***Aeromonas* spp.**																																					
	76		**77**		**78**		**79**		**80**		**81**		**82**		**83**		**84**		**85**		**86**		**87**		**88**		**89**		**90**		**91**		**92**		**93**		**94**	
W	26	S	17	S	16	S	20	S	15	I	20	S	17	S	18	S	20	S	20	S	15	I	15	I	15	I	20	S	16	S	16	S	16	S	17	S	16	S
C	25	S	25	S	30	S	30	S	22	S	17	I	30	S	29	S	30	S	27	S	25	S	25	S	30	S	29	S	26	S	28	S	25	S	22	S	25	S
FOS	19	S	0	R	25	S	29	S	25	S	21	S	22	S	21	S	25	S	23	S	22	S	20	S	21	S	25	S	0	R	0	R	0	R	26	S	0	R
TE	0	R	12	R	25	S	25	S	10	R	10	R	20	I	20	I	21	I	23	S	21	I	20	I	20	I	23	S	22	I	20	I	21	I	10	R	22	I
S	20	S	19	S	20	S	20	S	12	I	11	I	12	I	11	I	16	S	20	S	12	I	10	R	10	R	11	I	15	S	12	I	15	S	0	R	15	S
MXF	25	S	27	S	22	S	20	S	20	S	20	S	20	S	20	S	20	S	24	S	24	S	20	S	22	S	25	S	22	S	22	S	27	S	20	S	22	S
CIP	26	S	30	S	25	S	27	S	25	S	25	S	28	S	30	S	24	S	25	S	30	S	28	S	30	S	30	S	28	S	30	S	30	S	20	S	26	S
AMP	16	R	0	R	0	R	0	0	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
FD	0	R	0	R	0	R	0	0	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
F	14	R	16	I	18	S	18	S	15	I	15	I	15	I	18	S	15	I	16	I	15	I	15	I	15	I	16	I	18	S	17	S	17	S	24	S	16	I
K	15	I	15	I	17	I	16	I	14	R	12	R	13	R	15	I	15	I	15	I	14	R	11	R	11	R	16	I	15	I	15	I	15	I	20	S	15	I
NA	20	S	20	S	0	R	0	R	0	R	0	R	25	S	26	S	0	R	0	R	26	R	23	S	25	S	26	S	27	S	25	S	25	S	0	R	25	S
CT	12	S	12	S	11	S	10	R	10	R	10	R	21	S	11	S	12	S	11	S	11	S	10	R	11	S	12	S	12	S	14	S	12	S	15	S	13	S
CN	20	S	18	S	20	S	22	S	20	S	20	S	20	S	17	S	20	S	20	S	20	S	17	S	20	S	22	S	20	S	20	S	20	S	24	S	20	S
KF	10	R	18	S	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	20	S	0	R
P	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
CRO	20	R	24	S	25	S	20	R	20	R	20	R	21	R	21	R	20	R	20	R	20	R	17	R	20	R	25	S	22	R	22	R	24	S	25	S	21	R
VA	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
DA	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R	0	R
CTX	21	R	30	S	30	S	22	R	24	I	23	I	30	S	30	S	30	S	25	I	25	I	25	I	25	I	30	S	28	S	27	S	30	S	33	S	27	S
ETP	26	S	21	S	11	R	10	R	11	R	10	R	24	S	24	S	10	R	10	R	20	S	17	I	20	S	23	S	22	S	21	S	22	S	19	S	20	S
AK	20	S	19	S	21	S	20	S	20	S	20	S	18	S	18	S	20	S	20	S	20	S	18	S	16	I	20	S	20	S	20	S	20	S	25	S	20	S
MEM	24	S	25	S	15	I	15	I	11	R	12	R	24	S	24	S	12	R	15	I	25	S	25	S	25	S	27	S	25	S	25	S	25	S	22	S	25	S
E	10	R	18	I	18	I	16	I	15	R	13	R	15	R	15	R	11	R	12	R	15	R	18	I	20	I	20	I	18	I	17	I	18	I	20	I	16	I

R-resistant S-Sensitive I-Intermediate

Table: Antimicrobial resistance/susceptibility determination of *Aeromonas* spp. (95-100) and *Listeria* (76-78) isolates.

AB	*Aeromonas* spp.											
	95		**96**		**97**		**98**		**99**		**100**	
W	18	S	17	S	16	S	20	S	20	S	17	S
C	19	S	21	S	25	S	30	S	32	S	26	S
FOS	30	S	27	S	22	S	32	S	30	S	28	S
TE	11	R	10	R	20	I	22	I	25	S	22	I
S	0	R	0	R	20	S	10	R	18	S	18	S
MXF	20	S	18	I	20	S	21	S	22	S	20	S
CIP	19	I	19	I	24	S	27	S	29	S	30	S
AMP	0	R	0	R	0	R	0	R	0	R	0	R
FD	0	R	0	R	0	R	0	R	0	R	0	R
F	22	S	18	S	18	S	21	S	20	S	18	S
K	18	S	18	S	15	I	18	S	19	S	18	I
NA	0	R	0	R	0	R	0	R	0	R	0	R
CT	14	S	15	S	10	R	12	S	14	S	12	S
CN	27	S	24	S	21	S	22	S	26	S	25	S
KF	20	S	12	I	0	R	0	R	0	R	0	R
P	0	R	0	R	0	R	0	R	0	R	0	R
CRO	27	S	25	S	24	S	26	S	25	S	21	R
VA	0	R	0	R	0	R	0	R	0	R	0	R
DA	0	R	0	R	0	R	0	R	0	R	0	R
CTX	31	S	30	S	28	S	30	S	31	S	27	S
ETP	18	I	19	S	0	R	20	S	20	S	10	R
AK	25	S	25	S	0	S	22	S	24	S	20	S
MEM	20	S	22	S	15	I	23	S	29	S	27	S
E	15	R	10	R	10	R	11	R	12	R	18	S

AB	*Listeria SPP.*					
	76		**77**		**78**	
W	0	R	0	R	0	R
C	25	S	19	S	21	S
FOS	23	S	23	S	23	S
TE	18	R	18	R	15	R
S	20	S	20	S	18	S
MXF	22	S	30	S	26	S
CIP	30	S	32	S	31	S
AMP	15	R	14	R	10	R
FD	26	S	24	S	26	S
F	17	I	15	I	15	I
K	0	R	0	R	15	I
NA	0	R	0	R	0	R
CT	11	S	15	S	14	S
CN	26	S	24	S	21	S
KF	30	S	36	S	35	S
P	0	R	0	R	0	R
CRO	16	I	15	I	14	I
VA	27	S	27	S	25	S
DA	25	S	0	R	27	S
CTX	24	I	24	I	24	I
ETP	20	S	21	S	20	S
AK	20	S	24	S	20	S
MEM	38	S	35	S	25	S
E	0	R	0	R	0	R

R-resistant S-Sensitive I-Intermediate

Antibiotic Resistance Index (ARI) OF *Aeromonas* spp.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
No. R	8	8	7	7	9	7	7	9	8	7	8	8	7	7	7	7	8	7	10	11	8	8	8	8	10	8	6	6
No. T	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24
ARI	0.33	0.33	0.29	0.29	0.38	0.29	0.29	0.38	0.33	0.4	0.33	0.33	0.29	0.29	0.29	0.29	0.33	0.29	0.4	0.46	0.33	0.33	0.33	0.33	0.4	0.33	0.25	0.25

	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
No. R	8	8	8	7	8	7	7	7	9	9	9	10	9	9	7	7	7	8	8	8	8	8	14	11	13	10	14	14
No. T	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24
ARI	0.33	0.33	0.33	0.29	0.33	0.29	0.29	0.29	0.38	0.38	0.38	0.4	0.38	0.38	0.29	0.29	0.29	0.33	0.33	0.33	0.33	0.33	0.58	0.46	0.54	0.4	0.58	0.58

	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84
No. R	13	13	12	13	11	12	7	8	9	9	9	9	6	9	8	11	12	13	13	11	7	8	9	14	14	9	8	11
No. T	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24
ARI	0.54	0.54	0.5	0.54	0.46	0.5	0.29	0.33	0.38	0.38	0.38	0.38	0.25	0.38	0.33	0.46	0.5	0.54	0.54	0.46	0.29	0.33	0.38	0.58	0.58	0.38	0.33	0.46

	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
No. R	10	10	10	9	6	8	8	7	8	8	9	9	10	9	8	9
No. T	24	24	24	24	24	24	9	24	24	24	24	24	24	24	24	24
ARI	0.4	0.4	0.4	0.38	0.25	0.33	0.33	0.29	0.33	0.33	0.38	0.38	0.4	0.38	0.33	0.38